THE ROLE OF FGFR3 IN SKELETAL REPAIR

by

Simon P. Kelley

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

Institute of Medical Science
University of Toronto

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2016

Abstract

Fibroblast growth factor receptor 3 (FGFR3) mutations cause skeletal dysplasias with disparate phenotypes. Gain-of-function mutations are characterized by disproportionate short stature, whereas loss-of-function mutations are characterized by tall stature. FGFR3 is a known negative regulator of chondrocyte proliferation yet we observed that individuals with achondroplasia showed enhanced intramembranous bone regeneration during limb lengthening surgery, thus we hypothesized that FGFR3 mutations affect osteoblast differentiation, in addition to chondrocyte differentiation, and thus impact fracture repair. Investigating bone fracture repair in genetically modified $Fgfr3^{+/−}$ mice we showed, firstly, that FGFR3 acts as a switch between intramembranous and endochondral ossification leading to abnormal bone healing with impaired structural integrity. Secondly, we found that in controlled in-vitro assays FGFR3 regulates number, proliferation and osteogenic differentiation of murine bone marrow SSCs. Thirdly we showed that bone marrow transplantation can be used to control the osteoblast response to fracture in-vivo, and osteoblast differentiation in-vitro, such that WT bone marrow transplant has the ability to rescue abnormal fracture repair in native $Fgfr3^{+/−}$ mice, and conversely $Fgfr3^{+/−}$ bone marrow transplantation disturbs fracture repair in WT mice. Our results demonstrate that FGFR3 is critical in coordinating the repair of bone. Modulation of FGFR3 signaling using bone marrow related therapies, may offer the ability to enhance the proliferation and differentiation of skeletal progenitor cells for osteogenesis in limb reconstruction therapy and also to direct progenitor cells towards particular bone regenerative pathways, which has enormous appeal for treating common musculoskeletal disorders such as fracture repair.
Acknowledgments

Most importantly I would like to recognize the immense love and support from my wife Suzanne, who never even flinched when I said I was going to take on a PhD in addition to a full surgical practice immediately on arriving in Canada to start a new life with our two young daughters. Despite my own concerns, Suzanne has never doubted that I would be able to pull it off, and her confidence in me has helped immensely.

I would like to thank my supervisor, and colleague Dr. Benjamin Alman, who has supported my goal to become an orthopaedic surgeon-scientist. Ben teaches me things that no one else can, and has inspired me to look at problems in a different way. “Just because it’s difficult, it doesn’t mean you shouldn’t do it”.

Working with the program advisory committee of Dr Gordon Keller, Dr Janet Rossant, Dr Lidan You, and previously Dr Jane Aubin, is an honour due to their collective international expertise in the field, and has inspired me to do my best work at all times. I always felt that if I could satisfy this committee’s exceptionally rigorous scientific standards, then I could afford to be proud of my work, and would give me the best possible start as a surgeon-scientist.

I am blessed to work with not only some of the most talented paediatric orthopaedic surgeons in the world, but also the most collegial and supportive group, who in order for me to complete my PhD have supported my clinical practice and protected my time consistently and without hesitation throughout the last 6 years. I therefore thank my Colleagues, Catharine Bradley, Dr Martin Gargan, Dr Mark Camp, Dr Unni Narayanan, Dr Lucas Murnaghan, Dr Sevan Hopyan, Dr Andrew Howard, Dr. Reinhard Zeller, Dr Stephen Lewis and Dr James Wright.

In particular, I would like to thank Dr John Wedge, an extraordinary surgeon, who in addition to all the mentorship one could ask for as a junior surgeon has even postponed his retirement to protect my clinical hip practice whilst I obtained my graduate research training. John’s selfless commitment to my success is an inspiration.

I wish to thank all the members of Dr Benjamin Alman’s laboratory, particularly Chunying Yu, who has helped me integrate into the world of lab science and taught me all the things I didn’t know I didn’t know!

I dedicate this thesis to my two daughters Maia and Saphia
Contributions

Dr Simon Kelley (author) solely prepared this thesis. All aspects of this body of work, including the planning, execution, analysis and writing of all original research contained herewith in was performed by the author. The contributions by other individuals are formally and inclusively acknowledged:

Dr Benjamin Alman (Supervisor and Committee Member) – Mentorship, laboratory space, equipment supplies, guidance on the planning, execution, analysis and presentation of all experiments in addition to manuscript and thesis preparation.

Dr Gordon Keller (Committee Member) – Mentorship, guidance in interpretation of results, and thesis preparation

Dr Janet Rossant (Committee Member) – Mentorship, guidance in interpretation of results, and thesis preparation

Dr Lidan You (Committee Member) – Mentorship, guidance in interpretation of results, and thesis preparation

Dr Jane Aubin (Committee Member) – Mentorship, guidance in interpretation of results within the first year of study

Chunying Yu – (Laboratory Technician) – assistance in planning and execution of experiments, and analysis of results for chapters 2, 3 and 4

Heather Whetstone – (Laboratory Technician) – assistance in planning and execution of experiments, and analysis of results for chapters 2, 3 and 4

Puvindran Nadesan - (Laboratory Technician) – assistance and guidance in animal care

Dr Thomas Willett – (Scientist) assistance and guidance in execution of micro-CT experiments including analysis of results

Dr Gurpreet Baht – Post-Doctoral Fellow – guidance and assistance in planning and executing bone marrow transplant experiments
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<tbody>
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<td>ACH</td>
<td>Achondroplasia</td>
</tr>
<tr>
<td>ALP</td>
<td>Alkaline Phosphatase</td>
</tr>
<tr>
<td>BMD</td>
<td>Bone Mineral Density</td>
</tr>
<tr>
<td>BMC</td>
<td>Bone Mineral Content</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone Morphogenetic Protein</td>
</tr>
<tr>
<td>BMPR</td>
<td>Bone Morphogenetic Protein Receptor</td>
</tr>
<tr>
<td>BMSC</td>
<td>Bone Marrow Stromal Cell</td>
</tr>
<tr>
<td>BMT</td>
<td>Bone Marrow Transplant</td>
</tr>
<tr>
<td>BrdU</td>
<td>5-Bromo-2’-Deoxyuridine</td>
</tr>
<tr>
<td>BSP</td>
<td>Bone Sialoprotein</td>
</tr>
<tr>
<td>CATSHL</td>
<td>Camptodactyly Tall Stature Scoliosis and Hearing Loss Syndrome</td>
</tr>
<tr>
<td>CFE</td>
<td>Colony Forming Efficiency</td>
</tr>
<tr>
<td>CFU-F</td>
<td>Colony Forming Unit–Fibroblast</td>
</tr>
<tr>
<td>CFU-O</td>
<td>Colony Forming Unit–Osteoblast</td>
</tr>
<tr>
<td>ColI</td>
<td>Collagen Type I</td>
</tr>
<tr>
<td>COMP</td>
<td>Cartilage Oligomeric Matrix Protein</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-Diamidino-2-Phenylindole</td>
</tr>
<tr>
<td>DO</td>
<td>Distraction Osteogenesis</td>
</tr>
<tr>
<td>ERK1/2</td>
<td>Extracellular Signal-Regulated Protein Kinases 1 and 2</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast Growth Factor</td>
</tr>
<tr>
<td>FGFR3</td>
<td>Fibroblast Growth Factor Receptor 3</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein Isothiocyanate</td>
</tr>
<tr>
<td>FIZ</td>
<td>Fibrous Interzone</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycans</td>
</tr>
<tr>
<td>HA</td>
<td>Hydroxyapatite</td>
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<tr>
<td>HS</td>
<td>Heparan Sulphate</td>
</tr>
<tr>
<td>IHH</td>
<td>Indian Hedgehog</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus Kinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>LRP6</td>
<td>Low Density Lipoprotein Receptor-Related Protein 6</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-Activated Protein Kinases</td>
</tr>
<tr>
<td>MCF</td>
<td>Microcolumn Formation</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Macrophage Colony Stimulating Factor</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix Metalloproteinase</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal Stem Cell</td>
</tr>
<tr>
<td>μCT</td>
<td>Micro Computed Tomography</td>
</tr>
<tr>
<td>OCN</td>
<td>Osteocalcin</td>
</tr>
<tr>
<td>OPG</td>
<td>Osteoprotegerin</td>
</tr>
<tr>
<td>OPN</td>
<td>Osteopontin</td>
</tr>
<tr>
<td>OSX</td>
<td>Osterix</td>
</tr>
<tr>
<td>PFD</td>
<td>Post-Fracture Day</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol-3-Kinase</td>
</tr>
<tr>
<td>PMF</td>
<td>Primary Mineralization Front</td>
</tr>
<tr>
<td>PMI</td>
<td>Polar Moment of Inertia</td>
</tr>
<tr>
<td>PTHrP</td>
<td>Parathyroid Hormone-related Protein</td>
</tr>
<tr>
<td>RANK</td>
<td>Receptor Activator of Nuclear Factor-κβ</td>
</tr>
<tr>
<td>RANKL</td>
<td>Receptor Activator of Nuclear Factor-κβ Ligand</td>
</tr>
<tr>
<td>RIP</td>
<td>Regulated Intramembrane Proteolysis</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of Interest</td>
</tr>
<tr>
<td>RUNX2</td>
<td>Runt-related transcription factor 2</td>
</tr>
<tr>
<td>SSC</td>
<td>Skeletal Stem Cell</td>
</tr>
<tr>
<td>SOX9</td>
<td>SRY (Sex Determining Region Y)-Box-9</td>
</tr>
<tr>
<td>STAT1</td>
<td>Signal Transducers and Activators of Transcription 1</td>
</tr>
<tr>
<td>TD</td>
<td>Thanatophoric Dysplasia</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming Growth Factor Beta</td>
</tr>
<tr>
<td>TMD</td>
<td>Tissue Mineral Density</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor Necrosis Factor-α</td>
</tr>
<tr>
<td>TRAP</td>
<td>Tartrate Resistant Acid Phosphatase</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>WNT</td>
<td>Wingless-related Integration Site</td>
</tr>
<tr>
<td>WT</td>
<td>Wild Type</td>
</tr>
</tbody>
</table>
CHAPTER 1 - BONE BIOLOGY AND FIBROBLAST GROWTH FACTOR SIGNALING
1.1 Bone Development

1.1.1 Introduction

The adult human skeleton comprises 206 bones, which are divided into the axial skeleton, (including the skull, ribs and spine) and the appendicular skeleton, (including the bones of the limbs and the pelvis). Despite their varied size, shape and function, all 206 bones develop in a remarkably tightly controlled fashion, via two distinct processes, that of intramembranous and endochondral ossification. Intramembranous ossification is a process where osteochondroprogenitor cells differentiate directly to osteoblasts, which form bone directly (David M Ornitz & Marie, 2002). This type of ossification is responsible for development of the bones of the dermocranium and the medial ends of the clavicles (Matsuoka et al., 2005). Endochondral bone formation is a more complex process whereby a cartilage template is formed as an intermediate step which is subsequently replaced by bone (Kronenberg, 2003). Endochondral ossification is responsible for the majority of skeletal bone growth including all of the appendicular bones, vertebral column, ribs and bones of the face. Although it is conventional to categorize bones as to whether they form by intramembranous or endochondral ossification, in fact bones that form primarily by endochondral ossification also incorporate elements of intramembranous ossification, for the purpose of forming a central bone collar for structural integrity and developmental morphology of the bone (Syftestad, Weitzhandler, & Caplan, 1985). When a fractured bone heals, both endochondral and intramembranous ossification occur in varying degrees based upon anatomic location in the fracture and the overall mechanical environment (Perren, Fernandez, & Regazzoni, 2015). Distraction osteogenesis (DO) is a bone regenerative process, which is distinct from development and fracture repair, and is commonly seen during bone lengthening surgeries. DO is primarily achieved by intramembranous ossification aided by elements of endochondral ossification in the earliest phases (Bouletreau, Warren, & Longaker, 2002). There is emerging evidence that a third type of ossification may also occur, which sits somewhere between intramembranous and endochondral ossification, where mature chondrocytes actually transdifferentiate into osteoblasts which lay down bone in specific developmental processes such as the formation of an ossific nucleus at the growing ends of the long bone (J. Park et al., 2015; L. Yang, Tsang, Tang, Chan, & Cheah, 2014; Zhou et al., 2014).
Taken together, what has long been considered to be two quite distinct methods of bone formation may actually be just two ends of a spectrum, that are more intrinsically linked than we had originally supposed. Whether bone forms for development, fracture repair or distraction osteogenesis, the same fundamental cells, signaling pathways and niches are present. Understanding the nuances of each process, the cellular interactions, variations in signaling and the importance of niche is essential for the advancement of fundamental knowledge in addition to the development of novel therapies for the treatment of genetic bone diseases, the enhancement of fracture repair and tissue regeneration.

1.1.2 Mesenchymal condensation

The first step in the development of a bone is the migration and condensation of mesenchymal progenitors. The mesenchymal progenitors that form the cranium, mandible and medial clavicles (intramembranous bones) are derived from the neural crest. The mesenchymal progenitors that form the “endochondral” facial bones, vertebrae and limbs are derived from neural crest, the paraxial mesoderm and the lateral plate mesoderm respectively. The mesenchymal condensation reflects the aggregation of mesenchymal cells rather than the proliferation of cells, and is mediated by cell-cell interactions including the transient expression of NCAM and N-cadherin (Oberlender & Tuan, 1994).

1.1.3 FGFR expression in limb mesenchyme

FGF signaling is critical for the coordination of the formation of the limb bud including the mesenchymal condensation. FGFR1 and FGFR2 are expressed early with FGFR1 distributed more diffusely and in the periphery of the condensation, whereas FGFR2 is expressed more centrally as the cells condense. The expression of both receptors persists well into limb development (Orr-Urtrreger, Givol, Yayon, Yarden, & Lonai, 1991). FGFR3 is expressed at a slightly later stage than FGFR1 and FGFR2 and initially only in the very central area of mesenchymal condensation, which will become the first area to undergo chondrogenesis, suggesting a focused role for FGFR3 in bone development (Delezoide et al., 1998; Peters, Ornitz, Werner, & Williams, 1993). Some reports have identified that the role of FGFR3 is to increase chondrocyte proliferation (Davidson et al., 2005; Iwata et al., 2000) in these early embryonic stages of bone development rather than the more commonly reported role of FGFR3 being considered a negative regulator of chondrocyte proliferation (Deng, Wynshaw-Boris,
Zhou, Kuo, & Leder, 1996; Henderson et al., 2000). It is yet to be determined exactly why this dichotomy exists. It is possible, although as yet unproven, that FGFR3 has different, and perhaps opposing roles on chondrocyte proliferation depending on the stage of development.

1.1.4 FGF signaling in limb mesenchyme

FGF signaling begins from the very first moments of embryonic limb bud growth, even before mesenchymal condensation (Sun, Mariani, & Martin, 2002). FGF8 is the first signaling molecule to localize to the apical ectodermal ridge (AER) and is considered the key regulator of AER function (Sun et al., 2002). FGF9 is subsequently expressed by cells within the condensing mesenchyme (Colvin, Feldman, Nadeau, Goldfarb, & Ornitz, 1999), whereas FGF2, FGF5, FGF6 and FGF7 are expressed peripherally outside the condensation (deLapeyrière et al., 1995; Finch, Cunha, Rubin, Wong, & Ron, 1995; Haub & Goldfarb, 1991; Mason, Fuller-Pace, Smith, & Dickson, 1994). As yet the role of these peripherally acting FGFs is incompletely understood, as mouse models deficient in many of these molecules have no significant limb anomalies (Guo, Degenstein, & Fuchs, 1996; Hébert, Rosenquist, Götz, & Martin, 1994). The only two FGFs so far that have been shown to have significant roles are FGF9 and FGF18. The FGF9⁻/⁻ mouse demonstrates a rhizomelic short limb phenotype similar to that of the FGFR3 achondroplasia mutation (Hung, Yu, Lavine, & Ornitz, 2007). FGF18 is expressed in perichondrium and joint spaces and in the early stages causes chondrocyte proliferation and differentiation (Zhonghao Liu, Xu, Colvin, & Ornitz, 2002).

1.1.5 Endochondral ossification

The next step in the development of a long bone occurs when the central cells of the mesenchymal condensation differentiate into chondrocytes. Not all the cells of the condensation will differentiate to chondrocytes, however, as there is second sub-population of mesenchymal cells, which differentiate directly into osteoblasts and are positioned peripherally, which demarcates the bone from the surrounding mesenchymal tissue. The differentiation of chondrocytes expands out from the central portion of the condensation both longitudinally and transversely patterning the shape of the bone in the process. The initiation of chondrogenesis is accompanied by the expression of a number of important molecules. SOX9 is the earliest identified molecule associated with chondrogenesis in the limb bud, and is considered the master regulator of chondrogenesis, which acts upon the newly differentiated chondrocytes to generate
the extracellular matrix proteins collagen type II (ColII), collagen type IX (ColIX) and collagen type XI (ColXI) in addition to aggrecan and cartilage oligomeric matrix protein (COMP) (Degnin, Laederich, & Horton, 2010). SOX9 is considered the master regulator of chondrogenesis not only because of its spatial and temporal expression, but also because SOX9 deficiency shows such profound developmental skeletal defects. Mice lacking both SOX9 alleles have a complete loss of chondrogenesis, whereas mice haploinsufficient for SOX9 show severe chondrodysplasia, which mimics the human disease of campomelic dysplasia seen in individuals with heterozygous SOX9 deficiency (Akiyama, Chaboissier, Martin, Schedl, & de Crombrugghe, 2002; Bi, Deng, Zhang, Behringer, & de Crombrugghe, 1999; Bi et al., 2001; Foster et al., 1994; Kist, Schrewe, Balling, & Scherer, 2002; Wagner et al., 1994).

FGFR1, FGFR2 and FGFR3 are expressed in the developing bone as chondrogenesis progresses at the earliest stages of embryological development. Each of the three receptors seems to have distinct roles at this stage, although the exact interplay between the FGFRs and their numerous FGF ligands is incompletely understood (Degnin et al., 2010). The expression of FGFR2 occurs early and centrally at the initiation of chondrogenesis. The strength of FGFR2 expression decreases as one approaches the periphery of the mesenchymal condensation, where increased expression of FGFR1 is seen. This increase in FGFR1 expression corresponds to the development of the zone of intramembranous ossification called the bone collar; the function of which is to demarcate the bone from surrounding mesenchymal tissue, to help coordinate the three-dimensional shape of the developing bone, acting somewhat like a corset, by directing the chondrocytic cell differentiation along a more longitudinal rather than a transverse axis, thus increasing the length of the cartilage anlage, and finally it may act as a signaling area to help direct vascular invasion of the central cartilaginous anlage. FGFR3 is expressed after that of FGFR1 and FGFR2 and is seen centrally, presumably to assist with the tightly controlled processes involved in chondrogenesis of the expanding bony anlage (Kronenberg, 2003; Long & Ornitz, 2013; David M Ornitz & Marie, 2002).

Soon after the undifferentiated mesenchymal cells have become chondrocytes, the centrally located chondrocytes stop proliferating, start to exit the cell cycle, and undergo terminal differentiation to become hypertrophic chondrocytes. Hypertrophic chondrocytes drive bone formation and are characterized by the production of collagen type X (ColX). They also secrete matrix metalloproteinase 13 (MMP13) and vascular endothelial growth factor (VEGF) (Degnin
et al., 2010). VEGF and MMP13 stimulate the ingrowth of blood vessels and cleavage of extracellular matrix proteins respectively, to facilitate further vascular ingrowth to the central area of chondrocyte hypertrophy, which delivers both osteoclasts and osteoblasts. The hypertrophic chondrocytes undergo apoptosis leaving gaps within the extracellular matrix. Osteoclasts further remove the deposited chondrocytic matrix to allow more space for osteoblasts and blood vessels to invade. Osteoblasts then lay down bone matrix composed primarily of collagen type I (Coll) and then mineralize this matrix to form the primary spongiosa in the centre of the developing bone (Berendsen & Olsen, 2015; Kronenberg, 2003; Long & Ornitz, 2013; David M Ornitz & Marie, 2002).

This spreading wave of chondrocyte terminal differentiation moves longitudinally away from the centre of the anlage towards both ends of the growing bone. Simultaneously the chondrocytes at the ends of the growing bone continue to proliferate thus increasing the bones length. The wave of cellular hypertrophic differentiation never actually reaches the end of the bone. Rather the cells slow their rate of proliferation and differentiation and organize themselves into a disc-like structure called the growth plate, which is bordered by the primary spongiosa on one side and the epiphysis, made of proliferative chondrocytes, in the juxtarticular region. The epiphyses are largely cartilaginous in most bones in the early post-natal period, but during growth a secondary ossification centre forms. This occurs when the central epiphyseal chondrocytes undergo terminal differentiation to hypertrophic chondrocytes, and via the same mechanisms outlined above encourage vascular invasion and the formation of an ossific nucleus, which expands to replace the cartilaginous epiphysis, leaving just a layer of highly specialized articular cartilage surrounding the end of the bone forming the joint surface. This bony epiphysis is separated from the metaphysis of the bone by the growth plate (Berendsen & Olsen, 2015; Deginin et al., 2010; Kronenberg, 2003; Zelzer & Olsen, 2003).

The growth plate structurally consists of a layer of round resting chondrocytes that lie oriented parallel to the joint, which form a ready supply of cells to enter a proliferative state during skeletal growth. Beneath the layer of resting cells are neatly organized columns of disc shaped proliferative cells. The proliferative layer then transitions into a prehypertrophic layer and subsequently a hypertrophic layer of chondrocytes that undergo apoptosis and direct bone formation as previously described. The rate of proliferation and differentiation in the growth plate determine how rapidly a bone gains length. In adolescence the growth plate disappears and
is replaced by bone thus ending the phase of skeletal growth (Kronenberg, 2007; Long & Ornitz, 2013).

1.1.6 Origin of osteoblasts in endochondral ossification

An emerging concept in bone development is that rather than the linear model whereby osteochondroprogenitors become specified to differentiate to either an osteoblast or chondrocyte lineage, that chondrocytes may actually be able to transdifferentiate to osteoblasts. This line of investigation came about due to differences in opinion about the fate of hypertrophic chondrocytes (Shapiro, Adams, Freeman, & Srinivas, 2005). It has long been known that chondrocytes undergo apoptosis or autophagy as their terminal event and as a key step in driving local osteogenesis (Aizawa, Kokubun, & Tanaka, 1997; Farnum & Wilsman, 1987; Srinivas & Shapiro, 2006; Zenmyo et al., 1996) but in-vitro observations suggested that hypertrophic chondrocytes might be able to transdifferentiate to osteoblasts (Descalzi Cancedda, Gentili, Manduca, & Cancedda, 1992). In addition it is known that hypertrophic chondrocytes express many of the same markers as osteoblasts such as Runx2, osterix (Osx), alkaline phosphatase (ALP), bone sialoprotein (BSP), osteopontin (OPN) and osteocalcin (OCN) (Lefebvre & Smits, 2005). It was not until very recently that a number of publications gave more weight to the hypothesis that transdifferentiation of hypertrophic chondrocytes actually occurs, with convincing in-vivo demonstrations of this phenomenon (J. Park et al., 2015; G. Yang et al., 2014; L. Yang et al., 2014; Zhou et al., 2014). Through lineage tracing experiments using conditionally labeled hypertrophic chondrocytes it has been shown that some hypertrophic chondrocytes are able to transdifferentiate through a newly discovered progenitor cell expressing osterix, termed the chondrocyte-derived osteoprogenitor (CDOP) cell, which further differentiate to osteoblasts. These cells are seen in the region of the lower hypertrophic zone at the mineralization front of the growth plate. The mature osteoblasts derived from these CDOP cells can be seen in the primary spongiosa of the bone marrow alongside osteoblasts derived from the perichondrium (J. Park et al., 2015). Not only does it appear that hypertrophic chondrocytes can form up to 60% of osteoblasts in endochondral bone both prenatally and postnatally (in one month old mice), the same process also occurs in fracture healing with the direct transdifferentiation of hypertrophic chondrocytes to osteoblasts in the healing callus, adding to the pool of osteoblasts differentiated from dedicated progenitors in the periosteum (Zhou et al., 2014).
1.1.7 Transcriptional control of growth plate function

Following the initial differentiation of mesenchymal cells to chondrocytes, which requires SOX9 expression, the subsequent terminal differentiation of chondrocytes to hypertrophic chondrocytes is then accompanied by a reduction in SOX9 expression (Akiyama et al., 2002). An increase in RUNX2 expression is also seen at this time, which positively regulates terminal chondrocyte differentiation (and osteoblast differentiation) (Komori et al., 1997; Otto et al., 1997). Because Runx2 is also a major transcriptional regulator of osteoblast differentiation, the expression of osterix is therefore more specific for differentiation of the osteoblast lineage (Franceschi, Ge, Xiao, Roca, & Jiang, 2009).

1.1.8 IHH/PTHrP Signaling Loop

The IHH/PTHrP signaling loop is critical for controlling the balance of chondrocyte proliferation and differentiation in the growth plate of the developing bone (Kronenberg, 2003). Parathyroid Hormone related Peptide (PTHrP) is expressed at the end of the developing bone in perichondrial cells, and in the growth plate by proliferating chondrocytes. The action of PTHrP positively regulates chondrocyte proliferation by acting on its own receptors on proliferating chondrocytes to maintain a proliferative state and prevent differentiation (K. Lee et al., 1996; Weir et al., 1996). Proliferating chondrocytes receive less signal from PTHrP as they move distally away from their resting progenitors through the growth plate, which would tend to cause them to stop proliferating and undergo differentiation, however they become increasingly signaled by Indian Hedgehog (IHH) (Vortkamp et al., 1996). IHH is expressed by prehypertrophic and hypertrophic chondrocytes, which acts through patched-1 (ptc-1) receptors on the proliferating chondrocytes directly to prevent further chondrocyte differentiation (St-Jacques 1999). In addition IHH also acts by signaling back to the proliferative chondrocytes at the ends of the bone to secrete PTHrP, thus further maintaining the proliferating pool and closing the feedback loop (Karp et al., 2000; Kronenberg & Chung, 2001; Vortkamp et al., 1996). Developmental control of the growth plate control is therefore modulated by the gradient of IHH and PTHrP, which exists because of the spatial organization of between the physeal zones, and the fact that these signaling molecules are expressed by cells at either end of the growth plate.
It is through this intricately controlled feedback loop that the developing bone is able to grow in length, by controlling and maintaining the pool of proliferating chondrocytes but also to determine the rate and extend of osteoblast differentiation which adds structure and integrity to the bone, by controlling the rate of terminal differentiation of chondrocytes to hypertrophic chondrocytes which, as discussed above stimulates osteogenesis following apoptosis or autophagy, or transdifferentiate directly into osteoblasts.

The first elements of osteogenesis that occur in the developing bone are during the formation of the bone collar as previously discussed. The perichondrial cells that secrete PTHrP and differentiate directly to bone to form the bone collar are also stimulated by IHH, secreted by the nearby differentiating chondrocytes, which occurs in the centre of the cartilage anlage (St-Jacques, Hammerschmidt, & McMahon, 1999).

1.1.9 FGFRs in the growth plate

The FGFRs all have a role to play in further endochondral bone development and the growth plate. The initial roles in mesenchymal condensation and chondrocyte proliferation have been described earlier. Once chondrocyte differentiation occurs in the central area of the cartilage anlage FGFR3 is expressed mainly in proliferating chondrocytes, prehypertrophic chondrocytes but also can be detected in early hypertrophic chondrocytes and osteoblasts (Naski, Wang, Xu, & Ornitz, 1996; Valverde-Franco et al., 2004; Xiao et al., 2004), FGFR1 is expressed in hypertrophic chondrocytes (Delezoide et al., 1998; David M Ornitz, 2005) and FGFR2 is expressed peripherally in the perichondrial cells (Zhang et al., 2006). Furthermore FGFR1 and FGFR2 are expressed in the primary ossification centres and in osteoclasts (Chikazu et al., 2001).

1.1.10 FGFR3 in the Growth Plate

FGFR3 is perhaps the best understood of the FGFRs in the growth plate. It is expressed principally in proliferating chondrocytes, to a lesser extent in prehypertrophic chondrocytes and only small amounts in hypertrophic chondrocytes. FGFR3 expression reduces the size of proliferating and differentiating zones, effectively reducing the rate of growth of the bone in FGFR3 gain-of–function mice (Naski et al., 1996) or conversely in FGFR3 null mice leading to increased zones of both proliferation and hypertrophy (Colvin, Bohne, Harding, McEwen, &
Alternatively it has been suggested that FGFR3 can actually increase the rate of chondrocyte terminal differentiation (Minina, Kreschel, Naski, Ornitz, & Vortkamp, 2002), which is discussed in detail below. Limitations of this particular finding are that the stimulating ligand used in these mouse experiments was FGF2, rather than a directly mutated FGFR3 model, and findings were thus based on the similarities of FGF2 stimulation to a gain-of-function FGFR3 phenotype. It is possible that the effects seen were due to an effect of a different FGFR stimulated by FGF2 or via an alternative pathway such as IHH/PTHrP or BMP. If on the other hand these findings are correct, then taken together this indicates that FGFR3 has a dual effect to limit endochondral bone growth of both reducing the pool of chondrocytes going through proliferation and differentiation as well as reducing the pool of chondrocytes at the other end of the process by increasing the terminal differentiation leading the cells to undergo programmed cell death.

1.1.11 Ligands binding FGFR3 in the growth plate

The ligands that have been implicated in FGFR3 signaling to date are FGF9 and FGF18 (as discussed earlier in the description of mesenchymal condensations). FGF9 and FGF18 are both expressed in the perichondrium, suggesting candidacy for signaling to the growth plate, where the FGFR3 expressing proliferative cells reside (Zhonghao Liu et al., 2002; Ohbayashi et al., 2002). Fgf18−/− mice show a phenotype that is very similar to that of the Fgfr3−/− mouse, although differences exist in that the FGF18−/− mouse also demonstrates delayed ossification and decreased expression of osteogenic markers, which is not seen in reports of the Fgfr3−/− mouse (Zhonghao Liu et al., 2002). Interestingly FGF18 tracks the disparate effects of FGFR3 in the early and late embryonic growth plate, such that in early endochondral ossification FGF18 and FGFR3 stimulate chondrocyte proliferation (Davidson et al., 2005; Iwata et al., 2000), whereas at later stages both seem to switch function and inhibit chondrocyte proliferation and differentiation (Colvin et al., 1996; Deng et al., 1996; Zhonghao Liu et al., 2002).

Fgf9−/− mice also phenocopy mutant Fgfr3 mice with a rhizomelic pattern of skeletal dysplasia, and similar to Fgf18−/− mice also demonstrate osteogenic abnormalities (Hung et al., 2007). It is thought that in-vivo periosteal FGF9 may work in concert with FGF18 and the IHH/PTHrP feedback loop utilizing FGFR3 as an intermediate to regulate growth plate function (Hung et al.,
This mechanism needs further investigation to fully understand the relationship of FGF9 and FGFR3 in growth plate regulation.

1.1.12 Relationship of FGF to IHH signaling

Although there are distinct and direct roles for both FGFR3 signaling and IHH signaling in the maintenance of chondrocyte proliferation in the growth plate, there is some evidence that these two pathways act both independently and dependently to help regulate each other during hypertrophic chondrocyte differentiation. Evidence comes from two significant papers by Chen and Minina who used a series of ACH metatarsal explant or WT limb explant cultures (treated with FGF2 to phenocopy ACH skeletal explants). (L. Chen, Li, Qiao, Xu, & Deng, 2001; Minina et al., 2002) Both groups showed the reduced zone of proliferating chondrocytes and hypertrophic chondrocytes identified previously. What FGF signaling effectively does is shortens the distance over which the IHH/PTHrP loop acts by bringing the IHH producing prehypertrophic chondrocytes nearer the PTHrP producing proliferating chondrocytes. This therefore brings forward the onset of hypertrophic differentiation. Co-treating such explants with PTHrP restores the proliferative growth plate zone and thus delays the onset of chondrocyte differentiation. In addition to other data, these groups concluded that FGF signaling must act upstream to the IHH/PTHrP signaling loop by directly regulating IHH expression (L. Chen et al., 2001; Minina et al., 2002). These papers are important as they offer a different model whereby FGF signaling inhibits endochondral bone growth at the growth plate. Previously it was thought that both proliferation and differentiation were reduced based on changes in the size of both proliferative and hypertrophic zones. Minina suggests, however, that the real effect of FGF signaling is to reduce proliferation, and due to the effect of shortening the chondrocyte zones and thus the IHH/PTHrP loop, to also advance hypertrophic differentiation. However in addition to the advancement of the onset of hypertrophic differentiation the actual process of differentiation is also increased, so that cells undergo apoptosis earlier, thus leading to smaller zones of hypertrophic chondrocytes overall (Minina et al., 2002). Thus the overall net effect is to balance the rate of differentiation top the rate of proliferation. FGF also balances the IHH/PTHrP loop by simultaneously, and directly, down regulating IHH production so that the IHH/PTHrP response is proportionally reduced, with the reduced distance over which its feedback loop acts.
Despite this fascinating insight into the FGFR3/IHH network in mice no abnormalities in IHH or PTHrP expression in growth plates obtained from human fetuses with achondroplasia or thanatophoric dysplasia were identified (Cormier et al., 2002).

1.1.13 BMP signaling in the growth plate

Bone morphogenetic proteins (BMPs) are members of the TGF-β superfamily of growth and differentiation factors which activate heterodimeric receptors with serine/threonine kinase activity (Kronenberg, 2003). BMPs are known to be potent drivers of endochondral ossification, readily forming ectopic endochondral bone when injected subcutaneously in rats (Wozney et al., 1988). As with FGFs, numerous BMPs and their receptors are involved in many aspects of bone development, the full description of which is outside the scope of this thesis. However based on the fact that they are intimately involved with FGFs in skeletal growth and regulation they will be discussed briefly here.

BMP signaling is critical in the formation of mesenchymal condensations, to the extent that when BMP signaling is repressed by noggin, a BMP inhibitor, in chick limbs then the mesenchymal condensations fail to form (Pizette & Niswander, 2000). BMP receptors are subdivided in to BMPR-1A, BMPR-1B, and BMPR-II. Deletion of BMPR-1A and BMPR-1B in chondrogenic cells results in failure of formation of cartilage elements in the embryonic limb, but the reinstatement of either one of these receptors partially rescues the ability to form intact cartilage elements (Yoon et al., 2005), thus the BMPR1 receptors are functionally redundant during early chondrogenesis and in addition it has been found that BMPs are essential for early cartilage development in three main areas, proliferation, cell survival and differentiation and act through SOX9, SOX5 and SOX6 (Yoon et al., 2005; Zou, Wieser, Massagué, & Niswander, 1997). Signaling to the growth plate from the perichondrium are numerous BMP ligands, BMP2, BMP3, BMP4, BMP5, BMP7. Hypertrophic chondrocytes express BMP2 and BMP6, whilst proliferating chondrocytes express BMP7 (Kronenberg, 2003). BMP increases the expression of IHH from prehypertrophic chondrocytes which in addition to it’s own direct mechanisms allows BMP ligands to increase chondrocyte proliferation through the IHH/PTHrP signaling pathway (Minina et al., 2002).
1.1.14 FGFR3 and BMP interactions in the growth plate

BMPs broadly act on the same pathways in the growth plate but interestingly offer antagonistic effects; BMPs increase proliferation, improve cell survival and increase differentiation (Yoon et al., 2005). By utilizing co-treatments of skeletal explant cultures the relationship between FGF and BMP signaling has been investigated, and have shown that both pathways are independent of each other (Minina et al., 2002), although this is both incompletely understood and controversial as Naski suggested a direct relationship demonstrating a downregulation of BMP4 in both growth plate chondrocytes and in the perichondrium by an activating mutation of FGFR3 in growth plate chondrocytes (Naski, Colvin, Coffin, & Ornitz, 1998). It is entirely possible that with the observation of such striking opposing effects on growth plate chondrocytes that the FGF and BMP pathways do in fact converge, but how and where remains to be seen.

1.1.15 Intramembranous ossification

During the development of endochondral bone, and despite the name, there is a role for direct intramembranous bone formation. As described above, an increase in FGFR1 expression corresponds to the development of the zone of intramembranous ossification, called the bone collar, which is formed by the differentiation of cells within the perichondrium to osteoblasts, that then lay down bone, which has numerous important functions (Syftestad et al., 1985).

There are, however, bones that form completely by intramembranous ossification, where no cartilage template is seen, and mesenchymal cells differentiate directly to bone. Examples of intramembranous bones (and their origins) include the bones of the dermatocranium (mesoderm and neural crest), the mandible (neural crest) and the medial ends of the clavicles (neural crest and mesoderm) (Matsuoka et al., 2005). The embryological origins of the cells that make up the intramembranous bones are not actually defined by the mode of ossification, but rather by the attachment of muscles and have varied through evolution (Graham, 2005; Matsuoka et al., 2005).

The intramembranous bones, as with endochondral bones, form from an initial condensation of mesenchymal cells, which can be of neural crest or mesodermal origin often sharing cells of these two sources (Matsuoka et al., 2005). Ossification occurs directly from osteoblasts that have differentiated from these mesenchymal condensations. The bones of the cranial vault
expand during development but do not fuse due to the presence of sutures, which are specialized fibrous joints separating the bony plates (Hall & Miyake, 2000). Osteoblastic differentiation of osteoprogenitor cells occurs mainly at the margins of the condensation at the suture line. The osteoblasts lay down matrix composing of mainly collagen type I. Other extracellular matrix proteins expressed by osteoblasts include osteopontin, bone sialoprotein and osteocalcin. Osteocalcin is the most specific marker of a terminally differentiated osteoblast. Following matrix deposition osteoblasts then mineralize the extracellular matrix to form bone (J E Aubin, 1998).

1.1.16 FGF signaling in intramembranous bone formation

FGFR1 and FGFR2 are well-established regulators of bone formation in the dermatocranium. FGFR1 and FGFR2 are both seen in the entire mesenchymal condensations that will form intramembranous bone, particularly along the sutures, which equates to the ossification front as the cranial bones grow radially (Delezoide et al., 1998). Although FGFR3 is expressed at lower levels it is not seen in the mesenchyme itself, but it can be seen in the osteoblasts at the periphery of the osseous tissue, in the loose periosteum (Delezoide et al., 1998). Early studies of the cranium suggested that there were no significant sutural or cranial defects related to intramembranous ossification due to abnormalities in FGFR3 function (Colvin et al., 1996; Deng et al., 1996), however more recent studies in mice and humans with FGFR3 related skeletal dysplasias showed that premature fusions of the synchondroses were seen as well as abnormally ossified dermatocranial bones suggesting that FGFR3 is indeed involved in intramembranous ossification (Di Rocco et al., 2014).

Whereas in endochondral ossification FGF9 and FGF18 seem to be the critical ligands signaling through the FGFRs, during intramembranous ossification FGF2 and FGF4 are also utilized in addition, being seen in the early mesenchymal stages of craniofacial development.

FGF2 is expressed primarily in the sutural mesenchyme, in contrast to the FGFRs, which are expressed in the ossification front (Rice et al., 2000). FGF2 has been shown to both stimulate (Canalis & Raisz, 1980; Shimoaka et al., 2002) osteoblast proliferation and inhibit differentiation, with the most likely explanation being that the effects of FGF2 are developmentally stage specific. Tang showed how FGF2 added to in-vitro osteoblast cultures increase the proliferative stage whilst inhibiting the nodule development seen in differentiation.
Mansukhani looked in more detail at the effect of FGF2 on osteoblasts at different stages of development and concurred with Tang that in early stages, FGF2 does indeed increase proliferation and decrease differentiation, but noted that when applying FGF2 to more differentiated osteoblasts that no increase in proliferation is seen, rather that these differentiating cells are induced to undergo apoptosis more readily (Mansukhani, Bellosta, Sahni, & Basilico, 2000). In vivo, calvarial osteogenesis is enhanced by FGF2 with increased numbers of osteoblasts (Montero et al., 2000). FGF18 has similar functions to FGF2 in intramembranous ossification. Fgf18 null mice show delayed calvarial suture closure and in vitro decrease osteoblast proliferation and differentiation (Ohbayashi et al., 2002).

### 1.1.17 Transcriptional regulation of intramembranous ossification

Intramembranous bone formation is transcriptionally controlled at different stages by different factors. The four most important are from (upstream to downstream) RUNX2, Osterix (OSX), ATF4 and MSX2 (Franceschi et al., 2009). RUNX2 is expressed in early mesenchymal condensations and is important for both osteoblast and chondrocyte differentiation, and thus defines a common progenitor (Ducy, Zhang, Geoffroy, Ridall, & Karsenty, 1997). Mice deficient in RUNX2 fail to develop cartilage or bony elements to their skeleton and die shortly after birth (Komori et al., 1997). SOX9 downregulates RUNX2 expression, which allows specification of the chondrocyte lineage, whereas unchecked RUNX2 along with the expression of OSX steers the osteochondroprogenitor down the osteoblast lineage. OSX is therefore considered the master regulator of osteoblast differentiation (Nakashima et al., 2002). OSX null mice develop cartilaginous components of their skeleton but which fail to ossify; furthermore RUNX2 is still expressed in the cartilaginous tissues of OSX null mice confirming that OSX is downstream of RUNX2 (Nakashima et al., 2002). ATF4 is downstream of OSX and is involved in expressing genes such as osteocalcin that are critical for terminal differentiation of osteoblasts (Xiangli Yang & Karsenty, 2004). ATF4 is partly dispensable in osteoblast differentiation as in ATF4 absence bone can still form albeit at a reduced rate and volume (Xiangli Yang et al., 2004). MSX2 is also an important transcriptional regulator for the commitment of mesenchymal cells into osteoblasts particularly in the setting of craniofacial bone formation, which is induced by BMPs (Ichida et al., 2004). Deficiencies in MSX2 cause defects in calvarial ossification in both mice and humans, and mice over expressing MSX2 show enhanced growth of calvariae showing and increased number of osteoprogenitor cells (E W Jabs et al., 1993).
1.1.18 Signaling pathways

Numerous pathways downstream of FGF signaling have been identified in skeletal development, and were discussed in detail earlier, however specific to intramembranous ossification, the ERK1/2 MAPK pathway seems to be the most important (Hurley, Marcello, Abreu, & Kessler, 1996; David M Ornitz & Marie, 2002; Shimoaka et al., 2002).
1.2 Fibroblast Growth Factor Receptor 3 Signaling

1.2.1 Introduction

Fibroblast growth factor receptor 3 (FGFR3) is a very important member of the fibroblast growth factor receptor (FGFR) Family. In the last 20 years much has been learned about its structure and function, including its critical importance as the mutated gene of numerous skeletal dysplasias, and also for its role in a diverse group of cancers. Despite such advances in knowledge of this receptor there is much to be learned. It is still incompletely understood as to exactly how FGFR3 signaling pathways activate transcription of target genes, and what are the interactions of FGFR signaling with other signaling pathways, the ligands that most importantly bind to it, and indeed how paracrine signaling activates receptors more than a few cells from the source ligand? We don’t fully understand the role of FGFR3 in normal development, nor its role in skeletal regeneration and repair, or why in the skeleton it inhibits chondrocyte proliferation, whereas in cancer it is pro-mitotic?

Mutations in FGFR3 affecting the skeleton are interesting to study as they are not typically known to cause new functions, rather they exaggerate normal physiologic function which provides a fascinating window into understanding the true physiologic role of FGFR3. The following section aims to summarize the current state of knowledge of the biology of FGFR3.

1.2.2 Structure of FGFRs

Fibroblast growth factors (FGF) signal through a range of four transmembrane tyrosine kinase receptors known as the fibroblast growth factor receptors, FGFR1, FGFR2 (Dionne et al., 1990), FGFR3 (Keegan, Johnson, Williams, & Hayman, 1991) and FGFR4 (Partanen et al., 1991).

Structurally the FGFRs comprise of an extracellular region incorporating three immunoglobulin-like (Ig-like) domains, a transmembrane domain and a split cytoplasmic tyrosine-kinase domain. The four FGFRs demonstrate remarkable homology sharing 55-72% amino acid structure (Johnson & Williams, 1993).

The IG-like II domain, the interloop region and the N-terminus of IG-like III domain are responsible for ligand binding. The C-terminus of the IG-like III domain is responsible for ligand specificity.
1.2.3 Receptor isoforms

The FGF Receptors exist in different isoforms, which vastly increases the complexity and sophistication of the FGF signaling network. The isoforms exist by virtue of alternative splicing, which occurs in the second half of the IG-like III domain leading to b and c isoforms of each of the first three FGFRs. This results in seven receptors in total, FGFR1b, FGFR1c, FGFR2b, FGFR2c, FGFR3b, FGFR3c, FGFR4. The b isoforms generally localized to epithelial tissues, whereas the c isoforms are localized to mesenchymal tissues (Beer et al., 2000; Goldstrohm, Greenleaf, & Garcia-Blanco, 2001; Miki et al., 1992; Orr-Urtreger et al., 1993; Wuechner, Nordqvist, Winterpacht, Zabel, & Schalling, 1996).

It is because of the different isoforms of FGFR3 that we see tissue specific effects of its mutations depending on which isoform is involved. For example in the skeletal dysplasia achondroplasia, skeletal effects are the prominent phenotype when FGFR3 is strongly expressed in brain tissue.

1.2.4 Tissue distribution of FGFR3

FGFR3 has been shown in the mouse to be expressed at various stages during development. During embryogenesis high levels of FGFR3c are seen in the hindbrain, vertebrae and bones of the appendicular skeleton. As embryogenesis continues FGFR3c expression is noted to be more widespread throughout the central nervous system, vertebrae ribs and spinal cord. In contrast FGFR3b expression is extremely sparse in embryogenesis and occurs only in the gastrointestinal tract and urinary tract. The highest levels of FGFR3c are found in the remaining unossified cartilage tissues of the developing bones (Wuechner et al., 1996). Expression profiles of the FGFRs have also been studied in mature adult tissues. The distribution of FGFR3 was found to be much more limited than for FGFR1 and FGFR2. Marked FGFR3 expression was seen in the appendix, colon, liver, sublingual gland, placenta and cervix, with no expression in the stomach. Duodenum, ileum, kidney, ureter and ovary. Musculoskeletal tissues were not examined in this study (Hughes, 1997). In addition FGFR3 has been shown to be expressed in bone marrow, blood and thymus, bone and cartilage.
1.2.5 Fibroblast growth factors

Fibroblast growth factors (FGFs) are structurally related signaling molecules with 18 known mammalian members that are able to differentially bind the four FGFRs. These are FGF1-FGF10 and FGF16-FGF23. The FGFs are further grouped into subfamilies. **FGF1** (FGF1, FGF2), **FGF4** (FGF4, FGF5, FGF6), **FGF7** (FGF3, FGF7, FGF10, FGF22), **FGF8** (FGF8, FGF17, FGF18), **FGF9** (FGF9, FGF16, FGF20), **FGF19** (FGF19, FGF21, FGF23) (Beenken & Mohammadi, 2009). The orphaned ligands FGF11-FGF14 are considered fibroblast homologue factors (FHF) rather than true FGFs as, despite sharing structural features with the FGFs, they do not activate the FGFRs (Olsen et al., 2003) and FGF15 is the mouse orthologue of FGF19. Five FGF subfamilies are act through a paracrine manner (FGF1, FGF4, FGF7, FGF8, FGF9) and one family (FGF19) acts through an endocrine mechanism.

FGFs play important roles in embryogenesis, development and growth as well as key roles in homeostatic and reparative mechanisms in the mature organism. Each ligand can bind multiple receptors and each receptor can be bound by multiple ligands. The FGF ligands require the presence of a cofactor to stably bind the FGFRs and cause them to dimerize, which is typically heparan sulphate (HS) glycosaminoglycan for the five paracrine FGF families or alternatively klotho proteins for the endocrine FGF19 family (Imamura & Mitsui, 1987; Urakawa et al., 2006). In the same way that FGFRs have distinct isoforms that are expressed in different tissues, notably b isoform in epithelial tissues and the c isoform in mesenchymal tissues (Beer et al., 2000; Goldstrohm et al., 2001; Orr-Urtreger et al., 1993; Wuechner et al., 1996), a similar phenomenon is true of FGFs being expressed in a range of different tissues, either epithelial or mesenchymal. Typically ligands produced in epithelium bind to and activate the c-isoform mesenchymal receptors and ligands produced in mesenchymal tissues typically bind and activate b-isoform epithelial receptors (Beenken & Mohammadi, 2009). An exception to this rule is FGF1, which can bind and activate both b and c isoforms of the FGFRs regardless of which tissue produces it (Beenken & Mohammadi, 2009). Each activating ligand-receptor dimer composes of a 2:2:2 FGF-FGFR-HSGAG complex. Each FGF ligand binds both sides of the FGFR equally at the IG2 domain (Schlessinger et al., 2000).
1.2.6 In-vitro FGFR3 specificity of the FGF ligand subfamilies

Ornitz, and subsequently Zhang, undertook detailed in-vitro studies outlining the receptor specificity of the FGF ligand families using engineered BaF3 cell lines, based on a mitogenic readout. It must be accepted that these in-vitro readouts may not accurately reflect that FGF-FGFR specificity in-vivo, due to the multiple other biological systems at play, but nevertheless due to the complexity of FGF signaling, their studies offer insight into the variability of the FGFR specificity and a focus for future research to tease out the true in-vivo relationships.

FGF1 is unique in that it is able to activate all FGFRs, including a particularly strong activation of FGFR3c. FGF2 is also able to activate FGFR3c, but not as strongly as FGFR1c. The FGF8 subfamily (FGF8, FGF17, FGF18) and the FGF9 subfamily (FGF9, FGF16, FGF20) all activate FGFR3c, and have a stronger effect on FGFR3c than the other FGFRs of either isoform. The FGF4 subfamily (FGF4, FGF5, FGF6) also has some FGFR3c activity, but at much lower levels than both FGFR1c and FGFR2c, and the endocrine subfamily FGF19 (FGF19, FGF21, FGF23) have only weak activity on FGFR3c. The FGFR3b isoform in epithelial tissues is only activated by the FGF8 and FGF9 subfamilies, and when activated is at a lower level than FGFR3c activity by the same ligands (D M Ornitz et al., 1996; Zhang et al., 2006). Based in these in-vitro experiments the FGF ligands that are of most interest to the function of FGFR3c signaling are (from strongest activity to weakest) FGF8, FGF17, FGF2, FGF1, FGF9, FGF20, and FGF18.

FGF18 is of particular interest as an FGFR3 ligand because it is expressed in the perichondrium of the mouse, close to the FGFR3-rich proliferating chondrocytes in the growth plate. The Fgf18−/− mouse demonstrates a phenotype with elongated zones of chondrocyte proliferation and differentiation, in the growth plate with a phenotype similar to that of an Fgrf3−/− mouse. However the long bones were noted to be of normal length (Zhonghao Liu et al., 2002). This murine data offers good evidence that FGF18 may be a key ligand for FGFR3, although the expression of FGF18 has not been shown in the perichondrium of humans. Krejci showed that FGF1, FGF2, FGF17 were the predominant ligands expressed in human foetal growth plate cartilage (Krejci, Krakow, Mekikian, & Wilcox, 2007).
1.2.7 How activated FGFR3 effects transcription of target genes

It is thought the FGFR3 signaling mechanism is via regulated intramembrane proteolysis (RIP) (Degnin, Laederich, & Horton, 2011). RIP is a recently discovered phenomenon, which hinges on the concept that transmembrane proteins can be cleaved within the plane of the membrane to liberate cytosolic fragments that enter the nucleus to control gene transcription (Brown, Ye, Rawson, & Goldstein, 2000). RIP is now understood to be a coordinated sequence of molecular events that occurs to a large number of membrane bound proteins, receptors and growth factors to modify their actions. RIP typically occurs by a two-stage process. Firstly ectodomain shedding (S1 cleavage) of an extracellular fragment, followed by S2 cleavage within the transmembrane domain by an intramembrane cleaving protease to release an intracellular fragment (Brown et al., 2000). In FGFR3 signaling ectodomain signaling had already been described following studies of the bovine rib physis. The FGFR3 ectodomain fragment was noted to be in higher concentrations in the extracellular matrix of the hypertrophic zone of the physis suggesting a mechanism for FGFR3 signal regulation be it modifying the FGF ligand availability or acting as decoy ligand or receptor (Pandit, Govindraj, Sasse, Neame, & Hassell, 2002). Building on this work Degnin demonstrated that FGFR3 undergoes RIP. Unique to FGFR3 is that following ligand activation, S1 cleavage occurs through cathepsins rather than metalloproteinases, after the whole receptor is internalized with the ectodomain undergoing endocytosis. S2 cleavage by then follows, by γ-secretase in the transmembrane region, and generates a soluble intracellular domain, which can then translocate to the nucleus as necessary to effect gene transcription (Degnin et al., 2011).

1.2.8 FGFR3 SIGNALING Pathways

1.2.8.1 MAPK/ERK Signaling

There is strong evidence to suggest that the key signaling pathway activated by FGFR3 in skeletally relevant cells is the MAPK/ERK (also known as the Ras-Raf-MEK-ERK) pathway (Krejci et al., 2004; Krejci et al., 2005; Matsushita et al., 2009; S Murakami, Kan, McKeehan, & de Crombrugghe, 2000; Shunichi Murakami et al., 2004; Nowroozi et al., 2005; Yasoda et al., 2004). Once activated FGFR3 phosphorylates the signaling adaptor FRS2, which allows recruitment of GRB2-SOS complexes to the cell membrane (Schulze, Deng, & Mann, 2005; Zarich et al., 2006). The FGFR3-FRS2-GRB2-SOS complex activates membrane bound GTPase
RAS (Marshall, 1996). RAS then activates the protein kinase activity of RAF kinase (Dougherty et al., 2005; Marshall, 1996), which phosphorylates and activates MEK 1/2, which phosphorylates and activates ERK 1/2. ERK then regulates the transcription of genes important in the cell cycle and thus exert an effect on cellular proliferation (Weinberg, 1995).

Murakami generated a mouse constitutively active for MEK1, and reported it to have a phenotype similar to that of achondroplasia (overactive FGFR3). The diminished hypertrophic zone of the long bone growth plate demonstrated a chondrocyte differentiation block, which was associated with excessive SOX9 expression, but no defect in chondrocyte proliferation was seen. Normal chondrocyte differentiation was restored, and the phenotype rescued, after crossing this MEK1 mouse with a mouse deficient in FGFR3 suggesting that the ERK/MAPK pathway was at least in some part responsible for the effect of FGFR3 activation defect in FGFR3 related skeletal dysplasias (Shunichi Murakami et al., 2004). Furthermore the authors also found that in a different mouse model, with over active FGFR3, demonstrating diminished proliferative zones of the cartilaginous growth plate, that STAT1 restored the phenotype, suggesting in that STAT1 might be a mechanism for disrupted chondrocyte proliferation in achondroplasia and related skeletal dysplasias (Shunichi Murakami et al., 2004).

Nowroozi used FGFR3\textsuperscript{K650E} transfected PC12 cells (as they, like chondrocytes undergo a program of proliferation then differentiation) and concurred that activating FGFR3 mutations caused ERK prolonged activation, which pushed chondrocytes into premature hypertrophic differentiation. Conversely they did not find STAT signaling to be implicated in FGFR3 signaling, although this may related to the experimentation with PC12 cells rather than chondrocytes themselves (Nowroozi et al., 2005).

Increased MAPK signaling has also been conclusively shown to account for premature synchondrosis closure of the spine and base of skull. This phenomenon occurs in ACH, TD and other skeletal dysplasias caused by gain-of-function mutations of FGFR3, and has been demonstrated in both pathological human fetal samples and mice with equivalent FGFR3 mutations (Matsushita et al., 2009). Conversely the genetic inactivation of ERK1/2 signaling in chondrocytes delays synchondrosis closure and enlarges the spinal canal (Sebastian et al., 2011).

Synchondrosis closure occurs when the two opposed growth plates (sharing a common zone of resting chondrocytes) are replaced by bone. The proposed model includes the upregulation of
BMP2 and BMP7 with the concomitant downregulation of BMP inhibitors noggin, chordin and
gremlin by mutant chondrocytes, which directly stimulate osteoprogenitors to differentiate to
osteoblasts and form bone. In addition, the excessive MAPK signaling increases VEGF
expression from the mutant chondrocytes, which increases vascular invasion and is also is a key
component of the replacement of cartilaginous growth plates with bone (Matsushita et al., 2009).

It has been shown that for the inhibition of chondrocyte proliferation via the activation of
ERK1/2 signaling a prolonged signal from FGFR3 activation is required (>24 hours) (Krejci et
al., 2004). This is in contrast to the effect of transient signaling of ERK, which encourages the
pro-mitotic effects typically seen in other tissues (Roovers & Assoian, 2000). Foldynova-
Trantikova proposes that the unusual anti-proliferative effects on chondrocytes caused by
mutations of FGFR3 rely on the prolonged activation of the ERK signaling which differentiate
the action of FGFR3 from pro-mitotic effects on other cells where ERK signaling is more briefly
activated (Foldynova-Trantirkova, Wilcox, & Krejci, 2012).

1.2.8.2 Signal Transducer and Activator of Transcription (STAT) signaling

JAK-STAT signaling is an alternative to the second messenger system. A number of ligands
including interferon, interleukin and growth factors activate a transmembrane receptor, which
leads to autophosphorylation of the JAK protein by activation of its kinase function.
Phosphorylated JAK then phosphorylates the STAT protein, which forms homodimers or
heterodimers and translocates to the cell nucleus. Inside the nucleus STAT protein binds to
promoters to effect transcription of target genes (Aaronson & Horvath, 2002).

In 1997 Su showed that the activating FGFR3 mutation in TDII transfected in to chondrocytes
upregulated STAT-1 and caused nuclear translocation of the same (W. C. Su et al., 1997)
suggesting for the first time, in a specific FGFR3-related disease, a pathogenic signaling
mechanism. In 1999 Sahni then demonstrated that the down-regulation of chondrocyte
proliferation by FGF signaling could be explained through an effect on STAT-1 signaling
through their investigations on chondrocyte cell lines (Sahni et al., 1999). Two years later Sahni
demonstrated, in genetically modified mice, a mechanism for the STAT-1 effect, not only was
proliferation reduced in growth plate chondrocytes, but also that apoptosis was increased
throughSTAT-1 (Sahni, Raz, Coffin, Levy, & Basilico, 2001). Legeai-Mallet used human
tissue samples from fetuses with FGFR3-related skeletal dysplasias to further bolster the
argument for the importance of STAT-1 signaling. In contrast with Sahni’s work it was demonstrated that increased amounts of STAT-1, STAT-5 and p21<sup>cip1</sup> proteins were present in differentiating chondrocytes with reduced amounts of STAT proteins in the proliferative zone of the fetal physes. Legeai-Mallet argued that not only was STAT signaling critical in the pathogenesis of FGFR3 chondrocyte abnormalities, but that it had more significant effect on differentiation of chondrocytes rather than proliferation (L Legeai-Mallet, Benoist-Lasselin, Munnich, & Bonaventure, 2004).

Nowroozi looked at the functional importance of both ERK1/2 and STAT signaling, and reported that despite both pathways being upregulated in FGFR3 gain-of-function mutations, the STAT pathway could be knocked down without alteration of mutant phenotype, concluding that ERK1/2 activation was the key regulator of increased transition to hypertrophic differentiation of the growth plate (Nowroozi et al., 2005). Krejci summarized the inherent problems with the hypothesis that increased STAT signaling was the mechanism behind reduced chondrocyte proliferation and differentiation in FGFR3 skeletal dysplasias. Firstly that the phenotype of STAT-1 knockout mice does not resemble that of FGFR3 knockout mice, that ERK1/2 activation can also be considered alternatives, and stronger candidates for induction of p21<sup>waf1</sup> and growth inhibition, and that crossing STAT-1 knockout mice with ACH mice did not rescue the ACH phenotype (Durbin, Hackenmiller, Simon, & Levy, 1996; P Krejci et al., 2008; Krejci et al., 2004; Shunichi Murakami et al., 2004; Nowroozi et al., 2005; Raucci, Laplantine, Mansukhani, & Basilico, 2004). Krejci demonstrated that although STAT-1 interacts with FGFR3 in the cell, FGF did not activate STAT-1 in chondrocytes and moreover that siRNA knockdown of STAT-1 or STAT-3 also did not rescue FGF-mediated growth arrest, whereas similar knockdown of ERK1/2 signaling did (P Krejci et al., 2008).

Krejci went on to compare the ability of six different FGFR3 activating mutations to activate STAT-1, and discovered that only the mutations associated with TDII and SADDAN, the K650E and K650M mutations respectively, caused STAT-1 activation. On the other hand all six different FGFR3 mutations strongly activated ERK1/2 signaling in multiple cellular environments including chondrocytes (Pavel Krejci et al., 2008). It is therefore likely that the disparate results reported in the literature to this point were an artefact of the different mutations studied and the different experimental systems employed. It is therefore possible that
differential STAT-1 activation by different FGFR3 mutations could be one reason for the variation in clinical severity of different FGFR3-related skeletal dysplasias.

Despite a lack of complete understanding of how mutant FGFR3 signaling, it is ERK1/2 signaling that, at this time, is considered the most likely candidate signaling pathway primarily responsible for pathologic chondrocyte proliferation and differentiation in diseases associated with FGFR3 mutation.

1.2.8.3 PI3K/AKT signaling

The phosphatidylinositol-3-kinase (PI3K) signaling pathway is very complex and incompletely understood. It is implicated in FGFR3 signaling but due to the complexities of the pathway and it’s limited role identified so far in skeletal biology it will be discussed only briefly.

IRS1 is bound to the phosphorylated receptor tyrosine kinase following activation of FGFR3. IRS1 serves as a binding and activation site for PI3K. PI3K may also bind directly to a phosphorylated tyrosine kinase. Phosphorylated PI3K then moves to the underside of the cell membrane to bind with PIP2, phosphorylating it to become PIP3K. PIP3K then activates AKT which has numerous downstream effect including the inhibition of apoptosis, the activation of protein translation through mTOR, also AKT can lower the concentration of the tumour suppressor FOXO thereby preventing the inhibitory effects of FOXO on cellular proliferation (Luo, Manning, & Cantley, 2003).

Mice deficient in AKT show a dwarfed phenotype with poor bone formation, hinting at an association with FGFR-related skeletal dysplasias (Peng et al., 2003). It was subsequently shown that AKT is downregulated in chondrocytes following FGF signaling (Krejci et al., 2004; Raucci et al., 2004), which is the opposite effect, of AKT activation, commonly seen in other cell types (Luo et al., 2003). This discrepancy in the proliferative effects attributed to PI3K/AKT signaling may help explain the phenomena that FGF signaling in chondrocytes causes inhibition of proliferation rather than the pro-mitotic effects seen in other cell types that lead to certain cancers. Foldynova-Trantikova has hypothesized that the PI3K pathway actually interacts and competes with the ERK1/2 pathway through the competitive binding of GAB1, which is phosphorylated by FGFR3 (Foldynova-Trantirkova et al., 2012). Compared to study of the
MAPK signaling pathway in FGFR signaling, knowledge concerning the importance of the PI3K pathway is still in its relative infancy and will be the subject of intense future research.

1.2.8.4 Canonical WNT/β-Catenin Signaling

WNT/β-catenin signaling utilizes the inactivation of glycogen synthase kinase 3 (GSK3) to phosphorylate β-catenin for degradation. Furthermore, inactivation of GSK3 is also a critical event in the phosphatidylinositol 3-kinase (PI3K)/AKT pathway, and indeed PI3K may facilitate WNT/β-catenin signaling via this pathway (Dailey, Ambrosetti, Mansukhani, & Basilico, 2005). However ERK/MAPK can also activate WNT/β-catenin signaling but rather than using the GSK3 intermediary, it does so via phosphorylation of WNT co-receptor low density lipoprotein receptor-related protein 6 (LRP6) (Červenka et al., 2011). FGFR3 and other receptor tyrosine kinases have since been shown to activate WNT/β-catenin signaling through this ERK/MAPK mediated phosphorylation of LRP6, independent of the PI3K/AKT pathway (Krejci et al., 2012). In addition to the ERK/LRP6 facilitated activation of WNT/β-catenin signaling, the same authors also reported that FGFR3 (and other RTKs) were capable of direct β-catenin phosphorylation causing a release of β-catenin into the cytoplasm for translocation into the nucleus for transcription of WNT/β-catenin target genes (Krejci et al., 2012). Emerging evidence has just been reported that the interaction of WNT/β-catenin and FGFR signaling is important mechanism in suppressing chondrocyte differentiation (Buchtova et al., 2015).

1.2.9 FGFR3 in Skeletal Dysplasias

1.2.9.1 Achondroplasia

With an incidence of 1 in 15,000 live births, achondroplasia is the most common skeletal dysplasia and by virtue of its prevalence it is also the most studied (Oberklaid, Danks, Jensen, Stace, & Rosshandler, 1979). It is a heterozygous disorder of autosomal dominant inheritance whereby individuals express both wild type and mutant FGFR3 (Rousseau et al., 1994; Shiang et al., 1994). Achondroplasia is phenotypically characterized by disproportionate rhizomelic short stature and a wide array of complications affecting many different organ systems (Oberklaid et al., 1979).
1.2.9.2 FGFR3 mutation causes achondroplasia

The locus of the achondroplasia mutation was identified to be on chromosome 4 (Velinov et al., 1994). Velinov also suspected that FGFR3 was the candidate gene but didn’t have the data to prove it. This assumption was based on work by Thompson suggesting that FGFR3 could have been the gene for Huntingdon’s Disease (HD), as it is expressed in the brain, and located near the HD locus on chromosome 4 (Thompson et al., 1991). Velinov knew that FGFR3 was near the locus for achondroplasia that his team had discovered. In 1993 Peters examined the role of FGFR3 in embryology and organogenesis, and showed its importance in cartilage remains of developing bone and in resting chondrocytes with an expression pattern distinct from FGFR1 and FGFR2 (Peters et al., 1993).

Le Merrer published the same findings simultaneously in the same issue of Nature using gene mapping and linkage analysis to identify the locus of ACH (Le Merrer et al., 1994). They hypothesized that 1 of 4 genes mapping to this locus could be the cause of achondroplasia, that of iduronidase A (IDUA) was discarded as this gene defect was known to cause storage disorders, including Hurlers syndrome, which share few characteristics with achondroplasia. Furthermore these diseases are inherited in an autosomal recessive fashion whereas achondroplasia is inherited in an autosomal dominant fashion. Other genes implicated were the c-GMP phosphodiesterase gene (PDE-B), the zinc finger protein ZNF 141, HOX 7 and FGFR3 (Le Merrer et al., 1994). They considered that FGFR3 was the likely candidate gene for the same reasons as Velinov, because of the study by Peters, showing FGFR3 expression in chondrocytes. Both authors further referenced two earlier papers that showed that basic FGF, in addition to being a mitogen for chondrocytes, also caused inhibition of terminal chondrocyte differentiation (Kato & Iwamoto, 1990), and that reduction of basic FGF is coupled with terminal differentiation of chondrocytes (Iwamoto, Shimazu, Nakashima, Suzuki, & Kato, 1991).

Francomano identified the distal 2.5MB of the short arm of chromosome 4 as being the locus for achondroplasia to further confirm earlier findings. In common with the two groups publishing a few weeks earlier in the month they also used short tandem repeat markers throughout the human genome to identify the region genetically linked to achondroplasia (Francomano et al., 1994). In common with Velinov and Le Merrer, Francomano also suggested that FGFR3 was the
likely gene implicated in achondroplasia. Francomano also hypothesized that the effect of mutant FGFR3 may be dominant negative one, because it was already known that Wolf Hirschhorn Syndrome is associated with a deletion in the short arm of chromosome 4 (Tommerup et al., 1993) which included the achondroplastic locus, yet Wolf Hirschhorn Syndrome does not share any phenotypic similarities with achondroplasia, leading to the conclusion that any FGFR3 mutation in achondroplasia was not merely a deletion, but a mutation with a dominant negative effect. As it turned out this hypothesis was incorrect.

In 1994 two groups simultaneously identified a specific mutation in FGFR3 causing achondroplasia. Both groups used RNA extracted from lymphoblast and fibroblast cultures to reverse transcribe cDNA. Sanger sequencing was used to identify a G380R point mutation, with a transition of glycine to arginine at residue 380 in the transmembrane domain of the tyrosine kinase-coupled transmembrane receptor (Rousseau et al., 1994; Shiang et al., 1994).

1.2.9.3 The mechanism of the FGFR3 point mutation in achondroplasia

It was not known, when the FGFR3 mutation was discovered, exactly how it acted to cause achondroplasia. It was hypothesized that it could be through the prevention of receptor migration through the membrane, the prevention of dimerization or by affecting the tyrosine kinase activity of the receptor (Shiang et al., 1994).

It was Webster in 1996 who subsequently proposed and proved that the G380R point mutation in the transmembrane domain of the FGFR3 receptor lead to constitutively activated receptor dimerization, which lead to high activity of phosphorylation of the tyrosine residues (Webster & Donoghue, 1996). At the current time this mutation is known to account for more than 98% of cases that have been characterized. A remaining 1% of cases are caused by a transversion of cytosine for glycine at the same nucleotide (Superti-Furga et al., 1995). It should be noted that both mutations result in the same glycine to arginine substitution in the FGFR3 gene product.

Further evidence for the mechanism of ligand independent activation of FGFR3 in achondroplasia came from the development of a mouse model with targeted disruption of the FGFR3 gene. These Fgr3/− mice showed a striking phenotype of elongated long bones, (effectively the reverse phenotype of the FGFR3 based skeletal dysplasias, achondroplasia, thanatophoric dysplasia and hypochondroplasia). In-situ hybridization studies in these mice
showed that FGFR3 was expressed in the proliferating zone of chondrocytes in the growth plate, thus leading to the finding that FGFR3 acts as a negative regulator of endochondral bone growth by inhibiting the proliferation of chondrocytes (Deng et al., 1996).

1.2.9.4 FGFR3 Related Skeletal Dysplasias

In addition to achondroplasia (ACH), which was the first skeletal dysplasia associated with an activating mutation in the FGFR3 gene (Shiang et al., 1994), numerous other related skeletal dysplasias have also been shown to be caused by activating mutations in the FGFR3 gene. These are thanatophoric dysplasia (TD), hypochondroplasia (HCH), severe achondroplasia with developmental delay and acanthosis nigricans (SADDAN), platyspondylic lethal skeletal dysplasia San Diego type (PLSD-SD), Muenke syndrome, crouzonodermoskeletal dysplasia.

In addition to the common activating mutations of FGFR3, loss of function mutations of FGFR3 have also been reported to cause skeletal dysplasias but are known to be extremely rare. They include camptodactyly, tall stature and hearing loss (CATSHL) syndrome and an unnamed syndrome described by Makrythanasis in 2014 (Makrythanasis et al., 2014; Toydemir et al., 2006).

FGFR3 was suspected to be harboring the mutations for thanatophoric dysplasia (TD) and hypochondroplasia (HCH) because of phenotypic similarities with achondroplasia.

1.2.9.5 Thanatophoric dysplasia

Thanatophoric dysplasia is the most common neonatal lethal skeletal dysplasia (Maroteaux, Lamy, & Robert, 1967; Orioli, Castilla, & Barbosa-Neto, 1986). Two distinct phenotypes exist which are classified by the shape of the femora (Norman, Rimmer, Landy, & Donnai, 1992). Thanatophoric dysplasia type 1 (TD1) is characterized by cloverleaf skull and short curved femurs, whereas thanatophoric dysplasia type 2 (TD2) is characterized by a cloverleaf skull and relatively longer and straighter femora. TD1 and TD2 are caused by a number of mutations in the FGFR3 gene. TD1 is caused by a number of missense mutations: R248C, S371C (Tavormina, Shiang, et al., 1995) and later S249C (Tavormina, Rimoin, et al., 1995). Rousseau also added Y373C and G370C (Rousseau et al., 1996) as well as the stop codon mutations X807R and X807C. Both Tavormina and Rousseau postulated that the critical element of the missense mutations was not exactly where in the extracellular domain of the receptor the
mutation occurred, but that they all generated a cysteine residue in the extracellular domain of the receptor, causing inter-receptor disulphide bonds which resulted in constitutive activation.

Thanatophoric Dysplasia type II is caused by the K650E in the tyrosine kinase domain of the receptor (Tavormina, Shiang, et al., 1995).

1.2.9.6 Hypochondroplasia

Hypochondroplasia (HCH) is a skeletal dysplasia that resembles achondroplasia but with a more subtle skeletal phenotype (Walker, Murdoch, McKusick, Langer, & Beals, 1971). Not all individuals with HCH have an identifiable mutation, although many that have been characterized have a mutation of FGFR3. The FGFR3 mutation in HCH is typically N540K in the tyrosine kinase domain (Bellus et al., 1995).

1.2.9.7 SADDAN

SADDAN Dysplasia is a rare disease strongly resembling TD in severity but with a distinct phenotype (Tavormina et al., 1999). All identified patients have been shown to harbor the same mutation K650M in the tyrosine kinase domain of FGFR3 (Tavormina et al., 1999; Zankl et al., 2008).

1.2.9.8 PLSD-SD

Originally thought to be a distinct skeletal dysplasia (Horton, Rimoin, Hollister, & Lachman, 1979) all cases of PLSD-SD have since been shown to have the same mutations found in TD1 (Brodie et al., 1999).

1.2.9.9 Muenke syndrome

Described in 1997, Muenke syndrome is a distinct craniosynostosis syndrome characterized by coronal synostosis, macrocephaly, midface hypoplasia and developmental delay with a specific mutation of P250R mutation in FGFR3 (Muenke et al., 1997).

1.2.9.10 Crouzonodermoskeletal dysplasia

Crouzon syndrome had always been associated with an FGFR2 mutation (Gorry et al., 1995; E. W. Jabs et al., 1994; Reardon et al., 1994) until 1996, when three unrelated families with Crouzon syndrome and Acanthosis Nigricans were discovered to harbor novel mutations in the
transmembrane domain of FGFR3, A391E, distinct from the mutations seen in the same region of FGFR3 causing ACH and HCH (Meyers, Orlow, Munro, Przylepa, & Jabs, 1995).

1.2.10 Loss-of-function mutations in FGFR3

1.2.10.1 CATSHL Syndrome

Despite the range and number of individuals identified with FGFR3 gain-of-function mutations causing skeletal dysplasias, there also exist a small and rare subset of known loss of function mutations of FGFR3 also causing skeletal dysplasias. CATSHL syndrome was first described in a family in Utah in 2006. Many of the family members had the phenotype of camptodactyly, tall stature, scoliosis and hearing loss (Toydemir et al., 2006). Using linkage analysis Toydemir localized the abnormality to the tip of the short arm of chromosome 4, and further sequenced the mutation, which is characterized as the missense mutation R261H in the tyrosine kinase domain.

Makrythanasis reported the most recently discovered FGFR3-related skeletal dysplasia, which is also likely to be one with a loss-of-function mutation, as identified by its phenotype of tall stature, scoliosis, curved long bones and hearing loss, similar to CATSHL syndrome, rather than by functional testing of the mutation directly. This Syndrome was described in two brothers of consanguineous birth and has been characterized as a homozygous missense T546K.

{Makrythanasis, 2014 #240}
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Table 1 – Summary of FGFR-related syndromes
1.2.11 Non-skeletal FGFR3 related diseases

It is something of a curiosity as to why FGFR3 has been shown to have inhibitory effects on the proliferation of chondrocytes in cartilage, when in other extraskeletal tissues the effects of FGFR3 mutations are in fact pro-mitogenic. Broadly speaking there are two categories of non-skeletal FGFR3 signaling disorders; skin disorders and cancer. Skin disorders include seborrheic keratosis, epidermal nevi and acanthosis nigricans, whilst the cancers include multiple myeloma, bladder cancer, cervical cancer and seminoma \{Chesi, 2001 #4; Li, 2001 #33; Williams, 2013 #111; Cappellen, 1999 #107; Goriely, 2009 #87; Logié, 2005 #77\}. Activating somatic mutations of FGFR3 have been identified in all of these conditions which correspond to those same mutations that cause skeletal dysplasias, yet in these instances cause excessive cellular proliferation and enhance cell survival. In bladder cancer, for example, activating point mutations in FGFR3 are implicated in 80% of low grade non-invasive and 40% of invasive cancers (di Martino, Tomlinson, & Knowles, 2012). The most common mutations are S249C, Y375C, R248C and G372C, many of which are identical to those mutations seen in TD. It also follows that these mutations, as in the skeletal dysplasias, leave cysteine residues, which form either disulphide bonds or hydrogen bonds leading to ligand independent activation of FGFR3 (Adar, Monsonego-Ornan, David, & Yayon, 2002). The disparate proliferative effects of activating mutations of FGFR3 on different tissues remains a fascinating but as yet an unsolved problem.

1.2.12 Disparate mechanisms of mutant FGFR3 signaling

Different FGFR3-related skeletal dysplasias have markedly different phenotypes, yet their respective mutations all activate the same receptor via dimerization. The difference in phenotype lies in the fact that FGFR3 is mutated at different locations and, based on the location of the mutation, activate FGFR3 using different mechanisms. This subsequently leads to different levels of FGFR3 activation thus accounting for the difference in phenotypes seen. Essentially the effects of FGFR3 signaling are dose-related.

The ACH mutation G380R leads to the formation of hydrogen bonds between the side chains of two arginine residues, which cause ligand independent dimerization and activation (Webster &
The TD1 mutations R248C and Y373C allow the receptor to form covalent disulphide bonds. These occur between free cysteine residues extracellularly between the IG2 and IG3 (R248C) regions or at the transmembrane region (Y373C) respectively (Naski et al., 1996). The intracellular mutations of TD1 (K650M) and TD2 (K650E) occur in the tyrosine kinase domain and they act by mimicking the conformational changes that normally occur in the tyrosine kinase domain that lead to autophosphorylation (Webster & Donoghue, 1996).

FGFR3 mutations do not lead to permanent activation of the receptors; rather they increase the likelihood of activation over levels expected in normal amounts of ligand in normal physiological situations. The different locations of mutation and their different mechanisms of FGFR3 activation determine the extent to which ligand binding is also required for activation, and how much effect ligand binding has on the strength of signal. For example the G380R mutation in ACH typically causes a modest increase in FGFR3 activation, which can occur in a ligand independent fashion, but typically additional ligand binding will further enhance the FGFR3 signal. Alternatively more strongly activating mutations such as those in TD1 (Y373C and 2248C) cause higher levels of FGF3 activation yet still require some facilitation from ligand binding. It is this differential effect on ligand dependent and independent activation of FGFR3 mutations that determines the severity of phenotype in the FGFR3-related skeletal dysplasias (L. Legeai-Mallet, Benoist-Lasselin, Delezoide, Munnich, & Bonaventure, 1998; Monsonego-Ornan, Adar, Feferman, Segev, & Yayon, 2000; Naski et al., 1996; W. C. Su et al., 1997).

1.2.13 Pleiotropic skeletal effects of FGFR3 mutations

Initially it was thought that FGFR1 and FGFR2 were important in bone development of the appendicular skeleton, the axial skeleton as well as craniofacial development. This belief was bolstered by the observation that mutations in FGFR1 and FGFR2 caused skeletal dysplasias such as Jackson-Weiss, Apert, Pfeiffer, and Crouzon syndromes with abnormalities of the axial and appendicular skeleton as well as craniosynostosis, (premature closure of the sutures of the flat bones of the skull) (Gorry et al., 1995; Ibrahimi, Zhang, Eliseenkova, Linhardt, & Mohammadi, 2004; Reardon et al., 1994), yet mutations of FGFR3 had only been identified to cause skeletal dysplasias affecting the appendicular skeleton, with no evidence of craniosynostosis. Seeing as craniosynostosis is likely due to a defect in intramembranous ossification, and long bone development mainly endochondral ossification it lead to the belief
that FGFR3 was only important in development through the mechanism of endochondral ossification. Crouzon syndrome had always been associated with an FGFR2 mutation (Gorry et al., 1995; E. W. Jabs et al., 1994; Reardon et al., 1994) until 1996 when three unrelated families with Crouzon syndrome and acanthosis nigricans were discovered to harbor novel mutations in the transmembrane domain of FGFR3 (A391E), distinct from the mutations seen in the same region of FGFR3 causing ACH, TD and HCH (Meyers et al., 1995). This highlighted the pleiotropic effects of the FGFR genes, and also suggested for the first time that FGFR3 may have a role in craniosynostosis syndromes and therefore perhaps abnormalities intramembranous ossification. Since then Muenke syndrome, another skeletal dysplasia associated with limb anomalies, developmental delay and coronal synostosis has also since been shown to be caused by a P250R mutation in FGFR3 (Muenke et al., 1997).

Another interesting phenomenon reflecting the complexity and nature of FGF signaling is that identical proline to arginine gain-of-function mutations on different FGFRs cause different skeletal dysplasias. The P252A mutation on FGFR1 causes Pfeiffer Syndrome, the P253A mutation on FGFR2 causes Apert syndrome and the P250A mutation on FGFR3 causes Muenke syndrome. Apert syndrome is associated with more severe limb anomalies than either Pfeiffer syndrome or Muenke syndrome. Through biochemical analysis it has been shown that despite the common structural mechanism of gain-of-function mutations, that FGF ligand binding is appreciably different between these three FGFR based syndromes presumably due to the difference in primary sequence of each receptor (Ibrahimi et al., 2004).

1.2.14 The Fgfr3<sup>−/−</sup> mouse

An Fgfr3<sup>−/−</sup> mouse was made by Deng et al by using a targeting construct, pFGFR-3neo, with a phosphoglycerate kinase (PGK) neo insertion used to disrupt the FGFR3 gene. The cassette targeted exon 3, which is part of the IG-II domain and is shared by all the FGFR3 isoforms. Heterozygous embryonic stem cell clones were injected into C57BL/6J blastocysts from which FGFR3 deficient mice were derived (Deng et al., 1996). These mice show a dramatic phenotype of the axial and appendicular skeleton. The tails in the mutant mice were longer than the WT littermates and contained a number of deformities or “kinks”. These deformities were apparent in only 10% of mice just after birth but by three months were demonstrated by all mutant mice. Furthermore the mutant mice developed a significant scoliosis which is reported to have a focus
at both the lower cervical and lower thoracic spine. Interestingly the height of the vertebral bodies was also larger in mutants than the WT littermates. The severity of the scoliosis is reported to account for a significant proportion of neonatal deaths in these mutant mice, presumably due to thoracic insufficiency that can occur with severe spinal deformities. The long bones in the mice showed elongation and bowing. This was most apparent in the femurs, tibias and humeri. As the mice grow the difference between the lengths of long bones in the mutant mice and the WT littermates become gradually more discrepant, suggesting that FGFR3 had an effect on endochondral bone growth. Findings that supported the mechanism of FGFR3 as being a negative regulator of chondrocyte proliferation were identified histologically. In samples of vertebrae and the ends of the long bones, the physes were shown to be longer than WT littermates with apparent increases in the height of the column of hypertrophic chondrocytes. In further analysis, proliferating cell nuclear antigen (PCNA) showed increased proliferation of chondrocytes in the proliferative zone (Deng et al., 1996).

As FGFR3 is known to be expressed in the central nervous system one would expect to see neurological deficit in \( Fgfr3^{-/-} \) mice, yet in Deng’s original analysis of the murine phenotype, no neurological abnormality was reported. Colvin published their groups findings in an \( Fgfr3^{-/-} \) mouse and found that in addition to the skeletal effects reported by Deng that there were significant neurological defects including cochlear defects and deafness which aligned with the knowledge that FGFR3 is expressed in central neurological tissue (Colvin et al., 1996).

1.2.15 Use of the \( Fgfr3^{+/} \) mouse

Mice homozygous for the FGFR3 deletion had a very low post-natal survival rate with most dying within first three weeks, therefore they were unable to be used for experimentation. The heterozygous \( Fgfr3^{+/} \) littermates had a much higher rate of survival. The cause of death of the homozygotes was presumed to be respiratory compromise due to thoracic insufficiency from the severe scoliosis, which was not apparent in the heterozygous mutants.
1.3 Fracture Repair

1.3.1 Introduction

Fifty four percent of all adults in the US are affected by musculoskeletal conditions with the annual cost for treatment and lost wages totaling $874 billion (5.7%GDP) in 2011 (National Health Interview Survey (NHIS) Adult Sample, 2013).

Injuries account for a significant proportion of these musculoskeletal conditions, which also includes arthritis, back pain and osteoporosis. The annual cost of treating musculoskeletal injuries totals $176.1 billion, which includes the treatment of 18.3 million fractures annually. Fifty percent of women and 25% of men will suffer an osteoporosis related fracture with a 20% mortality rate within 12 months of a hip fracture (Bone and Joint Initiative USA, 2015).

Bone fracture healing is a complex reparative process utilizing multiple cell types that is tightly coordinated both spatially and temporarily, yet is incompletely understood. It is most efficient in children, who demonstrate relatively rapid and successful bone healing, but as a person ages healing capacity gradually reduces (along with a greater tendency to fracture), leading to longer time to fracture union (Edwards & Nilsson, 1969; Nilsson & Edwards, 1969). Four to ten percent of all fractures fail to heal, developing established nonunion (Hak et al., 2014; L. A. Mills & A. H. Simpson, 2013; L. A. Mills & A. H. R. W. Simpson, 2013). Many factors may participate in the development of nonunion including age of person, anatomic site of injury, mechanism of injury, presence of infection or systemic disease (Tzioupis & Giannoudis, 2007). Fracture nonunions cause significant morbidity from a biological, social, psychological and economic perspective and often require advanced surgical techniques and biological agents to establish union. Due to the length of time even routine fractures take to repair, and the extended period of rehabilitation needed to regain full function, all fractures would likely benefit from the enhancement of healing with significant benefits to the individual, family and population at large.

Although fracture healing necessarily occurs through a continuum, it can also be thought of as comprising different stages, which include inflammation, intramembranous ossification, endochondral ossification, and remodeling. These stages will be discussed below.
1.3.2 Inflammatory Stage

Following a trauma of such magnitude that a bone is fractured it must also be acknowledged that the surrounding tissues such as periosteum, muscle and blood vessels are also damaged, and it is these tissues that lead directly to the first phase of fracture healing; inflammation.

1.3.2.1 Haematoma

The fractured bone ends and surrounding soft tissues bleed causing a haematoma, which coagulates around the fracture in the shape of the future callus. The haematoma is comprised of cells from local bone marrow as well as blood cells from the peripheral and local circulation. Cells included in the haematoma at various time points include neutrophils, monocytes, macrophages and T-lymphocytes, however B-lymphocytes are not seen in appreciable numbers (Bastian et al., 2011). Removal or debridement of early fracture haematoma in animal models results in reduction of periosteal proliferation, delayed or nonunion of the fracture with a resultant reduction in the strength of fracture repair (Grundnes & Reikeras, 1993; Ozaki, Tsunoda, Kinoshita, & Saura, 2000; S.-H. Park, Silva, Bahk, McKellop, & Lieberman, 2002). Removal of periosteum also results in abnormal fracture healing with reduction in early chondrogenesis within the fracture haematoma (Ozaki et al., 2000). It is quite clear then, that the early stages of fracture repair rely on a complex interaction of injured tissues, cellular accumulation and their specific signaling cascades organized spatially and temporally to adequately heal the fractured bone.

The fracture haematoma is a dynamic entity with a changing cellular and chemical composition over time. In an animal model, haematoma extracted at post-fracture day 4 and injected intramuscularly readily forms endochondral bone, yet haematoma extracted at post-fracture day 2 does not (Mizuno et al., 1990). The importance of the fracture haematoma, and its composition, has become an area of intense investigation of late, as its exact role and mechanism of action is poorly understood.

Within the haematoma a field of inflammatory signals is propagated by the cellular accumulation focused on the point of origin of the fracture (Louis C Gerstenfeld, Cullinane, Barnes, Graves, & Einhorn, 2003). The inflammatory response is maximal at 24 hours after
injury and lasts for approximately 7 days, and is crucial for proper progression of fracture healing.

1.3.2.2 Inflammatory Cells

Neutrophils are the first cells to accumulate in appreciable numbers, within 24 hours after injury. Although neutrophils have a phagocytic function that may assist in prevention of infection, it is likely that their main role is to secrete powerful chemo-attractants CCL2 (MCP-1) and IL-6 (Hurst et al., 2001; Kasama, Strieter, Standiford, Burdick, & Kunkel, 1993) which cause the accumulation of numerous macrophages, maximal at 48-72 hours after injury (Bastian et al., 2011).

There is emerging evidence that macrophages may be a master regulator of fracture healing with numerous roles including the phagocytosis of cellular debris, production of cytokines and growth factors, stimulation of angiogenesis, initiation of cellular migration and control of osteoblast differentiation and thus bone formation (Bastian et al., 2011; Sinder, Pettit, & McCauley, 2015; Vi et al., 2015). Macrophages propagate the inflammatory cascade secreting IL1, IL6 and TNF-α (Ai-Aql, Alagl, Graves, Gerstenfeld, & Einhorn, 2008; Louis C Gerstenfeld et al., 2003; Kon et al., 2001). TNF-α acts through its receptors TNFR1 and TNFR2 to induce a secondary inflammatory signaling cascade at the fracture site and has been shown to induce osteogenic differentiation of MSCs in vitro (Ai-Aql et al., 2008; Louis C Gerstenfeld et al., 2003; Kon et al., 2001; Marsell & Einhorn, 2011). Mice null for TNF-α, show a significant delay in intramembranous and endochondral bone formation (L C Gerstenfeld et al., 2001). IL-1 expression mirrors that of TNF-α both in the inflammatory phase of fracture healing as well as in the later stages of bone formation and remodeling. IL-1 stimulates osteoblasts to produce IL-6, induces callus formation and stimulates angiogenesis (Kon et al., 2001; Marsell & Einhorn, 2011). IL-6 expression is only seen in the inflammatory stages of fracture healing, is expressed by both macrophages and osteoblasts and is also critical in the stimulation of angiogenesis, and the differentiation of mesenchymal cells (Xu Yang et al., 2007). Levels of the cytokines IL-1, IL-6 and TNF-α diminish rapidly after the first few days of injury (Ai-Aql et al., 2008).

Mice ubiquitously depleted of macrophages fail to heal fractures satisfactorily with a reduction in callus size and bone volume and demonstrate an overall slowing of the pace of fracture
healing (Vi et al., 2015). Furthermore conditional depletion of macrophages before fracture, and even 3 or 7 days post-fracture also have deleterious effects in fracture healing, again with reduced callus size and reduced bone formation (Vi et al., 2015). Macrophages had long been thought to be important in the early phases of fracture healing, indirectly proven by the haematoma debridement experiments. The recent work of Vi et al with conditional depletion of macrophages in a mouse model shows that these cells are indeed crucial in the early stages of fracture healing, but furthermore, they also play a significant role in the later stages of fracture healing, evidenced by the fact that mice depleted of macrophages 7 days after fracture also show defects in bone healing. Osteoclasts, like macrophages, also originate from the haematopoietic lineage, and appear in large numbers early in fracture healing. Vi showed that macrophages have an independent role to osteoclasts, despite their similarity in origin, by the fact that they fail to co-localize in fracture callus and that as macrophage numbers relatively reduce as fracture healing progresses, osteoclast numbers increase, reflecting their role in fracture remodeling.

One further emerging concept is that there may be different types of macrophage that have highly specialized roles in bone homeostasis and fracture repair. The factor that distinguishes between these two types of macrophage is based on the expression of an inflammatory marker, mac-2 (Alexander et al., 2011). Macrophages that express mac-2 are described as inflammatory tissue macrophages, and are recruited to the fracture site and act peripherally to take part in endochondral ossification, but not intramembranous ossification (Raggatt et al., 2014). On the other hand mac-2 negative macrophages, termed “OsteoMacs” are shown to localize on the endosteal tissues in close proximity to osteoblasts and engage specifically in the control of intramembranous ossification in fracture repair, and are thought not to play a role in the inflammatory cascade, which I have described in the preceding paragraphs (Alexander et al., 2011; Chang et al., 2008; Raggatt et al., 2014).

Taken together it is clear that macrophages play crucially important roles in many of the stages of fracture healing, that there may be specific subsets of macrophages that have individualized roles in fracture care, and that further studies need to be done to understand the mechanisms whereby the cells of these specialized cells of the haematopoietic lineage coordinate fracture healing.
1.3.2.3 Periosteal response

The injured periosteum has a crucial role in the inflammatory stage of fracture healing. BMP-2 is expressed by periosteum and osteoblasts, which induces the differentiation of progenitor cells into osteoblasts, which stimulates osteogenesis. Furthermore BMP-2 triggers the expression of other downstream members of the BMP subfamily, which are more active at later stages of fracture repair. It appears however that despite such profound effects of stimulating osteoblast differentiation, BMP-2 does not have a role in the recruitment of MSCs to the fracture site (Bais et al., 2009). On the other hand stromal cell-derived factor-1 (SDF-1) is expressed by periosteal cells after injury and has been shown to strongly recruit MSCs to the fracture site to promote endochondral ossification (Kitaori et al., 2009).

1.3.2.4 Molecular regulation

The TGF-β superfamily is a group of structurally related polypeptides that includes the BMPs, GDFs and TGF-β1-3, and are differentially involved throughout fracture repair. During the inflammatory phase BMP-2, GDF-8 and TGF-β1 are the most highly expressed of this large family and peak at day 1 (T.-J. Cho, Gerstenfeld, & Einhorn, 2002). TGF-β1 is expressed by the degranulation of platelets (Bostrom & Asnis, 1998) and is probably involved in cellular proliferation and callus initiation (Ai-Aql et al., 2008). GDF-8 (myostatin) is expressed in the myotome compartment and bone marrow derived MSCs (T.-J. Cho et al., 2002; Hamrick et al., 2007). Myostatin has long been known as a negative regulator of muscle mass, and in mice null for myostatin show greatly increased muscle bulk, but in addition fracture healing is also significantly enhanced (Kellum et al., 2009), which while interesting is difficult to interpret at present in the light of the observation that myostatin expression is increased in the inflammatory stage of fracture healing (T.-J. Cho et al., 2002).

The final group of important factors expressed in the inflammatory stage are those related to the recruitment and activation of osteoclasts and macrophages of the haematopoietic system including receptor activator of nuclear kappa factor β ligand (RANKL), osteoprotegerin (OPG) and macrophage-colony stimulating factor (M-CSF) (Kon et al., 2001).
1.3.3 Intramembranous Ossification

The next stage in fracture healing, which occurs from post fracture day 3 to 10 is that of intramembranous ossification, whereby osteoblasts differentiate directly from osteoprogenitor cells to lay down bone without a prior cartilage template. Intramembranous ossification is a distinct stage which is characterized by not only its timing within the process of fracture repair, but also its localization to the point of injury and its controlling mechanisms. Histologically intramembranous ossification can be seen as the deposition of woven bone in the peripheral periosteal region opposed to the cortex of the fractured bone, which rapidly bridges the fracture gap by the shortest distance (Einhorn, 1998). Intramembranous ossification is also specifically responsible for the repair of bone marrow defects, as has been shown in experimental animal models (L C Gerstenfeld et al., 2001).

Osteogenic progenitors that contribute to intramembranous ossification in fracture repair are activated and mobilized from two distinct sources; firstly, and most importantly the periosteum itself, which is a strong reservoir of osteoprogenitor cells that have the ability to differentiate to osteoblasts or chondrocytes (Céline Colnot, 2009; Christa Maes et al., 2010). The second important reservoir of osteoprogenitors is the bone marrow and endosteum in the vicinity of the fracture. These osteoprogenitor cells are quite distinct from the osteoprogenitors in the periosteum because they only have the ability to differentiate into osteoblasts and not chondrocytes, even when exposed to an environment that supports chondrogenesis in vivo, which has been shown in a series of elegant transplantation experiments (Céline Colnot, 2009). Circulating mesenchymal progenitor cells from distant bone marrow sites have long been supposed to contribute to fracture healing and other tissue repair, and have been used in numerous translational therapies to enhance fracture repair (Tögel & Westenfelder, 2007). However the evidence suggesting that they actually localize and contribute to tissue repair is very weak (C Colnot, Huang, & Helms, 2006; Céline Colnot, 2009; C. Maes, 2013). Although it has been shown that some circulating mesenchymal progenitors may be recruited under the influence of SDF-1 from the periosteum (Kitaori et al., 2009), the importance and role of these particular cells is not yet clear, and current understanding suggests that they may have little importance (Dirckx, Van Hul, & Maes, 2013). As was discussed in detail earlier there is a significant contribution from macrophages to the control of intramembranous ossification,
specifically locally based resident macrophages termed osteomacs, which co-localize with osteoblasts (Alexander et al., 2011).

Although it is seen as a distinct repair process the molecular mechanisms underlying the formation of intramembranous ossification in fracture repair are poorly understood compared to the wealth of knowledge we now have concerning endochondral ossification. Due to the fact that intramembranous bone forms very early in fracture repair it is likely that it is under the control of the regulatory elements outlined in the inflammatory phase including IL-1, IL-6, TNF-α and BMP-2 (Ai-Aql et al., 2008).

Taken together current evidence suggests that the mechanisms for the intramembranous ossification during fracture healing rely on specific local populations of progenitor cells, which have the ability to differentiate to osteoblasts based upon their location, type of injury, mechanical environment and subsequently repair local damage. The signals controlling intramembranous ossification may be by both locally based inflammatory signaling pathways and coordinated by specific local resident and circulating haematopoietic cells such as osteal macrophages in addition to other locally based signaling pathways. How macrophages control osteoblast differentiation is, as yet, unknown but a crucial piece of the puzzle in understanding how fractures heal.

1.3.4 Endochondral Ossification

Endochondral ossification is a critical process for regenerating new bone during fracture repair. The central principle is that a cartilaginous template, called callus, is formed from the proliferation and hypertrophy of chondrocytes, which then undergoes resorption and replacement with bone from invading osteoblasts coupled with blood vessel invasion.

1.3.4.1 Cartilaginous Callus formation and mineralization

The first stage of endochondral ossification occurs with the differentiation of mesenchymal progenitors to proliferating chondrocytes. As was discussed previously endosteal progenitor cells do not have the ability to differentiate to chondrocytes (Céline Colnot, 2009), and circulating mesenchymal progenitors are rare and not though to contribute significantly to the reparative process (Dirckx et al., 2013). The main repository for mesenchymal progenitors that differentiate to chondrocytes is therefore thought to be the periosteum (Céline Colnot, 2009).
Following proliferation, these chondrocytes then differentiate to hypertrophic chondrocytes. The chondrocytes form a cartilaginous soft callus consisting of type II collagen and proteoglycan. (Einhorn, 1998). This occurs only in the extra-osseous, and indeed extra-periosteal, area of the fracture. This cartilaginous callus bridges the fracture gap and sits upon the buttresses of “hard” callus, formed simultaneously by intramembranous ossification (Hadjiarisyrou & Oapos;Keefe, 2014). The cartilaginous callus is then mineralized, which is choreographed by the hypertrophic chondrocytes. Mitochondria accumulate calcium-based granules, which are then packaged within matrix vesicles in the cytoplasm of the hypertrophic chondrocytes. These matrix vesicles then bud from the hypertrophic chondrocytes into the extracellular matrix releasing their calcium contents and proteolytic enzymes to make way for mineralization. Of particular importance is the release of phosphatases, which degrade phosphodiesters in the callus matrix to mobilize phosphate ions, which then precipitate with the calcium to form apatite crystals and thus mineralize the callus (Einhorn, 1998; Ketenjian & Arsenis, 1975; Marsell & Einhorn, 2011). Callus mineralization is merely a short-lived transitional phase and acts a cue for true endochondral ossification. This newly calcified cartilage callus proceeds to stimulate its own resorption and replacement with woven bone via a second wave inflammatory cascade characterized by elevated levels of M-CSF, RANKL and OPG. Elevated levels of TNF-α are also seen but towards the end of this resorptive phase. The rise of TNF-α acts to recruit mesenchymal progenitor cells to the callus in addition to further stimulating hypertrophic chondrocytes to undergo apoptosis, indeed in mice deficient for TNF-α a significant delay in endochondral cartilage resorption is seen (L C Gerstenfeld et al., 2003). At the end of this phase all of the mineralized cartilaginous callus has been replaced by a large bridge of woven bone, spanning the fracture site and is blended with the periosteal intramembranous bone formation that occurred at an earlier stage.

1.3.4.2 Signaling pathways

The molecular signature of this stage of fracture healing includes some of the factors previously activated during the inflammatory phase such as BMP-2 and OPG (Ai-Aql et al., 2008). BMP-2 is considered the critical BMP (and indeed molecule) for the initiation of fracture repair, which stimulates both intramembranous ossification and callus formation of endochondral ossification. It has been shown that complete absence of BMP-2 in a conditional knock-out mouse model, is associated with absolutely no signs of fracture healing, despite corresponding normal levels of
all other important modulators of early fracture repair, including BMP-4 and BMP-7, IL-1 and RANKL (Tsuji et al., 2006). Interestingly the conditional knockout of endogenous expression of BMP-2 in osteoblasts seems to have no effect on fracture healing yet conditional knockout of BMP-2 in chondrocytes delays endochondral ossification by slowing the differentiation of hypertrophic chondrocytes, and thus prolonging the cartilage phase of endochondral ossification (Mi et al., 2013). These data suggests that BMP-2 has different spatial and temporal roles in fracture repair, which requires further investigation.

During chondrocyte proliferation large number of other members of the TGF-β superfamily also show a dramatic increase in expression including BMP-4, BMP-5, BMP-6, TGF-β2, TGF-β3 and GDF-5. Angiogenic factors are also on the rise with increases in vascular endothelial growth factor D (VEGF-D) ad angiopoietin-1 (Ai-Aql et al., 2008).

### 1.3.4.3 Vascular response

For osteoblasts and osteoclasts to invade and replace the calcified cartilage callus with bone a network of blood vessels are required to deliver them to the site of action. During fracture healing there is thus a considerable vascular response, which accompanies both intramembranous and endochondral ossification. The growth of new blood vessels into the callus requires some space for them in which to grow, necessitating a coordinated resorption of the mineralized callus. Blood vessels are derived by two mechanisms; firstly by angiogenesis, where new blood vessels extend from existing blood vessels surrounding the fracture, particularly from the periosteum, alternatively blood vessels may be generated via a process called vasculogenesis whereby vessels are established de-novo from the differentiation of endothelial mesenchymal cells and grow in a vascular plexus (Lehmann et al., 2005; Marsell & Einhorn, 2011; Suri et al., 1996). The new blood vessels, however derived, invade the avascular cartilage callus from the proximal and distal ends carrying with them the osteoblasts and osteoclasts necessary for cartilage resorption and new bone formation. The vascular response is under the control of two main angiogenic pathways, an angiopoietin-dependent pathway and vascular endothelial growth factor (VEGF) – dependent pathway (Lehmann et al., 2005; Marsell & Einhorn, 2011; Suri et al., 1996). Both hypertrophic chondrocytes and osteoblasts are potent sources of VEGF (Deckers et al., 2002; Yeh & Lee, 1999). Following the calcification of cartilage callus, the subsequent apoptosis of hypertrophic chondrocytes is thus a critical stage,
which allows the release of large amount of proteolytic enzymes to degrade the callus and the release of VEGF to drive new vessel growth into this zone. Seeing as TNF-α is a key regulator of hypertrophic chondrocyte apoptosis, it has been shown that the expression of angiopoietin and VEGF are part of a tightly regulated system coordinated by the inflammatory cytokine TNF-α (Céline Colnot, Thompson, Miclau, Werb, & Helms, 2003; Louis C Gerstenfeld et al., 2003; Lehmann et al., 2005). The second main source of VEGF is from osteoblasts. The expression of VEGF from osteoblasts is greatly increased by the action of BMPs (Yeh & Lee, 1999), which also see a massive rise during the callus mineralization and resorption phase of fracture repair (Tsuji et al., 2006). It can thus be considered that osteoblasts along with hypertrophic chondrocytes are key regulators of the vascular response (Louis C Gerstenfeld et al., 2003).

1.3.4.4 Origin of osteoblasts for bone repair

Following osteoclast mediated resorption of the calcified callus, osteoblasts are required to lay down the new woven bone in its place and indeed numerous osteoblasts are seen in the callus after mineralized callus resorption. Lineage tracing studies show that the origin of these osteoblasts is from the periosteum in the region of the fractured bone, rather than from the peripheral tissues or circulation. Furthermore these migratory cells that have travelled from the periosteum are in fact osteoblast precursors and not mature osteoblasts. The osteoblast precursors find their way to the callus via an elegant intimate co-localization to new invading blood vessels in a pericyte-like fashion (Christa Maes et al., 2010). Despite this intuitive model of the origin of osteoblasts within the healing fracture callus, others have disputed these findings and have provided alternative hypotheses as to the origin of osteoblasts in the healing fracture callus. Zhou and Yang have both shown in independent studies using lineage-tracing techniques in mice that hypertrophic chondrocytes can in fact transdifferentiate into osteoblasts. Both groups determined that these findings held true for bone development as well as bone repair, and Zhou has suggested that in bone development as many as 60% of trabecular osteoblasts originate from chondrocytes (L. Yang et al., 2014; Zhou et al., 2014).

1.3.5 Remodeling

The bridge of hard callus that spans the fracture site is not the end of bony healing despite its significant improvement in mechanical stability. The haphazard woven bone must now be
replaced through a process of remodeling to return the bone to its pre-injury state, where it consists of layers of lamellar bone with its haversian canals, and a central marrow filled medullary canal. The aim is to recreate a bone that can withstand the full range of physiological forces. The remodeling phase takes a relatively long time to occur compared to all other phases of fracture healing and is characterized by the intricate and quite remarkable ability of osteoclasts and osteoblasts to work together in a tightly coupled process of bone resorption and formation modulated by receptor activator of nuclear factor-κβ (RANK), RANK ligand (RANKL) and osteoprotegerin (OPG) (Boyle, Simonet, & Lacey, 2003).

1.3.5.1 Bone remodeling unit

To action normal remodeling osteoclasts and osteoblasts must work together in a coupled fashion, and indeed are often referred to as bone remodeling units. Osteoclasts are tissue specific multinucleated macrophages of haematopoietic origin, and they define remodeling in skeletal development, homeostasis and fracture repair. Osteoclasts, once activated, resorb bone by approaching and attaching to a bone surface, forming a tight seal at its edge through specialized areas called podosomes, attaching to bone matrix using integrins. This essentially forms an extracellular compartment within which localized bone resorption occurs. The osteoclast cellular membrane within the sealed zone reorganizes itself by developing a ruffled border, which massively increases its active surface area. Then the localized environment is acidified by the release of hydrogen ions, through the action of carbonic anhydrase, to degrade the mineral elements of bone. Demineralization then allows the second wave of bone resorption by exposing the organic bone matrix, which is enzymatically digested by numerous proteases including tartrate resistant acid phosphatase (TRAP), cathepsin K and matrix metalloproteinases (MMPs). The osteoclast processes the breakdown products of bone within its cytoplasm and then releases these into the surrounding tissues. The resulting depressions caused by resorption at the bone surface are called Howship’s Lacunae and serve as the point for osteoblasts to migrate and lay down new bone (Boyle et al., 2003).

1.3.5.2 RANK/RANKL/OPG Axis

The osteoclast/osteoblast functional relationship occurs through the RANK/RANKL/OPG axis. RANK is a transmembrane signaling receptor on the surface of osteoclasts that when activated by RANKL stimulates osteoclasts to become active and resorb bone (J. Li et al., 2000). RANKL
is a surface bound soluble polypeptide that is expressed on the surface of osteoblasts and proteolytically released, and thus forms the basis of the tightly coupled relationship between these two cells (Boyle et al., 2003; Wong et al., 1997). Once osteoclasts have resorbed an area of bone within the Howship’s lacunae then osteoblasts secrete an organic matrix consisting primarily of collagen type 1 (Col1), and other extracellular matrix proteins, followed by the mineralization of this matrix with the precipitation of calcium and phosphate in the form of hydroxyapatite crystals (Einhorn, 1998). Osteoprotegerin (OPG) is a circulating protein also expressed by osteoblasts, which acts as a decoy receptor. It inhibits the activation of osteoclasts by binding RANKL, leaving decreased ligand to bind to RANK, thus inhibiting osteoclast activation (Udagawa et al., 2000).

1.3.5.3 Matrix Metalloproteinases

This second period of resorption is quite distinct from the initial period of calcified cartilage resorption in that it has a different molecular signature. No longer are M-CSF, RANKL and OPG directing the process, but rather II-1 and IL-6 are the key cytokines that are once again elevated along with continued expression of TNF-α and BMP-2 (Louis C Gerstenfeld et al., 2003). Other BMPs are diminished during remodeling, as is the vascular response driven by VEGF (Ai-Aql et al., 2008). Angiogenesis, still essential for bringing reparative cells to the fracture site and to restore normal lamellar anatomy, does still occur, but may be under the more specific control of angiopoietin. TNF-α retains its coordinating role during fracture remodeling by controlling the expression of the matrix metalloproteinases (MMPs) and further vascular response. TNF-α null mice that have undergone fracture studies show dramatically inhibited fracture remodeling with reduced expression of MMPs (Lehmann et al., 2005). MMPs secreted by osteoblasts and osteoclasts are critical regulators of bone remodeling. Over 20 MMPs have been identified but the key players in remodeling are thought to be MMP-13, MMP-9, and MMP-2 (Ortega, Behonick, & Werb, 2004). MMP-13 is a potent collagenase expressed by hypertrophic chondrocytes and osteoblasts, which are co-localized with tartrate resistant acid phosphatase (TRAP) secreting osteoclasts, and serves to degrade the organic matrix of woven bone. MMP-13 is able to degrade nearly all collagen types including Col1, Col2, Col3 and Col10. MMP-13 null mice show increases in trabecular bone during development and fail to remodel fractures appropriately (S. Y. Tang, Herber, Ho, & Alliston, 2012). MMP-9 is a gelatinase secreted by
osteoclasts that degrades Col4 and Col5 as well as gelatin in the matrix of bone. The role of MMP-9 may be more profound than that of MMP-13, in that it has been shown to not only control remodeling directly by the degradation of matrix, but also act to recruit haematopoietic cells, and mesenchymal cells to the fracture site (Heissig et al., 2002). MMP-9 also acts to direct vascular invasion of callus through it’s action on VEGF and may even act as a fate switch to direct mesenchymal progenitor cells from the periosteum to differentiate to chondrocytes or osteoblasts and thus the balance of intramembranous and endochondral bone directly (Céline Colnot et al., 2003; Ortega et al., 2004). MMP-2 is expressed by osteoblasts and has a much more specific role in fracture remodeling in that it only facilitates bone remodeling and not resorption of calcified cartilage (Lieu et al., 2011; Mosig & Martignetti, 2013). MMP-2 null mice show normal levels of the other critical MMPs in bone remodeling (MMP-13 and MMP-9), and has no effect on osteoclast recruitment nor vascular invasion (Lieu et al., 2011).

1.3.6 FGF and FGFR3 signaling in fracture repair

1.3.6.1 FGF gene expression

Considering that fracture repair recapitulates many of the developmental processes in skeletal formation it is no surprise that the FGF genes play a key role in fracture repair and are implicated in all stages from the initiation of fracture healing through to the latter stages of bone remodeling. It is interesting to note that while numerous FGFs have been shown to be expressed during fracture healing exact temporospatial relationships have yet to be determined due to variability in animal models, surgical technique, modalities of measurement (Bolander, 1992; Bourque, Gross, & Hall, 1993; A. Nakajima et al., 2001; Arata Nakajima, Shimizu, Moriya, & Yamazaki, 2003; Rundle et al., 2002; Schmid, Kobayashi, Sandell, & Ornitz, 2009; Song, Hutmacher, Nurcombe, & Cool, 2006). As fracture repair is typically divided up into an initial inflammatory response, endochondral ossification and then remodeling phase, it follows that describing the expression of the FGF genes in terms of early, mid and late expression is appropriate (Schmid et al., 2009). During the early inflammatory phase, from post fracture day (PFD) 1 onwards, elevations in expression of FGF1, FGF2, FGF5, FGF6 and FGF9 are seen, which corresponds with the findings of other groups (Bourque et al., 1993; Rundle et al., 2002). Interestingly Schmid comments that elevations in FGF1, FGF2, FGF6 and FGF9 at this early
stage are thought to be due to experimental artefact and non-significant, thus only FGF5 seemed to have a true early increase in expression secondary to fracture (Schmid et al., 2009). During the mid-phase of endochondral ossification FGF16 and FGF18 have been shown to be elevated (Zhonghao Liu et al., 2002; Rundle et al., 2002; Schmid et al., 2009). FGF2, FGF5 and FGF6 are also raised at PFD9, and, at this time, are significantly elevated over controls (Schmid et al., 2009). During the later remodeling phase significant increases in expression was seen for FGF1 and FGF17 (Rundle et al., 2002; Schmid et al., 2009). In addition FGF2, FGF9 FGF16 and FGF18 remained elevated although these levels were seen to be declining from a peak at an earlier time point (Rundle et al., 2002; Schmid et al., 2009).

1.3.6.2 FGFR gene expression

FGFR1, FGFR2 and FGFR3 are all expressed in fracture repair, but FGFR4 is not thought to be involved (Rundle et al., 2002; Schmid et al., 2009). FGFR1 is expressed in periosteum throughout fracture healing, then subsequently expressed in chondrocytes and osteoblasts at PFD7 and finally throughout the later stages of healing, suggesting its involvement with intramembranous and endochondral ossification in a rat model of fracture repair (Rundle et al., 2002). Schmid found, however, that in a mouse tibia fracture model FGFR1 has been shown to be elevated from day 1 in fracture repair but only diverges from control values later in fracture repair at PFD9 when expression continues to rise to a peak at PFD21. FGFR2 expression follows FGFR1 expression closely, albeit more strongly in chondrocytes than osteoblasts when compared to FGFR1. Temporally FGFR2 also expression rises immediately after fracture, but does not significantly exceed control values until PFD9, with a peak at PFD21 (Rundle et al., 2002).

FGFR3 expression rises more slowly than FGFR1 and FGFR2 after fracture, and in contradistinction to FGFR1 and FGFR2 peaks at PFD9, before reducing in expression as fracture healing enters the remodeling stage (Schmid et al., 2009). The rise in expression of FGFR3 at PFD9 corresponds to the proliferation and differentiation of chondrocytes from which it has been shown to be maximally expressed (Rundle et al., 2002). Although most authors describe the importance of FGFR3 in chondrocyte proliferation and differentiation (Arata Nakajima et al., 2003), Rundle also described the expression of FGFR3 from osteoblasts in the healing callus suggesting a potential role for FGFR3 in intramembranous as well as
endochondral ossification (Rundle et al., 2002). Nakajima on the other hand was not able to show the expression of FGFR3 in osteoblasts (Arata Nakajima et al., 2003). Despite the variation in reporting of the expression in both timing and location of the FGF and FGFR genes it is quite clear that they are crucially involved in the fracture healing process and more detailed study is required to support or refute their respective roles.

1.3.6.3 FGFR3 mutations and fracture repair

A single study exists exploring the effects of FGFR3 mutations on fracture repair. Su showed that in a tibial fracture in a mouse with a gain-of-function mutation of FGFR3, that fracture healing was delayed, with more cartilage being visible on histomorphometric analysis in the fracture callus at later stages of healing. This finding was thought to be due to a delay in the differentiation of chondrocytes as they progress through the stages of differentiation (N. Su et al., 2008). No mention is made of the effect of this mutation on osteogenesis by either endochondral or intramembranous pathways nor its mechanism of action. Due to these preliminary findings further investigation into the exact role of FGFR3 in fracture healing is warranted to identify how FGFR3 affects the initial inflammatory and proliferative response after fracture, how FGFR3 may affect both intramembranous and endochondral ossification due to the fact that FGFR3 has been shown to be expressed in both chondrocytes and osteoblasts and by which mechanism it exerts such effects.
1.4 Distraction Osteogenesis

1.4.1 Introduction

Distraction osteogenesis is a regenerative phenomenon whereby a bone is initially divided in two and stabilized in a mechanical device, such as an external fixator, following which the bone ends are then distracted at a specific rate and rhythm, which causes regenerate bone to form in the enlarging distraction gap, thereby lengthening the limb segment (Ilizarov, 1990). The new regenerate bone gradually consolidates over many weeks until it has enough structural integrity that the stabilizing mechanical device can be removed. The new bone at this stage is considered to be able to withstand physiologic forces but still improves its physical properties via a process of remodeling. Distraction osteogenesis is a distinct biological process that differs from fracture healing, mainly because it occurs primarily through a process of intramembranous ossification, rather than endochondral ossification (J Aronson, Harrison, Stewart, & Harp, 1989). Distraction osteogenesis is most often used surgically to lengthen abnormally short limb segments in individuals with congenital limb deficiencies, skeletal dysplasias such as achondroplasia, or post traumatic limb deformities (Ilizarov, 1990). Three distinct biological phases of distraction osteogenesis; latency, distraction and consolidation are detailed below (J Aronson et al., 1989; Ilizarov, 1990).

1.4.2 Surgical preparation

The bone is completely divided in a low-energy fashion to prevent thermal necrosis of the cut bone ends. Typically this involves the use of a low speed drill and osteotome (bone chisel) rather than an oscillating saw. A mechanical device such as a monolateral or circular external frame is applied to the bone (limb) segment in question to stabilize both of the bone ends, which at this time are still in direct contact with each other.

1.4.3 Latency

Following the surgical procedure a period of latency is necessary, where no distraction of the bone ends is allowed. The purpose of latency is for the initial inflammatory response to reduce and for osteoprogenitors to become primed to form bone in the distraction gap, when distraction of the bone ends is finally allowed to occur (Ai-Aql et al., 2008; Hamdy, 2014). The inflammatory response occurs secondary to surgical division of the bone, similar to that which
occurs following a traumatic fracture. At this early stage there is an accumulation of inflammatory cells derived from the haematopoietic lineages, as well as proliferation of cells within the periosteum (J Aronson et al., 1989) (Bouletreau et al., 2002). Some early chondrocyte differentiation and cartilage formation is seen at the extraperiosteal edges of the osteotomy, which if left undistracted would progress through the stages of endochondral ossification, much as in fracture repair. These small areas of cartilage formation are soon to disappear once distraction commences (J Aronson, Good, Stewart, Harrison, & Harp, 1990; J Aronson et al., 1989; J Aronson & Shen, 1994).

1.4.3.1 Molecular regulation of latency

A rapid elevation in the expression of inflammatory markers IL-1 and IL-6 from haematopoietic cells and osteoblasts occurs, yet in contrast to fracture repair no early increase in TNF-α has been demonstrated (T. J. Cho et al., 2007). IL-6 inhibits the proliferation of mesenchymal progenitor cells in-vitro but enhances osteoblastic differentiation (T. J. Cho et al., 2007). RANKL and OPG are elevated, appearing at the end of latency. This elevation follows that of IL-1 and IL-6, which are known to stimulate the expression of RANKL and OPG (Pérez-Sayáns, Somoza-Martín, Barros-Angueira, Rey, & García-García, 2010). BMP-2 and BMP-4 are also elevated in the periosteum during the latency phase, and they appear at approximately day 4 after osteotomy (Sato et al., 1999). Seeing as BMP-2 and BMP-4 are critical regulators of the initiation of bone healing and of osteoblast differentiation, this relationship helps to explain the importance of the latency phase, in that it allows for the upregulation of BMP-2 and BMP-4, which will help stimulate rapid osteoblast differentiation in the distraction gap. TGF-β1 is also expressed at the end of latency and localizes to the inflammatory cells at the site of bone injury. TGF-β1 then remains elevated throughout distraction. The role of TGF-β1 is to promote collagen production and upregulate other extracellular matrix proteins whilst inhibiting MMPs that would otherwise degrade the newly formed matrix (Bouletreau et al., 2002) (Z Liu, Luyten, Lammens, & Dequeker, 1999).

1.4.4 Distraction

During the distraction phase the bone ends are transported away from each other using the preassembled mechanical device, under highly controlled conditions. Of critical importance are the rate and rhythm of distraction. If the bone ends are distracted too quickly only fibrous tissue
will form in the gap, whereas if the bone ends are distracted too slowly the bone ends will heal together, which is termed a premature consolidation. The principle of the optimum rate and rhythm is to ensure that a consistent strain is applied to the cells within the distraction gap. Theoretically, continuous distraction is ideal, however due to the limits and practicalities of a manual mechanical fixator construct, four episodes of distraction per day are considered appropriate for excellent bone regeneration. The distraction rate varies by animal, type of bone and age of the subject. The typical distraction rate is 1mm per day, and has been shown to be suitable in both dogs and humans to form excellent bone regenerate. In very young children, occasionally a rate of 0.75mm per day is appropriate (Ilizarov, 1990).

1.4.4.1 Histology of distraction

As the bone ends separate the distraction gap comprises of three histologically distinct zones of tissue. These three zones all use a series of parallel bundles of collagen fibres as a template, which attach to and span the gap between both cut bone ends. These collagen fibres mainly comprise collagen type 1 fibres (Vauhkonen, Peltonen, Karaharju, Aalto, & Alitalo, 1990). The zone closest to the cut bone ends is the zone of microcolumn formation (MCF), where osteoblast differentiation and mineralization of the collagen fibres has occurred leaving bone columns that taper towards the central area of the distraction gap. The central area is known as the fibrous interzone (FIZ) which is composed of fibroblasts, osteoblasts, chondrocytes-like cells and oval cells (J Aronson, 1994; J Aronson et al., 1990; J Aronson et al., 1989; J Aronson & Shen, 1994; Vauhkonen et al., 1990). Between the bone columns in the MCF are longitudinal vascular channels, but these channels do not cross the FIZ. The third zone is a watershed between the MCF and FIZ where a high concentration of proliferating cells reside and is known as the primary mineralization front (PMF) and it is here that a large number of osteoblasts mineralize the collagen fibres of the FIZ (Ai-Aql et al., 2008; J Aronson et al., 1990).

1.4.4.2 Molecular regulation of distraction

The molecular control of the distraction phase sees to the rapid and elaborate formation of new regenerate intramembranous bone and a key player in this process are the BMPs. BMP-2, BMP-4 and BMP-6 are massively upregulated throughout distraction (Ai-Aql et al., 2008; G. Li, Berven, Simpson, & Triffitt, 1998; Sato et al., 1999). Due to the drop in BMP expression when distraction ceases it is thought that strain imparted on the osteoblastic cells in the distraction gap
through mechanotransduction is the primary driving force in BMP upregulation. Numerous publications have identified the sources of BMP-2 expression to be mesenchymal cells such as osteoblasts and chondrocytes, but Matsubara has identified that endothelial cells and vascular smooth muscle cells in the distraction gap and in the surrounding soft tissues express BMP-2, leading to the conclusion that vascular cells and osteoblasts are intimately coupled in the distraction osteogenesis process, and that BMP-2 may be a key player in the cross-talk between angiogenesis and osteogenesis in bone repair (Matsubara et al., 2012). An additional finding of Matsubara was that shortly after a peak in BMP-2 expression there was also a peak in two Wnt antagonists, sclerostin and DKK. Matsubara hypothesized that the purpose of the Wnt antagonist expression was to inhibit premature mesenchymal terminal differentiation and mineralization, because with unchecked BMP-2 expression stimulating MSC differentiation, premature calcification may occur which may prevent blood vessels from growing through the regenerating tissues. In this regard BMP-2 may also have control of not only vasculogenesis but also on Wnt activity (Matsubara et al., 2012).

As with fracture repair a strong vascular response is required for the regeneration of new bone. VEGF-A, VEGF-D, neuropilin, angiopoietin-1 and angiopoietin-2 are all upregulated in distraction osteogenesis and are maximal throughout the distraction phase. The vascular response is seen to localize to the distraction gap, particularly in the MCF and the PMF, rather than the FIZ, and further more also localize to the maximal areas of BMP-2 expression suggesting a paracrine effect of linking vasculogenesis to osteogenesis (Ai-Aql et al., 2008; Matsubara et al., 2012; Pacicca et al., 2003).

RANKL and OPG expression is also seen during distraction as in the latency phase, but is thought to correspond to the need to resorb the small amounts of mineralized cartilage that form alongside the ends of the cut bone in latency, and areas of cartilage formation in the distraction gap where small areas of endochondral ossification occur (Ai-Aql et al., 2008; Pérez-Sayáns et al., 2010; Zhu, Wang, Wang, & Wang, 2007).

1.4.5 Consolidation

Once the bone has been distracted to the desired length a period of consolidation is required. The bone ends are held firmly within the mechanical fixator device. Over time the regenerate bone gains structural integrity, the progress of which is typically monitored by serial
radiographs. Consolidation is said to be complete when the density of the regenerate bone approaches that of the native bone, and corticalization of the regenerate bone can also be seen on radiographs. The period of consolidation is the longest of the three phases of distraction osteogenesis, typically accounting for two thirds of the total time for tissue regeneration. At the end of consolidation the mechanical fixator is removed and the limb returned to physiologic function, such as weight bearing and ambulation in the setting of lower limb lengthening (Ilizarov, 1990).

1.4.5.1 Molecular regulation of consolidation

A significant drop in BMP expression is seen in the consolidation phase, as the rapid period of bone formation is complete, although elevated levels of BMP-2 and BMP-4 are still seen compared to control tissues (Sato et al., 1999). RANKL and OPG levels are seen to peak during consolidation (Zhu et al., 2007) as the rather homogenous mineralized bony tissue begins to corticalize and develop a medullary cavity, and this is accompanied by a modest vascular response (Carvalho et al., 2004).

The investigation of the systemic response in humans to distraction osteogenesis was performed by Weiss, and identified that when compared to a control group of human patients who had undergone bone osteotomy and rigid fixation, FGF2, MMP-1, ALP and bone specific ALP all showed significantly raised levels in the serum, particularly during the distraction and consolidation phase. Significant serum elevations in TGF-b, IGF-1, IGFBP3 and hGH levels were also seen, but only during the distraction phase. Weiss surmised that the likely source of these growth factors strain-dependent osteoblasts ((Weiss, Baumgart, Jochum, Strasburger, & Bidlingmaier, 2002).

1.4.6 FGF signaling in distraction osteogenesis

1.4.6.1 FGF and FGFR expression

Limited studies exist concerning the expression of the FGFs and no studies have been performed to quantify the expression of the FGFRs in distraction osteogenesis. Of the studies that have looked at the FGFs, none have been able to identify the expression of any of the FGFs during the latency phase; rather FGF expression always starts following mechanical distraction. FGF2 is the most studied of the FGFs in distraction osteogenesis and has been shown to be upregulated
throughout distraction phase in a sheep mandibular distraction model (Farhadieh, Dickinson, Yu, Gianoutsos, & Walsh, 1999) and in a goat model (Yeung, Lee, Fung, & Leung, 2001). Haque showed in a rabbit model that FGF2 is expressed in osteoblasts, chondrocytes and fibroblasts in the FIZ of the distraction gap, which was maximal in the distraction phase but then diminished in the consolidation phase. FGF1 also showed a similar temporal expression to FGF2, but its cellular localization differed in that it was seen expressed mainly in chondrocytes rather than osteoblasts. FGF18 on the other hand was expressed mainly in chondrocytes and fibroblasts with minimal expression in osteoblasts, but not only in distraction, but also in the prolonged consolidation phase (Haque, Amako, Nakada, Lauzier, & Hamdy, 2007). The prolonged high expression throughout distraction osteogenesis of FGF18 and FGF2 are quite unique amongst growth factors from any biological family, as most tend to diminish significantly in expression after distraction finishes, and further work is therefore warranted to understand the importance of FGFs in bone regeneration. Interestingly FGF18 is known to be one of the more specific ligands for FGFR3. We have observed that children with achondroplasia (FGFR3 gain-of-function mutation) who undergo limb lengthening for their short stature by distraction osteogenesis regenerate excellent bone, indeed bone that consolidates faster than children without the FGFR3 genetic mutation (Fig 1-1). These personal observations brought about the initial concept for this thesis that increased FGFR3 signaling in distraction osteogenesis may enhance osteoblast differentiation, and taken with previous literature described here, it is therefore possible that FGF18 and FGFR3 are key regulators of bone regeneration in distraction osteogenesis.
Figure 1-1. Children with achondroplasia undergoing distraction osteogenesis form faster regenerate bone than normal children
A) Radiographs of a normal human tibia in distraction phase of distraction osteogenesis. B) Radiographs of a normal human tibia in consolidation phase of distraction osteogenesis. C) Consolidation Index (time from end of distraction phase to appearance of three cortices of bone on orthogonal radiographs consistent with clinical consolidation) of normal and achondroplastic children expressed in days per centimeter of tibial regenerate bone. Achondroplastic children have shorter bone regenerate consolidation time. n=4 patients per genotype, data represent mean values +/- 95%CI. **p<0.005 (2-sided, 2-sampled Student’s t-test).

1.4.6.2 FGF enhancement of distraction osteogenesis

Only FGF2 has been applied in therapeutic animal experiments to attempt to enhance bone regeneration in distraction osteogenesis. Jiang found that BM-MSCs transfected with FGF2 and inserted into the distraction gap of rabbit mandible increased bone mineral content (BMC) and bone mineral density (BMD) compared to BM-MSCs alone (Jiang et al., 2010). Aronson showed that endogenous FGF2 levels were diminished in old rats undergoing tibial distraction osteogenesis that also showed defects in endosteal bone formation compared to young rats. He applied FGF2 to old rats and found that it rescued the age-related decrease in endosteal bone formation (James Aronson, 2004).
1.5 Research Aims, Hypotheses and Specific Aims

1.5.1 Research Aims

The overarching aim of this body of research is to investigate the role of FGFR3 signaling on skeletal regeneration and repair, based on a novel clinical observation that I have made with respect to the excellent bone regeneration that forms in children with achondroplasia undergoing limb lengthening surgery. These children form bone faster than normal children, and in addition, one typically lengthens the limb over a greater length than in normal children. Limb lengthening surgery, as discussed earlier in this chapter, utilizes the biological process of distraction osteogenesis, through which regenerate bone forms using the intramembranous pathway. Intramembranous bone forms from osteoblasts directly differentiated from progenitor cells, without a cartilage intermediate step, as would be seen in endochondral ossification. Thus pathways that affect distraction osteogenesis likely have an effect on the ability of mesenchymal progenitor cells to differentiate to osteoblasts, and alter the ability of osteoblasts to form bone. The skeletal phenotypes seen in achondroplasia and other genetically related skeletal dysplasias have been explained by the effects of mutant FGFR3 signaling in disrupting chondrocyte proliferation and differentiation. The observation that children with achondroplasia make such excellent regenerate bone during limb lengthening through distraction osteogenesis cannot be clearly explained by the current knowledge on the molecular function of FGFR3 in relation to its effects on chondrocytes, rather they may be due to a stimulation in osteoblast proliferation and differentiation leading to the enhancement of regenerate bone formation.

I believe that there is fundamental new knowledge to be gained as to the role of FGFR3 in skeletal biology; by taking a new approach to investigating the function of FGFR3 and examining how it affects bone fracture repair in genetically modified mice. Broadly speaking the content of this research has implications for the potential of the FGFs, and particularly FGFR3, as a target for novel therapeutics to enhance bone regeneration and repair. Furthermore, this research also suggests an exciting new paradigm, which is that cells of the haematopoietic lineage may have a critical role coordinating the behavior of mesenchymal cells such as osteoblasts, that rebuild fractured bone, and that FGFR3 signaling might be critical to this process, and thus open up a whole new avenue to target enhancements in skeletal repair.
1.5.2 Hypothesis

FGFR3 signaling modulates bone fracture repair. Specifically abnormalities in FGFR3 signaling affect osteoblast differentiation, which leads to abnormal bone fracture repair, and furthermore that FGFR3 signaling in multiple cell types from the bone marrow niche regulate osteoblast differentiation.

1.5.3 Specific Aims

I aim to test the hypothesis described above by answering the following three questions:

1. How does FGFR3 regulate the size and structure of healing fracture callus?

2. How does FGFR3 affect mesenchymal progenitor proliferation and osteoblast differentiation?

3. Does FGFR3 signaling in bone marrow cells affect osteoblast differentiation and thus act as a mechanism through which FGFR3 can modulate bone fracture repair?
CHAPTER 2 – FGFR3 REGULATES THE COMPOSITION OF FRACTURE CALLUS
2.1 Abstract

FGFR3 is known to be a negative regulator of chondrocyte proliferation affecting long bone endochondral growth. However we observed that individuals with achondroplasia showed enhanced bone regeneration during distraction osteogenesis for limb lengthening, leading us to hypothesize that FGFR3 also affects intramembranous ossification, and that FGFR3 related abnormalities in intramembranous ossification would manifest in the abnormal healing of bone fractures. Using an established murine semi-stabilized tibia fracture model in genetically modified $Fgfr^{3+/−}$ knockout mice and their WT controls we have demonstrated that FGFR3 deficiency results in more rapid fracture healing based on the temporal changes in fracture callus size, but also that this abnormally rapid healing corresponds to a loss of structural integrity at PFD21. On further analysis we show that FGFR3 is controls the balance between the two major pathways of bone formation; deficiency in FGFR3 leads to an inhibition of intramembranous (direct) bone formation but at the same time increasing bone formation through the endochondral (indirect) pathway, thus FGFR3 effectively acts as a bone switch. In addition FGFR3 deficiency also increases bone fracture remodeling in $Fgfr^{3+/−}$ mice, which is effected by an increase in osteoclast number and osteoclast activity. Taken together we show that through alteration in both bone formation and bone remodeling FGFR3 regulates the size and structure of healing fracture callus.
2.2 Introduction

Bone fracture healing is a complex reparative process utilizing multiple cell types that is tightly coordinated both spatially and temporarily, yet is incompletely understood. Four to ten percent of all fractures fail to heal; developing established nonunion (Hak et al., 2014; L. A. Mills & A. H. Simpson, 2013; L. A. Mills & A. H. R. W. Simpson, 2013). Fracture nonunions cause significant morbidity and often require additional surgery, bone grafting and biological agents to establish union. Due to the length of time even routine fractures take to repair, and the extended period of rehabilitation needed to regain full function, all fractures would benefit from therapies and techniques to enhance bone healing.

Considering that fracture repair recapitulates many of the developmental processes in skeletal formation, including both intramembranous and endochondral ossification, the fibroblast growth factor (FGF) ligands and their receptors (FGFRs) are thought to play key roles in fracture repair, and are implicated in all stages from the initiation of fracture healing through to the latter stages of bone remodeling. It is interesting to note that while numerous FGFs have been shown to be expressed during fracture healing, exact temporospatial relationships and their exact roles have yet to be determined (Bolander, 1992; Bourque et al., 1993; Arata Nakajima et al., 2003; Rundle et al., 2002; Schmid et al., 2009; Song et al., 2006).

With an incidence of 1 in 15,000 live births, achondroplasia is the most common skeletal dysplasia (Oberklaid et al., 1979). Achondroplasia is phenotypically characterized by disproportionate rhizomelic short stature, with limb growth being inhibited more than that of the spine (Oberklaid et al., 1979). It is a heterozygous disorder of autosomal dominant inheritance whereby individuals express both WT and mutant overactive fibroblast growth factor receptor 3 (FGFR3) (Rousseau et al., 1994; Shiang et al., 1994). Human disease with loss-of-function of FGFR3 (CATSHL syndrome) is an uncommon condition with a phenotype of tall stature and scoliosis (abnormal curvature of the spine), but confirms the importance of FGFR3 on skeletal development (Toydemir et al., 2006). FGFR3 has been shown to act as a negative regulator of endochondral bone growth, by inhibiting the proliferation and differentiation of growth plate chondrocytes (Colvin et al., 1996; Deng et al., 1996; Naski et al., 1996). In gain-of-function mutations such as in achondroplasia, this has been reported to account for many of the developmental effects seen in the skeleton, and in particular the inhibition of limb growth (Deng
et al., 1996). In endochondral bone formation in the developing skeleton, FGFR3 expression is seen in the chondrocytes of the resting zone and the proliferative zone of the long bone growth plate (Naski et al., 1996).

Although most authors describe the importance of FGFR3 in chondrocyte proliferation and differentiation (Colvin et al., 1996; Deng et al., 1996; Arata Nakajima et al., 2003; Naski et al., 1996), Rundle also described the expression of FGFR3 from osteoblasts in the healing fracture callus suggesting a potential role for FGFR3 in intramembranous as well as endochondral ossification (Rundle et al., 2002). Despite earlier studies of the cranium suggesting that there were no significant sutural or cranial defects related to intramembranous ossification due to abnormalities in FGFR3 function (Colvin et al., 1996; Deng et al., 1996), more recent studies in mice and humans with FGFR3 related skeletal dysplasias showed that premature fusions of the synchondroses were seen, as well as abnormally ossified dermatocranial bones, and alterations in osteoblast differentiation in-vivo, suggesting that FGFR3 may indeed be involved in intramembranous ossification (Di Rocco et al., 2014; Valverde-Franco et al., 2004).

Distraction osteogenesis is a process whereby bone is formed primarily through intramembranous ossification, without first requiring a cartilage template. Limb lengthening and reconstruction surgery utilizes the phenomenon of distraction osteogenesis to regenerate bone (J Aronson et al., 1989; J Aronson & Shen, 1994; Ilizarov, 1990). Individuals with achondroplasia have been noted to form excellent regenerate bone when undergoing surgical limb-lengthening procedures. Considering that distraction osteogenesis regenerates bone through intramembranous ossification this clinical finding adds further weight to the hypothesis that FGFR3 signaling may be important in osteoblast differentiation.

Taken together the evidence from mouse and human studies indicates that the profound effects of FGFR3 on skeletal development, regeneration and repair cannot be solely explained by its effect on chondrocyte biology and endochondral ossification. We hypothesized that FGFR3 alters both endochondral and intramembranous ossification, and that these effects are present in the process of tissue repair and would lead to altered fracture healing. To address this question we analyzed the process of bone fracture healing in FGFR3-deficient mice.
2.3 Materials and Methods

2.3.1 Mice

_Fgfr3^+/^-_ mice were purchased from Jackson Laboratory (B6; 129S- _Fgfr3^tm1Dor_/J, Stock #004234) and maintained at the Toronto Center for Phenogenomics (TCP). Animal protocols were approved by the animal care committee of the TCP, in accordance with the regulations and guidelines from the Canadian Council on Animal Care. Genomic DNA from skin samples of harvested mice were used to genotype animals by PCR.

2.3.2 Tibia fracture generation

Semi-stabilized tibial fractures were generated on 12-week-old male _Fgfr3^+/+ _ and _Fgfr3^+/^- _ mice following a protocol previously described (Y. Chen et al., 2007). In brief, the mice were anaesthetized using inhalational general anaesthesia. The left hind limb of each mouse was surgically prepared, by shaving and cleaning with disinfectant. A small anterior midline incision was made over the knee joint and proximal tibia. A 0.7 mm pilot hole was made in the proximal tibial epiphysis just medial to the insertion of the patella tendon using a hollow needle. A 0.7mm Anticorrollo insect pin (Fine Science Tools, http://www.finescience.ca) was then inserted into the medullary cavity of the intact tibia and advanced to the distal tibia. A transverse fracture was then induced at the mid-shaft of the tibia using blunt scissors. The insect pin was cut 5-7mm proud of the proximal tibial epiphysis and the skin incision closed with a series of absorbable vicryl rapide sutures, and metallic wound clips. Analgesic given subcutaneously (buprenorphine, 0.1mg/kg/twice per day) was administered for 3 days after surgery. Previous data shows that a fracture generated in this manner heals through both intramembranous and endochondral ossification (Hiltunen, Vuorio, & Aro, 1993; Le, Miclau, Hu, & Helms, 2001). The animals were allowed to maintain full weight bearing in their cages following surgery. At specific time points, PFD3, PFD7, PFD14 and PFD21 following fracture, samples of fractured and unfractured tibiae were harvested following euthanasia with inhaled carbon dioxide. A minimum of 4 mice per genotype, per time point, were used for further analysis.

2.3.3 Micro-CT

Fractured and unfractured tibiae in both _Fgfr3^+/^- _ and _Fgfr3^+/+ _ mice were analyzed using micro-CT based static histomorphometric analysis. Prior to micro-CT scanning the insect pin was
removed from each fracture with great care not to disrupt the fracture callus. Each tibia was wrapped in saline soaked gauze and inserted into a falcon tube with an interference fit. Each tibia was scanned using a Skyscan 1174 compact X-Ray micro-CT scanner (Skyscan, Belgium) with the beam set at 50kV and 800μA, a 0.25mm Al filter and an 11.6μm isometric voxel size. Prior to each scanning session calibration data of bone mineral density was performed for future analysis using phantom bones composed of 250mgHA/cm³ and 750mgHA/cm³. All reconstructions were performed using Skyscan NRecon software. All measurements, analyses and post-processing imaging were performed using Skyscan CTan (version 1.5.0, Skyscan, Bruker, MicroCT). Measurements of bone mineral density (BMD) for tissue in each fracture callus were normalized to the BMD of the same animal’s intact, unfractured, contralateral tibia, to account for mouse-to-mouse variability in bone mineral density. The region of interest (ROI) of the intact control tibia was a 250-slice section centered on the mid-diaphysis in the sagittal plane. On an axial view a ROI was hand drawn using the outermost border of the tibial bone. The maximum density of the cortical bone was subsequently determined, excluding tissue occupying <0.01% of the total cross-sectional area to diminish edge effect. The minimum density of intact cortical bone was calculated at a threshold of 60% of the maximum density (Nicholls et al., 2013). Five mice per genotype, per time point, were used for analysis.

For callus analysis the ROI in the sagittal plane for each fracture callus corresponded to a 250-slice section centered on the coincident mid-diaphysis and maximum diameter of callus. On an axial view the ROI was hand drawn and corresponded to the outer most aspect of the fracture callus. Using semi-automated interpolation and segmentation techniques the native bone was digitally subtracted from the surrounding callus so as not to add artefact to measurements of callus tissue densities, as cortical native bone is indistinguishable in CT analysis from healing bone in the fracture. Within the callus, tissue was either considered non-mineralized (0-35% maximum cortical density), mineralized callus (35-60% maximum cortical density), bone (60-100% maximum cortical density) or total mineralized tissue (35-100% maximum cortical density). Total callus volume was determined for each fracture sample, as was determination of tissue density of the mineralized callus, bone and total mineralized tissue (mineralized callus and bone combined). Comparisons between Fgfr3+/- and Fgfr3+/+ were expressed as either mean tissue mineral density (TMD) in g/mm³, or as a proportion of tissue (of interest) volume per callus volume (BV/TV). The effective polar moment of inertia (J), was also calculated as an
indirect measure of the callus’s ability to resist torsion, by reflecting how points of mineral measurement are distributed about its axis (Bouxsein et al., 2010; Morgan et al., 2009; O'Neil et al., 2012). Five mice per genotype, per time point, were used for analysis.

2.3.4 Histology

Fractured tibiae were harvested on PFD7, PFD14 and PFD21, fixed in 10% formalin following by decalcification with 20% (w/v) EDTA pH 8.0 or formic acid bone decalcifier (Decal Chemical Corp, Tallman, NY) and embedded in paraffin. Serial 5µM sections of paraffin embedded tissues were deparaffinized, and rehydrated through an alcohol gradient to water. Sections were stained with Safranin-O and counter stained with fast green/Mayer’s haematoxylin. Here red staining confirms the presence of proteoglycans, which indicates cartilaginous tissue, and green staining indicates bone (Camplejohn & Allard, 1988), or stained with Tartrate Resistant Acid Phosphatase (TRAP) (manufacturers protocol, TRAP Staining-387A-1KT, Sigma, St. Louis, MO) to evaluate osteoclast activity.

2.3.5 Histomorphometry

Callus composition was analyzed using computer–assisted histomorphometry (Adobe Photoshop, San Jose CA, USA). The cross-sectional area of each section of callus was measured using digital–subtraction techniques to exclude native bone. The relative abundance of bone and cartilage were expressed as a percentage of the total callus tissue area. Four to six mice per genotype, per time point, and 5 sections per mouse were used for analysis. For osteoclast analysis, measurements were made of TRAP stained sections using ImageJ (NIH Bethesda, MD USA). The prevalence and functional activity of TRAP-positive osteoclasts, in the woven bone of the callus, were determined, by the numbers of osteoclasts per bone surface area, and the erosion surface of osteoclasts per bone surface, on each FOV. A minimum of 3 mice with 5 sections, for each of 5 fractured tibiae, per genotype were used for this analysis. Twelve FOVs at 40x magnification were analyzed per mouse.

2.3.6 Gene expression analysis

Fracture calluses (n=5) and control unfractured tibial bone (n=5) were harvested at PFD3, PFD7, PFD14, PFD 21 and flash frozen in liquid nitrogen. Tissues were pulverized into powder under continuous cooling with liquid nitrogen and then further homogenized by Mini-Beadbeater
(Biospec Products, model# 2412PS-12W-B30, Bartlesville, OK) in TRIzol reagent (ambion by Life Technologies, Cat # 15596018). Total RNA from homogenized tissue or culture cells were extracted by TRIzol reagent and purified by RNeasy Mini kit (QIagen, Cat#74106) according to the manufacturer’s protocol. cDNA were synthesized by using Superscript II kit (Invitrogen by life technologies, Cat # 18064-014) with random hexamer primer. Gene expression in fracture tissue and unfractured control tibia was analyzed by real-time quantitative PCR using ∆∆Ct methods relative to the expression of the control housekeeping gene β-actin. Taqman Universal PCR master mix and Taqman Gene Expression Assays (Applied Biosystems) were used for the reaction.

2.3.7 Osteoclast differentiation

Femora and Tibiae from 12-week old female Fgfr3+/− and WT mice were isolated and debrided of soft tissue. Bone marrow was flushed into BMSC culture media containing α-modification of eagle’s medium (α-MEM) (Wisent Inc, Cat # 310-012-CL, St. Bruno, CA), 10% FBS (Gibco Life Technology, Cat # 16000-044, Burlington, ON) and 1x antibiotic and antimycotic (Wisent Inc, Cat # 450-115-EL). At 24 hours after incubation non-adherent cells were harvested and resuspended in osteoclast differentiation media, which consisted of media described above with the addition of 50ng/ml Recombinant Mouse RANKL (R&D Systems, Cat # 462-TR-010, Minneapolis, MN) and 30ng/ml Recombinant Mouse M-CSF (R&D Systems, Cat # 416-ML) onto cover slips in 6-well plates. Cells grown on coverslips were fixed with 10% formalin for 10 min, then fixed with ethanol-acetone (50:50 v/v) for 1 min and stained with TRAP following the manufacturer’s protocol (TRAP Staining-387A-1KT, Sigma). TRAP-positive cells containing at least 3 nuclei were considered osteoclasts and the numbers of osteoclasts per coverslip were counted using light microscopy. Quantification performed with 5-6 mice per genotype.

2.3.8 Osteoclast functional analysis

For osteoclast activity analysis, cells were cultured as above then harvested using trypsin on day 10 post-differentiation and continued in cell culture on 24-well Corning Osteo Assay plates with a bone biomimetic synthetic surface (Sigma, Cat # CLS3987-4EA) for a further 7 days at an initial density of 3x10⁴ cells per well. Osteo Assay plates were incubated with double distilled water to lyse the cells. Plates were then stained using the Von Kossa method, with 5% silver nitrate for 5 min on a light box to detect the resorption pits, characterized by the absence of
staining. After washing and drying, digital scanned images were taken of the Osteo Assay plates. The areas of the resorption pits in each well were analyzed using Photoshop Extended CS6 (Adobe Photoshop, San Jose CA, USA). Quantification performed with a minimum 3 mice per genotype with 3 wells per mouse.

2.3.9 Statistical analysis

Data are expressed as mean, +/-95% confidence intervals and were calculated for each sample. Student’s t-test was used to compare data sets. The data were considered to be statistically significant at a confidence level of 95% (p<0.05).
2.4 Results

To investigate the role of FGFR3 in fracture healing we used an established murine semi-stabilized tibia fracture model. Firstly we sought to determine that FGFR3 is indeed expressed in normal bone fracture repair, and furthermore to ascertain the temporal relationship of FGFR3 expression throughout each stage of fracture repair from the inflammatory phase, through intramembranous and endochondral ossification to the remodeling phase. We used quantitative real-time PCR (qPCR) to compare FGFR3 expression in WT fracture calluses compared to unfractured contralateral tibiae (Fig.2-1). FGFR3 mRNA expression increased rapidly in fracture callus compared to uninjured tissues reaching a peak at post-fracture day (PFD) 7 (10-fold increase).

![Figure 2-1. FGFR3 mRNA expression is elevated in normal fracture repair](Image)

Expression of FGFR3 mRNA in both unfractured control murine tibia bone and semi-stabilized fractured murine tibia callus tissue is measured using qPCR at time-points PFD3, PFD7, PFD14 and PFD21. Callus tissue showed peak FGFR3 mRNA expression at PFD7, which although declining by PFD14 and PFD21, remained elevated compared to unfractured control bone. n= 5 mice per genotype, per time-point, data expressed as FGFR3 expression relative to unfractured control tissue at PFD3, +/- 95% confidence intervals, *p<0.05, **p<0.0005 (2-sampled, 2-sided Student’s t-test).
The increased FGFR3 mRNA expression then rapidly declined over the following week to PFD14, at which point it was still expressed at significantly elevated levels compared to unfractured control bone, and furthermore remained significantly elevated through PFD21 which marks the remodeling phase. This data suggests an important role for FGFR3 in fracture repair, particularly the early phases of cellular proliferation and early bone and callus formation in addition elevated expression at the later stages of fracture repair when remodeling is taking place.

Now that we had established that FGFR3 was indeed elevated in normal fracture repair, we then investigated the effect that FGFR3 had on callus structure by comparing the fracture healing response in genetically modified Fgfr3+/− heterozygous knock-out mice to their WT litter mates at critical time points representing distinct phases of fracture repair, namely early callus formation and intramembranous ossification (PFD7), endochondral ossification (PFD14) and fracture remodeling (PFD21) respectively.

Histological sections of fracture callus stained with Safranin-O (for cartilage) and Fast Green (for bone) and were examined using histomorphometry at PFD7, PFD14 and PFD21 and measurements of fracture callus area were taken, (Fig. 2-2). Fgfr3+/− fracture calli were noted to have a significantly increased callus area at PFD7 when compared to WT calli. By PFD14 however, the Fgfr3+/− fracture calli were significantly smaller in area than WT, and continued to decrease in area through to PFD21 where they remained significantly smaller than WT controls. These data suggested an increase in the rapidity of fracture healing.
To assess whether diminished callus area in $\text{Fgfr3}^{+/--}$ fractures reflected an altered three-dimensional structure and composition, and also to predict the physical properties of this altered fracture callus, PFD21 fractures were analyzed using micro-computed tomography (micro-CT) for volume, and polar moment of inertia (PMI) (Fig. 2-3). PMI is a quantity used to predict an objects ability to resist torsion, and thus predict strength of fracture repair. Paired uninjured contralateral tibiae from each mouse were micro-CT scanned and used to control for variations in mouse-to-mouse bone mineral density. Native bone was digitally subtracted form the...
surrounding callus to allow assessment of callus morphology. \textit{Fgfr3}^{+/−} callus demonstrated markedly diminished volume at PFD21 and also demonstrated lower PMI.

\textbf{Figure 2-3. FGFR3 regulates fracture callus volume and structural integrity}

A) Representative sagittal plane (left) and axial plane (right) 3D reconstructed micro-CT images of WT and \textit{Fgfr3}^{−/−} murine tibial fractures at PFD21. Axial plane images have central native bone digitally removed using semi-automated image segmentation leaving callus tissue for analysis. Line on sagittal image represents plane of axial section at maximum diameter of callus. B) 3D micro-CT analysis of WT and \textit{Fgfr3}^{−/−} tibia fracture callus at PFD21 shows mineralized callus volume (BV), total callus volume (TV), total mineral density (TMD), callus mineralized volume fraction (BV/TV), bone mineral content (BMC) and polar moment of inertia (J). \textit{Fgfr3}^{−/−} calli had reduced TV, BV, BMC and J, when compared to WT calli, but no difference in TMD or BV/TV. \( n = 5 \) mice per genotype, data represent mean values +/-95% CI, ***\( p<0.0005 \) (2-sided, 2-sample Student’s t-test).

Taken together with the histomorphometry results described above these data suggest that FGFR3 deficiency results in more rapid fracture healing based on temporal changes in fracture callus size, but also that this abnormally rapid healing corresponds to a loss of structural integrity at PFD21. The mouse tibial fracture model allows a detailed phenotypic analysis of the
contribution of new bone formation from both the intramembranous and endochondral pathways in fracture repair, due to their differing spatial characteristics. Knowing that FGFR3 deficiency impacts on the structure, rapidity and strength of overall fracture healing we next sought to investigate and contrast the role of FGFR3 in fracture repair in more detail by examining its effect on both intramembranous and endochondral ossification.

We first examined intramembranous bone formation in-vivo using detailed histomorphometric techniques. At the very edges of fracture repair the injured subperiosteal zone gives rise to intramembranous bone formation, which can be visualized and quantified at PFD7. No cartilage template precursor is seen in this subperiosteal zone unlike in the larger central area of fracture healing which heals with endochondral ossification. Histological sections of WT and Fgfr3+/− mouse tibial fracture callus were stained with Safranin-O (cartilage) and Fast Green (bone) and analyzed using digital subtraction histomorphometric techniques to isolate areas of intramembranous ossification. Segments for analysis correspond to the boxed area in the diagram. Here we show a significantly reduced percentage of bone area per callus area in Fgfr3+/− mutant samples representing reduced intramembranous ossification (Fig. 2-4).

Next we investigated the effect of FGFR3 on endochondral ossification whereby bone is laid down using a cartilage template. This process forms the mainstay of bone formation in fracture healing. Histological sections of WT and Fgfr3+/− mouse tibial fracture callus corresponding to the boxed area in the diagram from PFD7 and PFD14 were stained with Safranin-O (cartilage) and Fast Green (bone) (Fig.2-5). The sections were analyzed using digital subtraction histomorphometric techniques to isolate areas of cartilage formation and bone deposition. Although demonstrating no difference in the percentage of cartilage per callus area at PFD7, by PFD14 Fgfr3+/− mutants demonstrated a markedly diminished percentage of cartilage per callus area.
The converse was true concerning quantification of bone within the callus. No difference in the percentage of bone per callus area was seen at PFD7 between WT and Fgfr3+/−, but by PFD14 Fgfr3+/− mutants demonstrated a significant increase in the percentage of bone per overall callus area. This shows that the process of endochondral ossification is faster in Fgfr3+/− fractures, as at PFD14 most of the cartilage template has already been replaced by bone. Taken together these results demonstrate that FGFR3 is critical in controlling the balance between the two major pathways of bone formation. Deficiency in FGFR3 leads to an inhibition of intramembranous
(direct) bone formation but at the same time increasing bone formation through the endochondral (indirect) pathway, thus FGFR3 effectively acts as a bone switch.

Figure 2-5. FGFR3 deficiency enhances endochondral ossification in fracture repair
A) Schematic diagram of a murine tibial fracture with surrounding callus. Boxed ROI containing light grey shaded region demonstrates large central area of callus, where endochondral bone formation occurs during normal fracture healing. B) Histological sections of PFD7 and PFD14 tibial fracture callus, digitally subtracted from native bone, from WT and Fgfr3+/− mice corresponding to boxed ROI shown in schematic diagram, stained with Safranin-O (cartilage) and Fast Green (bone). C) Quantification of mean cartilage area per callus area, and bone area per callus area, of histological samples shown above. Both WT and Fgfr3+/− samples show similar proportion of cartilage and bone in the whole fracture callus at PFD7, but at PFD14 Fgfr3+/− samples show significantly reduced proportion of cartilage per callus area compared to WT. Conversely Fgfr3+/− samples show a significantly increased proportion of bone per callus area at PFD14.

Based on the findings that Fgfr3+/− callus volume was diminished at PFD21 when compared to WT, and despite demonstrating that endochondral ossification is accelerated which may explain the small callus volume at PFD21, we also sought to determine whether FGFR3 signaling had
any effect on fracture callus remodeling. An alternate hypothesis as to why fracture callus volume was smaller in \( \text{Fgfr3}^{+/+} \) at PFD21 than WT samples is that fracture callus remodeling is enhanced in the mutants. Further histomorphometric analysis of fracture calli was therefore performed on PFD21 samples, and stained with Safranin-O and Fast Green as previously described.

![Image](image.png)

**Figure 2-6. FGFR3 deficiency increases fracture callus remodeling**

A) Schematic diagram as previous. Boxed ROI demonstrates area of histomorphometric analysis. B) 20x magnified views of WT and \( \text{Fgfr3}^{+-} \) callus stained with Safranin-O (cartilage) and Fast Green (bone) at PFD21 demonstrated barely detectable amounts of proteoglycan in \( \text{Fgfr3}^{+-} \) compared to more abundant proteoglycan in WT. In addition trabecular bone structure was more sparsely distributed in \( \text{Fgfr3}^{+-} \) than WT. C) Histological sections of PFD21 tibial fracture callus from WT and \( \text{Fgfr3}^{-/-} \) mice corresponding to boxed ROI shown in diagram, stained with Safranin-O and Fast Green. Images on left show unmodified callus. Images on right show same callus but with digital subtraction of cartilage tissue to reveal extent of bone. D) Quantification of mean absolute cartilage area, and bone area per callus area, of histological samples shown above. More cartilage remained in WT callus compared to \( \text{Fgfr3}^{-/-} \), and a larger bone area per callus area was also demonstrated in WT compared to \( \text{Fgfr3}^{+-} \) calli. \( n = 4-6 \) mice per genotype, minimum 4 samples per mouse, data represents mean values +/- 95% CI, ***p<0.0005 (2-sided, 2-sampled Student’s t-test).

\( \text{Fgfr3}^{+-} \) calli revealed significantly less bone area per callus area. Furthermore there was also a reduction in cartilage content in \( \text{Fgfr3}^{+-} \) samples (Fig. 2-6). These findings when taken in context with the increase in bone from endochondral ossification seen at PFD14 in the \( \text{Fgfr3}^{+-} \) mutants, suggests that there is also increase in fracture remodeling in \( \text{Fgfr3}^{+-} \) mice.
Fracture remodeling is performed by the coupled action of osteoclasts and osteoblasts. Osteoclasts (of haematopoietic lineage) are the primary cells implicated in bone resorption for fracture remodeling, and so one would expect there to be an increase in number and/or activity of osteoclasts in the $Fgfr3^{+/−}$ fracture calli if remodeling is indeed enhanced. TRAP stained sections of callus were analyzed using histomorphometry, which showed an increase in the number of osteoclasts per bone surface, and also an increase in osteoclast erosion surface per bone surface in $Fgfr3^{+/−}$ calli compared to WT calli, indicating not just an increase in number of osteoclasts in $Fgfr3^{+/−}$ calli but also an increase in osteoclast activity (Fig. 2-7).

Osteoclast cultures were also performed in-vitro using BM aspirate from $Fgfr3^{+/−}$ mice and their WT littermates. $Fgfr3^{+/−}$ samples demonstrated increased numbers of TRAP stained osteoclasts in culture and using a resorption pit assay demonstrated that the increased numbers of osteoclasts were also more functional (Fig. 2-8). Taken together these data demonstrate that FGFR3 deficiency increases bone fracture remodeling in addition to acting as a bone switch, which reduces intramembranous bone formation and enhances endochondral ossification, and therefore that smaller callus size in PFD21 fractures is likely to be due to a combination of enhanced bone formation and increased bone resorption.
Figure 2-7. FGFR3 deficiency increases osteoclast number and activity in bone fracture healing

A) Schematic diagram of a healing murine tibial fracture. Boxed ROI containing demonstrates representative area shown in histological panels and that used for analysis. B) Magnified 10x (left) and 40x (right) views of WT and Fgfr3+/− callus stained for TRAP at PFD14. C) Histomorphometric analysis showed an increased number of osteoclasts per bone surface (Oc/BS) and increased activity of osteoclasts (increased erosion surface per bone surface, ES/BS) in Fgfr3+/− calli compared to WT calli at PFD14. n = 3 mice per genotype, minimum 5 sections per mouse, and 12 ROI per mouse, data represents mean +/- 95% CI, ***p<0.0005 (2-sided, 2-sampled Student’s t-test).
Figure 2-8. FGFR3 deficiency increased osteoclast differentiation and function in-vitro

A) Representative images of in-vitro osteoclast formation assay using WT and Fgfr3<sup>+/−</sup> BM aspirate, demonstrating large multinucleate (>3) TRAP-positive osteoclasts. Quantification of in-vitro differentiation assay shows that Fgfr3<sup>+/−</sup> samples contained more osteoclasts per field of analysis. B) Representative images of in-vitro osteoclast functional assay using WT and Fgfr3<sup>+/−</sup> BM aspirate cultured on Osteo Assay plates. Resorption pits (white) reflect osteoclast activity and are shown by the absence of Von Kossa staining. Quantification of area of resorption pits were calculated and shown to be greater in Fgfr3<sup>+/−</sup> samples compared to WT. n = 3 mice per genotype, 3 wells per mouse. Data expressed as mean values +/-95% CI, *p<0.05, **p<0.005 (2-sided, 2-sampled Student’s t-test).
2.5 Discussion

Our findings suggest that FGFR3 signaling has a critical role in the regulation of bone fracture healing; specifically FGFR3 acts to regulate fracture repair by altering the balance of intramembranous and endochondral ossification. Inhibition of FGFR3 diminishes intramembranous ossification whilst at the same time enhancing endochondral ossification. In addition to the primary finding that FGFR3 regulates the balance of bone formation, FGFR3 signaling also appears to be important in the remodeling phase of fracture healing, as inhibition of FGFR3 leads to more rapid remodeling. Taken together, and based upon micro-CT analysis, these alterations in bone formation and remodeling lead to a loss of structural integrity of the healing fracture callus.

*Fgfr3*^+/−^ mice initially showed a rapid increase in the size of fracture callus at PFD7, but at PFD14 and PFD21 the callus was then smaller than WT controls. This suggests that reduced FGFR3 signaling leads to accelerated fracture healing overall. Our data are consistent with a previous study, which showed delayed fracture healing in a mouse with gain-of-function FGFR3 mutation (N. Su et al., 2008), in which the authors hypothesized that this finding was due to a delay in the differentiation of chondrocytes in fracture callus.

To understand the structural and compositional consequences of the accelerated fracture healing we proceeded to analyze PFD21 fractures with micro-CT scanning. It has been previously reported that the micro-CT based measurements of changes in fracture callus structure and composition, such as BV, BV/TV, TMD, BMC, and PMI are able to predict the mechanical properties of the callus, and constitute a basis for non-invasive testing of fracture healing (Morgan et al., 2009). Furthermore it has also been shown that measures of callus size and structure (TV, PMI) are more sensitive to changes in callus over time post-fracture than assessing callus substance (for example BV/TV, TMD, BMD) (O'Neill et al., 2012), and indeed the same authors also showed that none of the mechanical testing performed was able to reliably distinguish between fractured and un-fractured bones (O'Neill et al., 2012). These studies offer evidence that micro-CT assessment of fracture healing is superior to mechanical testing, and in addition is less invasive and uses fewer animals. To attempt to ensure that any differences in structure and composition of the callus were not due to inherent abnormalities in the bone mineral content of each mouse (Valverde-Franco et al., 2004), we analyzed the contralateral
unfractured tibia in each animal, to control for bone density of each subject mouse. Our findings confirmed a smaller bone volume (BV), total callus volume (TV) and bone mineral content (BMC) in $Fgfr3^{+/−}$ mutants supporting our histological findings of accelerated fracture healing. A fracture heals with callus, as an elegant way of rapidly forming a temporary biological and structural splint that acts as a stimulus and template for new bone to form, and thus repair the bony defect. This process enables the limb to resist physiologic forces and allow early mobilization and ambulation, which is to the survival advantage of the animal. With this in mind, accelerated fracture healing is only desirable if the callus and healing bone that forms is of sufficient strength to resist physiologic forces. We found that, despite accelerated fracture healing in $Fgfr3^{+/−}$ mice, the polar moment of inertia was reduced. The polar moment of inertia (PMI) is a measurement of an object’s ability to resist a torsional force, and in a healing fracture is considered a measure of its integrity (Morgan et al., 2009). We were not able to determine from this study whether the reduction in PMI in $Fgfr3^{+/−}$ fractures was great enough to constitute insufficient stiffness for physiological purposes in our mice. Therefore whilst we have identified accelerated fracture healing in $Fgfr3^{+/−}$ mice in this study, we were not able to conclude if this was a true biological enhancement and advantage, but the findings certainly speak to the concept that faster healing does not necessarily mean better healing, in terms of the quality of bone repair.

Bone fracture healing is a complex reparative process utilizing multiple cell types that is tightly coordinated both spatially and temporally, and thus serves as an excellent in vivo system to examine the differentiation and interaction of osteoblasts and chondrocytes in the healing process, and to understand their relative contribution to bone formation. Of particular interest is that bone formation comes from two sources, intramembranous and endochondral ossification. Our original hypothesis questioned the contribution of FGFR3 signaling to the differentiation of osteoblasts in the healing fracture, and thus we examined both intramembranous and endochondral ossification within the fracture samples. We identified that intramembranous ossification is inhibited in $Fgfr3^{+/−}$ mice, despite the initial observation that fracture repair was enhanced as a whole. Despite earlier conflicting reports concerning the effect of FGFR3 signaling on the function of osteoblasts (Colvin et al., 1996; Deng et al., 1996), our data supports more recent studies of FGFR3 related skeletal dysplasias, which have shown that abnormally ossified dermatocranial bones are related to defects of FGFR3 signaling in
osteoblasts affecting intramembranous ossification (Di Rocco et al., 2014). Our findings of FGFR3 signaling regulating intramembranous ossification in fracture healing are novel, and furthermore reinforce the principles of fracture repair recapitulating the same pathways that are seen in skeletal development (Louis C Gerstenfeld et al., 2003).

Distraction osteogenesis occurs primarily through intramembranous bone formation. Our FGFR3-deficient mice show diminished intramembranous ossification, and therefore our data may offer an explanation as to why children with achondroplasia (gain-of-function FGFR3 mutation) who undergo limb-lengthening surgery form such excellent regenerate bone.

We also identified that endochondral ossification was enhanced in fracture healing in Fgfr3+/- mice as demonstrated by the rapid accumulation of cartilage-based callus at PFD7 and the rapid turnover of the central callus area to bone by PFD14. Cartilaginous callus formation occurs from the abundance of proliferating chondrocytes and their subsequent differentiation to hypertrophic chondrocytes, thus an acceleration of callus formation and replacement by bone is likely due to increases in chondrocyte proliferation and differentiation. FGFR3 has long been known as a negative regulator of chondrocyte proliferation and differentiation, thus being a gene critical for normal skeletal development (Colvin et al., 1996; Deng et al., 1996; Naski et al., 1996; Valverde-Franco et al., 2004; Xiao et al., 2004). The short-limbed phenotypes of individuals with FGFR3-related skeletal dysplasias, such as achondroplasia, are primarily explained by reduced chondrocyte proliferation and differentiation in the growth plate (Deng et al., 1996). Our data, using a fracture repair model, are therefore consistent with the findings of previous developmental studies using the same Fgfr3 deficient mouse, leading to an increase in endochondral ossification. In addition our study demonstrates how the function of FGFR3 in fracture healing recapitulates its function in development through gross abnormalities in chondrocyte biology.

A smaller callus at PFD21 can occur for a number of reasons; more rapid endochondral ossification, leading to a smaller callus at an earlier time point, it could be due to a poor reparative response where throughout fracture healing a diminutive callus is formed, or it could be because of rapid remodeling, where a normal callus is remodeled to lamellar bone in a shorter time frame. Therefore, despite our initial result demonstrating increased endochondral ossification in the Fgfr3+/- we proceeded to examine whether enhanced remodeling could be a
factor in explaining the smaller size of PFD21 calli. We demonstrated an increased number and activity of osteoclasts in the healing fracture callus as well as the enhanced differentiation and function of osteoclasts in-vitro, which are consistent with the findings of an increase in fracture callus remodeling. Our data are aligned with that of Valverde-Franco who demonstrated an increase in TRAP positive osteoclast number lining the bone surfaces of \( Fgfr3^{-/-} \) mice in a bone mineralization study (Valverde-Franco et al., 2004). However it is not clear from our data whether the increased activity of osteoclasts is a cell autonomous effect of reduced FGFR3 signaling, or whether the osteoclasts are in fact normal but responding to increased signaling as a consequence of increased endochondral ossification in the reparative setting.

Our findings demonstrate that defects in FGFR3 signaling have wide ranging effects on both mesenchymal and haematopoietic lineage cells that control skeletal homeostasis and repair, but our interpretation of these data are limited in that we are not able to determine the hierarchy of this relationship in the current study. It is possible that FGFR3 signaling from mesenchymal cells coordinates a change in activity of cells of the haematopoietic lineage, or alternatively the cells of the haematopoietic lineage may exert a dominant effect. In light of recent studies implicating macrophages as a key regulator of fracture healing (Alexander et al., 2011; Baht et al., 2015; Raggatt et al., 2014; Vi et al., 2015) this poses a fascinating and crucial question to further understand the role of FGFR3 in skeletal biology and forms the basis of further work in this thesis (See Chapter 4).

Our study sheds more light on the role of FGFR3 in skeletal repair by identifying that it effectively acts as a bone switch, regulating the tightly coordinated balance of intramembranous ossification and endochondral ossification, with FGFR3 deficiency inhibiting intramembranous ossification and enhancing endochondral ossification. The role of FGFR3 in directing the differentiation of these multipotent osteoprogenitor cells may prove to be an important advance in the field of skeletal tissue regeneration for therapeutic purposes. The successful regeneration of high quality, functional tissue will depend strongly on the ability to understand and replicate the critical steps in the differentiation of mesenchymal cells that occurs under normal development and repair. Furthermore our data suggests that FGFR3 may also prove to be a useful target for novel biological agents in enhancing fracture healing.
CHAPTER 3 – FGFR3 REGULATES
OSTEOPROGENITOR NUMBER, PROLIFERATION
AND DIFFERENTIATION
3.1 Abstract

FGFR3 affects the size and structure of healing murine fracture callus, and acts as a bone switch. Specifically, deficiency in FGFR3 signaling inhibits intramembranous ossification and enhances endochondral ossification. Considering that one of the key steps in intramembranous ossification is the direct differentiation of osteoprogenitor cells to osteoblasts to form bone, we hypothesized that there is a defect in osteoblastic differentiation in mice deficient for FGFR3. Using a series of colony forming unit–fibroblast (CFU-F) assays, we have demonstrated that skeletal stem cells (SSCs) in $Fgfr3^{+/−}$ bone marrow are scarcer, are associated with a reduced capacity for proliferation of BMSC colonies to which they contribute, and show reduced proliferation in the periosteum of healing fractures in-vivo. Furthermore, using colony forming unit-osteoblast (CFU-O) assays and gene expression analysis we also showed that these defective SSCs (and BMSC colonies) have a reduced capacity for osteoblast differentiation. Taken together these findings offer an explanation for the in-vivo clinical findings of defective intramembranous ossification in fracture healing in mice deficient in FGFR3.
3.2 Introduction

Osteoblasts are the primary bone forming cells and a detailed understanding of their proliferation-differentiation sequence and the factors that control it are critical to be able to utilize osteoblasts for purposes of bone tissue engineering. Creating functional osteoblasts that mimic their differentiation in normal development will be important for a wide range of applications in musculoskeletal regenerative medicine such as for developing cell-based therapies for treating genetic skeletal diseases, for enhancing fracture repair and stimulating bone regeneration.

Fibroblast growth factor receptor 3 (FGFR3) has a key role in normal skeletal development. Gain-of-function mutations in FGFR3 cause numerous skeletal dysplasias including achondroplasia, thanatophoric dysplasia and hypochondroplasia and are characterized by a short-limbed phenotype (Oberklaid et al., 1979) (Rousseau et al., 1994; Shiang et al., 1994) (Maroteaux et al., 1967; Orioli et al., 1986) (Walker et al., 1971). Human disease with loss-of-function of FGFR3 (CATSHL syndrome) is an uncommon condition with a phenotype of tall stature and scoliosis (abnormal curvature of the spine), but confirms the importance of FGFR3 on skeletal development (Toydemir et al., 2006). FGFR3 has been shown to act as a negative regulator of endochondral bone growth, by inhibiting the proliferation and differentiation of growth plate chondrocytes (Colvin et al., 1996; Deng et al., 1996; Naski et al., 1996). Although most authors describe the importance of FGFR3 in chondrocyte proliferation and differentiation (Colvin et al., 1996; Deng et al., 1996; Arata Nakajima et al., 2003; Naski et al., 1996), Rundle also described the expression of FGFR3 from osteoblasts in the healing fracture callus suggesting a potential role for FGFR3 in intramembranous as well as endochondral ossification (Rundle et al., 2002). Despite earlier studies of the cranium suggesting that there were no significant sutural or cranial defects related to intramembranous ossification due to abnormalities in FGFR3 function (Colvin et al., 1996; Deng et al., 1996), more recent studies in mice and humans with FGFR3 related skeletal dysplasias showed that premature fusions of the synchondrosis were seen, as well as abnormally ossified dermatocranial bones, and alterations in osteoblast differentiation in-vivo, suggesting that FGFR3 may indeed be involved in intramembranous ossification (Di Rocco et al., 2014; Valverde-Franco et al., 2004).
Bone marrow stromal cells (BMSCs) are a heterogeneous group of cells and contain a subgroup of multipotent skeletal stem cells (SSCs) that have the ability to differentiate into osteoblasts, chondrocytes and adipocytes, which form bone, cartilage and fat respectively (Bianco, Riminucci, Gronthos, & Robey, 2001; Friedenstein, 1980; S. A. Kuznetsov et al., 2007). BMSCs have the ability to form colonies in vitro, and each colony is considered to form from a single-cell precursor, the colony forming unit-fibroblast (CFU-F) (Friedenstein, 1976, 1980; Friedenstein, Gorskaja, & Kulagina, 1976; Friedenstein et al., 1978; Latsinik, Gorskaia Iu, Grosheva, Domogatskii, & Kuznetsov, 1986). The number of BMSC colonies that form therefore represents the number of CFU-Fs in a sample, and the number of CFU-Fs per number of cultured cells gives a calculation of colony forming efficiency (CFE), which equates to the number of CFU-Fs in the bone marrow of any given individual (Sergei A Kuznetsov, Mankani, Bianco, & Robey, 2009). CFU-Fs have been shown to be a heterogeneous hierarchy of progenitors, some of which are SSCs with multipotent characteristics, whereas some are more committed progenitors (Bennett, Joyner, Triffitt, & Owen, 1991; Berry, Grant, McClure, & Rooney, 1992; Bianco et al., 2001; Herbertson & Aubin, 1997). Nevertheless, considering that there is a lack of consensus on biochemical or morphological markers for what truly constitutes a CFU-F (OR SSC), then the CFE of any given bone marrow sample is currently the best approximation to the number of SSCs in a marrow sample (Bianco et al., 2001; Sergei A Kuznetsov et al., 2009).

Consistent with the difficulty in identifying markers of multipotent mesenchymal progenitors in bone marrow, the identification of specific osteoprogenitors is also challenging, yet under specific in vitro conditions cells within BMSC colonies can differentiate to osteoblast-like cells, which form bone nodules identifiable by the production of bone matrix and mineral (Bellows, Ciaccia, & Heersche, 1998; Bhargava, Bar-Lev, Bellows, & Aubin, 1988; Herbert, Lecouturier, Masquelier, Hauser, & Remacle, 1997; Nefussi, Boy-Lefevre, Boulekbache, & Forest, 1985). The number of bone nodules, termed colony forming units osteoblast (CFU-O), represents the number of osteoprogenitors in a bone marrow sample as each colony again forms from a single cell precursor (J E Aubin, 1998). The number of bone nodules per number of cultured cells gives a calculation of the osteogenic efficiency of a sample, and serves as measure of the bone forming capacity of the bone marrow of an individual. CFU-F and CFU-O assays therefore form
an important method for systematically investigating the characteristics, and ability to differentiate, of the mesenchymal progenitor population.

We previously showed that FGFR3 affects the size and structure of healing murine fracture callus, and acts as a bone switch. Specifically, deficiency in FGFR3 signaling inhibits intramembranous ossification and enhances endochondral ossification. Considering that one of the key steps in intramembranous ossification is the differentiation of osteoprogenitor cells to osteoblasts, to form bone, we hypothesized that deficiency in FGFR3 decreased the number, or proliferative ability, of osteoprogenitors in bone marrow and furthermore that impaired FGFR3 signaling reduces the ability of osteoprogenitors to differentiate to functional osteoblasts. We investigated these questions by examining the characteristics and osteogenic potential of mesenchymal progenitors in bone marrow and periosteum of Fgfr3+/− mice.
3.3 Materials and Methods

3.3.1 Mice

Fgfr3\textsuperscript{+/-} mice were purchased from Jackson Laboratory (B6; 129S-Fgfr3\textsuperscript{tm1Dor}/J, Stock #004234) and maintained at the Toronto Center for Phenogenomics (TCP). Animal protocols were approved by the animal care committee of the TCP, in accordance with the regulations and guidelines from the Canadian Council on Animal Care. Genomic DNA from skin samples of harvested mice were used to genotype animals by PCR.

3.3.2 Tibia fracture generation

Semi-stabilized tibial fractures were generated on 12-week-old male Fgfr3\textsuperscript{+/-} and WT mice following a protocol previously described (Y. Chen et al., 2007). In brief, the mice were anaesthetized using inhalational general anaesthesia. The left hind limb of each mouse was surgically prepared, by shaving and cleaning with disinfectant. A small anterior midline incision was made over the knee joint and proximal tibia. A 0.7 mm pilot hole was made in the proximal tibial epiphysis just medial to the insertion of the patella tendon using a hollow needle. A 0.7mm Anticorrot insect pin (Fine Science Tools, http://www.finescience.ca) was then inserted into the medullary cavity of the intact tibia and advanced to the distal tibia. A transverse fracture was then induced at the mid-shaft of the tibia using blunt scissors. The insect pin was cut 5-7mm proud of the proximal tibial epiphysis and the skin incision closed with a series of absorbable vicryl rapide sutures, and metallic wound clips. Analgesic given subcutaneously (buprenorphine, 0.1mg/kg/twice per day) was administered for 3 days after surgery. Previous data shows that a fracture generated in this manner heals through both intramembranous and endochondral ossification (Hiltunen et al., 1993). The animals were allowed to maintain full weight bearing in their cages following surgery. At PFD3 following fracture, samples of fractured and unfractured tibiae were harvested following euthanasia with inhaled carbon dioxide. A minimum of 4 mice per genotype were used for analysis.

3.3.3 Histology

Fractured tibiae were harvested at PFD3 fixed in 10% formalin following by decalcification with 20% (w/v) EDTA pH 8.0 or formic acid bone decalcifier (Decal Chemical Corp, Tallman,
NY) and embedded in paraffin. Serial 5µM sections of paraffin embedded tissues were deparaffinized, and rehydrated through an alcohol gradient to water.

3.3.4 In-vivo BrdU assay

For assessment of periosteal cell proliferation \( Fgfr3^{+/−} \) and WT mice that had previously undergone tibial fracture surgery, as described above, received an intraperitoneal injection with 150µg/g body weight Bromodeoxyuridine (BrdU) (Sigma) in 0.9% saline 3 hours prior to fracture harvest on PFD3. Limbs were then harvested and decalcified as above. Sections were digested with 1% (w/v) pepsin in 0.5M acetic acid (Sigma) for 30 min at 37°C and endogenous peroxide activity was blocked with 3% (v/v) peroxide in methanol. Nonspecific binding was blocked with 2% (v/v) normal horse serum (Vectorlabs, Burlingame, CA) in PBS and primary antibody to BrdU (Roche 11170376001, Germany) was incubated for 1 hour at 25°C. Primary antibody binding was detected using biotinylated secondary antibody and avidin-linked peroxidase (Vectastain Universal Elite ABC kit, Vectorlabs, Burlingame, CA). Periosteal cell proliferation was assessed using computer assisted enumeration (Adobe Photoshop, San Jose CA, USA) by counting the number of BrdU-positive cells per mm² of periosteal tissue, with measurements were taken at 8 ROI per section of callus tissue, 5 sections per mouse, 4-5 mice per genotype.

3.3.5 Colony forming units – fibroblast (CFU-F)

Femora and Tibiae from 12-week old female \( Fgfr3^{+/−} \) and WT mice were isolated and debrided of soft tissue. Bone marrow was flushed into BMSC culture media containing α-modification of eagle’s medium (α-MEM) (Wisent Inc, Cat# 310-012-CL, St. Bruno, CA), 10% FBS (Gibco Life Technology, Cat# 16000-044, Burlington, ON) and 1x antibiotic and antimycotic (Wisent Inc, Cat# 450-115-EL). Where required FGFR3 inhibitor 10µM SU5402 (Santa Cruz Biotechnology Inc. Cat# sc-204308) diluted with 1:10,000 DMSO was added to the media. The cell suspensions were passed through an 18G needle and 70µm cell strainer to dissociate clumps of cells. Single cell suspensions were plated at a density of 1x10⁶ cells/cm² surface area on 6-well plates, in BMSC culture medium for 7 days, with 50% of the media changed at day 4. BMSC colonies arising from Colony forming units-fibroblasts (CFU-F) were analyzed at day 7 by staining colonies with 0.5% (w/v) Crystal Violet (Sigma, Cat#: C3886) in 25% Methanol for 15 minutes at room temperature. CFU-Fs were counted manually using a microscope. A CFU-F
derived BMSC colony was defined as a discrete colony that contained 50 or more cells. A minimum of 3 wells per mouse in 3 mice per genotype, or condition, was used. BMSC proliferation was evaluated by BrdU assay. Cells grown on coverslips were pulsed by adding 1:100 diluted BrdU Labeling Reagent (Zymed/Life Technologies, Cat# 00-0103) for 16 hours. Incorporated BrdU was detected by mouse monoclonal anti-BrdU antibody (Roche, Cat# 11170376 001, Germany) following the manufacturers protocol. Cell proliferation was analyzed using computer assisted enumeration with Photoshop Extended CS6 (Adobe Photoshop, San Jose CA, USA) by calculating the ratio of BrdU-positive cells to BrdU-negative cells per field of view. Three mice were used per genotype, 4 colonies examined per mouse.

3.3.6 Limiting Dilution

Limiting dilution was performed as standardized technique (Bellows & Aubin, 1989). Whole bone marrow aspirate was obtained and prepared for cell culture as for CFU-F assay (above). Cells were plated in 96-well plates with α-modification of eagle’s medium (α-MEM) (Wisent Inc, Cat# 310-012-CL, St. Bruno, CA), 10% FBS (Gibco Life Technology, Cat# 16000-044, Burlington, ON) and 1x antibiotic and antimycotic (Wisent Inc, Cat# 450-115-EL). Cell numbers were adjusted to give a starting concentration of $3.33 \times 10^6$ cells/ml for WT, and $4 \times 10^6$ cells/ml for $Fgfr3^{+/−}$, from which serial dilutions were made. Final cell dilutions ranged from $1 \times 10^6$ cells per well to $26 \times 10^3$ cells per well for WT, and from $1.2 \times 10^6$ cells per well to $31.2 \times 10^3$ cells per well for $Fgfr3^{+/−}$ in 0.3 ml aliquots. A total of 10 serial dilutions with 24 replicates per dilution were set. Cultures were left undisturbed for 4 days. Half the media was exchanged for fresh media on day 4. On day 7 all the media was changed for fresh media and non-adherent cells were removed. Cell culture continued for a further 7 days with change of media every 2-3 days. BMSC colonies were identified by staining cells with 0.5% (w/v) Crystal Violet (Sigma, Cat#: C3886) in 25% Methanol for 15 minutes at room temperature. BMSC colonies were counted manually using a microscope. A BMSC colony was defined as a discrete colony that contained 50 or more cells. The fraction of wells not containing a colony for each cell plating density was calculated, and these points were plotted against the number of cells plated per well. The proportion of CFU-Fs in whole marrow aspirate is reflected by the number of cells required to form one colony, assuming each colony originates from a single CFU-F, and was determined by the point at which the line of best fit crossed the y-axis at 0.37 (Bellows & Aubin, 1989). The fraction of wells with no nodules is related to the mean number of CFU-Fs
based upon the following formula: \( F_0 = e^{-x} \), where \( F_0 \) is the fraction of wells with no colonies, and \( x \) represents the mean number of CFU-Fs per well. Based on a Poisson distribution, \( F_0 = 0.37 \) corresponds to the dilution at which there is 1 CFU-F per well.

3.3.7 Colony forming unit – osteoblast (CFU-O)

Bone marrow cells were cultured as for the CFU-F assay, described above, until day 7, at which time the media was changed for osteoblastic differentiation media, which consisted of BMSC culture media, as described above, with the addition of 50\( \mu \)g/ml Ascorbic Acid (Sigma, Cat# A4544), \( 10^{-8} \)M Dexamethasone (Sigma, Cat# D8893), 8mM \( \beta \)-Glycerol phosphate (Sigma, Cat# G9891). The osteoblastic differentiation media was subsequently changed every 2-3 days for the duration of the experiment. At day 7, 14 or 21 cells were rinsed with PBS and fixed with 10% formalin for 30 mins and rinsed twice with water. Alkaline phosphatase staining (Fast Red TR/Naphthol AS-MX Tablets: Sigma#F4523, manufactory protocol) and Von Kossa staining (2.5% (w/v) Silver Nitrate: Sigma #S8157, 30 minutes at room temperature on light box) were performed to assess for osteoblastic differentiation and mineralization. CFU-Os were counted manually using a microscope. A CFU-O was defined as a discrete colony that contained 50 or more cells with the majority of cells in each colony staining for ALP or Von Kossa respectively. All quantification performed with 3 wells per mouse, and minimum of 3 mice per genotype.

3.3.8 Gene expression analysis

Total RNA from culture cells were extracted by TRIzol reagent (ambion by Life Technologies, Cat # 15596018) and purified by RNeasy Mini kit (Qiagen, Cat#74106) according to the manufacturers protocol. cDNA was synthesized by using Superscript II kit (Invitrogen by life technologies, Cat # 18064-014) with random hexamer primer. Target and endogenous control genes were amplified with TaqMan MGB probes labeled with 6-FAM (Applied Biosystems) for Runx2, Sox9, Osx, Coll, Bsp, Opn, Ocn, ALP, Coll2, ACTB. Reactions were performed in triplicate in a 96-well plate using OneStep Plus (Applied Biosystems) for 40 cycles. Gene expression was analyzed by real-time quantitative PCR using \( \Delta \Delta C_t \) methods relative to the expression of control house-keeping gene \( \beta \)-actin.
3.3.9 Statistical analysis

Data are expressed as mean, +/-95% confidence intervals and were calculated for each sample. Student’s t-test was used to compare data sets. The data were considered to be statistically significant at a confidence level of 95% (p<0.05). For limiting dilution, the ELDA: Extreme Limiting Dilution Analysis Software was used (Hu & Smyth, 2009).
3.4 Results

The mesenchymal cells that contribute to both intramembranous and endochondral ossification during bone fracture repair; namely osteoblasts, the primary bone-forming cells, and chondrocytes, the primary cartilage-forming cells, are believed to originate from a common progenitor cell, the skeletal stem cell (SSC), which are thought to be a discrete subpopulation of bone marrow stromal cells (BMSCs), a proportion of which undergo proliferation and then differentiation into either osteoblasts or chondrocytes at the earliest stages of fracture repair. We demonstrated, in Chapter 2, that during fracture repair in \( Fgfr3^{+/−} \) mice the balance of intramembranous and endochondral ossification is altered, with a decrease in intramembranous ossification and an increase in endochondral ossification.

To investigate the cellular mechanism underpinning this altered mesenchymal behaviour, we first examined the number and proliferative capacity of BMSCs aspirated from the long bones of both \( Fgfr3^{+/−} \) and WT mice in-vitro. A colony forming unit-fibroblast (CFU-F) assay was performed (Fig. 3-1A). In addition to WT and \( Fgfr3^{+/−} \) samples, a third condition was established with WT cells also being treated with the FGFR inhibitor SU5402 (Mansukhani et al., 2000). Each BMSC colony forms from a single cell precursor (the CFU-F) with the colony number and size reflecting the number and proliferative capacity of CFU-Fs present in the sample, and thus SSCs in the animal (Bianco et al., 2001; Friedenstein, 1976; S. A. Kuznetsov et al., 1997; Owen & Friedenstein, 1988). Crystal violet staining of the colonies demonstrated that both genetic and chemical inhibition of FGFR3 (\( Fgfr3^{+/−} \) BMSCs and WT BMSCs +SU5402 respectively) reduced BMSC colony count in-vitro compared to WT BMSC controls. To investigate the effect of plating density on BMSC colony formation, the CFU-F assay was then repeated at different seeding densities (0.5 x 10^6, 0.75 x 10^6, and 1 x 10^6 cells per cm^2) (Fig. 3-1B). Here, we found that the number of BMSC colonies formed per million cells plated remained constant, with consistently reduced CFU-F efficiency (CFE) from \( Fgfr3^{+/−} \) bone marrow compared to WT.
Figure 3-1. FGFR3 regulates BMSC colony formation
A) Representative wells showing colonies stained with crystal violet from CFU-F assay of WT, Fgfr3−/− and WT+SU5402 (FGFR3 inhibitor) murine bone marrow aspirate after 7 days of culture. Quantification shows reduced mean BMSC colony count per well in both Fgfr3−/− and WT+SU5402 samples compared to WT. n=3 mice per genotype or condition, with 3 wells per mouse. B) Quantification of mean BMSC colony count per well from a CFU-F assay performed using WT and Fgfr3−/− bone marrow aspirate under same conditions as A, but at a range of different seeding densities. Fgfr3−/− samples show proportionally reduced colony number at each seeding density compared to WT samples. C) Limiting dilution analysis of CFU-Fs. WT and Fgfr3−/− bone marrow cells were inoculated into plates at concentrations ranging from 2.6x10^4 to 1.2x10^6 cells per well in 96 well plates and maintained in vitro for 14 days. Wells were stained with crystal violet and screened for the presence of BMSC colonies. The fraction of wells without colonies was plotted against cell number per well. Based upon a Poisson distribution, the probability of no colonies at the 0.37 level shows that approx. 1 of every 0.47x10^6 WT cells and 1 of every 1x10^6 Fgfr3−/− cells is a CFU-F. All data show mean colonies per well ± 95%CI. Statistics for A) and B) show *p<0.05, ** p<0.005, ***p<0.0005 (2-sided, 2-sample Student’s t-test). Statistics for differences in CFU-F frequencies in C) p<0.0005 (Chi square).
The finding that $Fgfr3^{+/−}$ bone marrow has a reduced CFE may represent a reduction in the number of SSCs in $Fgfr3^{+/−}$ animals. It is difficult to enumerate SSCs based on biochemical and morphological characterization, due to a lack of consensus on criteria for identification (Bellows & Aubin, 1989), so therefore we performed a limiting dilution assay to answer the question of progenitor number (Fig. 3-1C). Assuming that each BMSC colony forms from a single CFU-F cell precursor, we used the CFE as an indicator of the number of CFU-Fs present in the aspirated marrow population. Based on the features of a Poisson distribution, the intersection of a value of 37% of wells not forming BMSC colonies with the line of best fit, we showed that fewer CFU-Fs are present in bone marrow aspirate of $Fgfr3^{+/−}$ compared to WT. We calculated that approximately 1 in $10^6$ plated $Fgfr3^{+/−}$ cells has the capacity to form BMSC colonies compared to approximately 1 in $0.47 \times 10^6$ WT cells.

Next, we used a BrdU assay to assess the proliferative capacity of the cells within the BMSC colony (Fig.3-2A). $Fgfr3^{+/−}$ colonies showed a reduction in the proportion of BrdU-positive cells to BrdU-negative cells compared to WT BMSC colonies (Fig.3-2B).

During fracture repair, mesenchymal progenitor cells residing in the periosteum migrate to the fracture site where they proliferate and differentiate into a rich and primary source of osteoblasts and chondrocytes to repair the injured bone (Céline Colnot, 2009; Christa Maes et al., 2010). To determine whether the in-vitro finding of reduced BMSC proliferation in $Fgfr3^{+/−}$ mice was recapitulated in mesenchymal progenitors in healing fracture calli, histological sections of WT and $Fgfr3^{+/−}$ mouse tibial fractures harvested at PFD3 were analyzed for BrdU incorporation into cells of the periosteum and extra-periosteal tissues (Fig. 3-2A, 3.2B). $Fgfr3^{+/−}$ healing fracture calli showed less BrdU incorporation, reflecting fewer proliferating cells, in the periosteum and extra-periosteal tissues at PFD3 compared to WT fracture calli (Fig. 3-2F). This demonstrated an impaired early proliferative response in the periosteum with FGFR3 deficiency.
Figure 3-2. FGFR3 regulates proliferation of BMSCs in-vitro and periosteal osteoprogenitors in-vivo

A) Representative images from an in-vitro CFU-F assay of BMSC colonies stained for BrdU to show proliferating cells (brown) at 2x magnification (upper) and 10x magnification (lower), using WT and \( \text{Fgfr3}^{+/} \) murine bone marrow, after 7 days of culture. B) Quantification of in-vitro CFU-F proliferation assay showed that there were fewer proliferating cells within the \( \text{Fgfr3}^{+/} \) BMSC colonies than WT colonies. \( n=3 \) mice per genotype. C) Schematic diagram of a murine tibial fracture with surrounding callus. Large box represents area shown in D. Small box containing dark grey shaded region demonstrates example of periosteal area analyzed quantitatively for the presence of BrdU positive osteoprogenitor cells and corresponds to area shown in E. D) Representative low magnification (2.5x) histological sections of PFD3 tibial fracture callus stained for BrdU. Box identifies area shown in E. E) Representative high magnification (10x) histological sections of PFD3 tibial fracture callus stained for BrdU to show cell proliferating cells (brown) in periosteum from WT and \( \text{Fgfr3}^{+/} \) mice. F) Quantification of in-vivo periosteal proliferation study showed fewer proliferating cells per area of periosteum examined within the \( \text{Fgfr3}^{+/} \) fracture callus than compared to in WT callus. 8 ROI per section of callus, 5 sections per mouse, \( n=4-5 \) mice per genotype. Data represent mean values +/- 95%CI. **\( p<0.005 \) (2-sided, 2-sample Student’s t-test).
Despite each BMSC colony arising from a single CFU-F precursor, cells comprising BMSC colonies show significant heterogeneity and, under appropriate experimental conditions, have the potential to differentiate to multiple cell types of the mesenchymal lineage, although not all CFU-Fs, or indeed BMSC colonies, have the ability to differentiate to all of the mesenchymal cell types (Bianco et al., 2001; Sergei A Kuznetsov et al., 2009). On the basis that during fracture repair $Fgfr3^{+/\text{-}}$ mice demonstrated reduced intramembranous ossification, and considering that BMSCs are the candidate precursors of the osteoblasts, which participate in the formation of fracture callus, we characterized the expression osteoblast genes in the cells comprising the BMSC colonies (Fig. 3-3). Interestingly, qPCR analysis showed reduced mRNA expression of genes associated with osteoblast differentiation; Runx2, collagen type1 (Col1), alkaline phosphatase (ALP) and bone sialoprotein (BSP), in $Fgfr3^{+/\text{-}}$ colonies when compared to WT, despite culture media not containing additives used to drive osteogenic differentiation.

![Figure 3-3. Osteoblast genes are downregulated in $Fgfr3^{+/\text{-}}$ BMSC colonies](image)

Expression of mRNA as markers of osteoblast differentiation in BMSC colonies during CFU-F assay from WT and $Fgfr3^{+/\text{-}}$ bone marrow at day 7 of culture. Expression of Runx2, Collagen type1, bone sialoprotein and alkaline phosphatase are shown. Data are mean values of relative expression of $Fgfr3^{+/\text{-}}$ compared to WT +/-95% CI. *p<0.05 (2-sided, 2-sample Student’s t-test).

Taken together these data suggest that skeletal stem cells (SSCs) in $Fgfr3^{+/\text{-}}$ bone marrow are scarcer, are associated with a reduced capacity for proliferation of colonies that they form in-vitro, reduced proliferation in the periosteum of healing fractures in-vivo, and in addition may have a predetermined limitation in potential for their subsequent differentiation into cells of the osteoblast lineage.
Due to the defects in \( Fgfr3^{+/—} \) CFU-F number, BMSC proliferation in-vitro and in-vivo, and due to the abnormalities in intramembranous ossification seen in fracture repair in \( Fgfr3^{+/—} \) mice, we next examined the osteogenic capacity of CFU-F derived BMSC colonies in-vitro using a colony forming unit-osteoblast (CFU-O) assay (Fig. 3-4A). The CFU-O assay quantified those colonies that stained positively for alkaline phosphatase (ALP) as a marker of bone matrix, and Von Kossa (VK) as a marker of bone mineralization following culture in osteogenic differentiation media. \( Fgfr3^{+/—} \) BMSC colonies were shown to generate fewer CFU-Os, as demonstrated by both a reduced number of ALP+ staining colonies and VK+ staining colonies compared to WT BMSC colonies (Fig. 3-4B)).

**Figure 3-4. FGFR3 deficiency reduces CFU-O formation of BMSCs in-vitro**
A) Representative wells from colony forming unit-osteoblast assay (CFU-O). BMSCs were aspirated from the tibiae of WT and \( Fgfr3^{+/—} \) mice, adhered to tissue culture plastic and differentiated in osteogenic media. After 14 days of differentiation, cultures were washed, fixed and stained for alkaline phosphatase (ALP) or mineral (Von Kossa). B) CFU-Os were quantified by counting ALP+ and VK+ colonies for matrix and mineral respectively. Graphs shows reduced mean ALP+ and VK+ (CFU-O) colony count per well in \( Fgfr3^{+/—} \) samples compared to WT. n=3 mice per genotype, with 3 wells per mouse. Data are mean values +/- 95%CI. **p<0.005 (2-sided, 2-sample Student’s t-test).
To determine whether the diminished capacity for forming CFU-Os from \( Fgfr3^{+/\text{-}} \) bone marrow was a merely reflection of reduced CFU-F efficiency (or CFE), we calculated the ratio of CFU-O to the total number of BMSC colonies, by over-staining the CFU-O proven colonies with crystal violet (Fig. 3-5A). The ratio of \( Fgfr3^{+/\text{-}} \) CFU-Os to BMSC colonies was also reduced compared to WT controls thus demonstrating a true reduction of osteogenic efficiency in \( Fgfr3^{+/\text{-}} \) BMSC colonies (Fig. 3-5B), which can be considered an approximation of the osteogenic potential of SSCs (Bianco et al., 2001; Sergei A Kuznetsov et al., 2009).

**Figure 3-5. Reduced CFU-O efficiency of \( Fgfr3^{+/\text{-}} \) BMSCs is not an artefact of reduced CFU-F efficiency.**

A) Representative wells from colony forming unit-osteoblast assay (CFU-O). BMSCs were aspirated from the tibiae of WT and \( Fgfr3^{+/\text{-}} \) mice, adhered to tissue culture plastic and differentiated in osteogenic media. After 14 days of differentiation, cultures were washed, fixed and stained for alkaline phosphatase (ALP) and quantified for CFU-O formation, next the same wells were overstained with crystal violet (CV) for quantification of overall BMSC colony formation B) Osteogenic efficiency was quantified by counting ALP+ colonies for matrix. Graphs shows reduced absolute mean ALP+ (CFU-O) colony count per well, and in addition show a reduction in the ratio of ALP+ colonies to total number of colonies (CV+) in \( Fgfr3^{+/\text{-}} \) samples compared to WT thus we demonstrated both reduced CFU-F efficiency and CFU-O efficiency. n=3 mice per genotype, with 3 wells per mouse. Data are mean values +/- 95%CI. **p<0.005 (2-sided, 2-sample Student’s t-test).
In addition we examined the expression of osteoblast genes Runx2, osterix (Osx), BSP, OPN, Alp, Col1 and OCN in the cells of the CFU-O colonies at day 10 and 21 of culture (Fig. 3-6). QPCR analysis of the mRNA expression showed decreased expression of Runx2, Osx, Col1, Alp and OPN at day 10 in Fgfr3+/− CFU-Os, and in addition at day 21, a reduction in BSP and OCN expression was also identified in Fgfr3+/− CFU-Os. These findings demonstrate not only a reduction in the expression of important osteoblast genes during forced osteogenesis in Fgfr3+/− CFU-Os, but in addition these results also align with the recognized in-vivo temporal relationship of gene expression in osteogenesis (J E Aubin, 1998).

Figure 3-6. Reduced expression of osteoblast genes in Fgfr3+/− CFU-O colonies
Levels of mRNA expression of osteoblast differentiation markers during CFU-O assay from WT and Fgfr3+/− BMSCs measured using qPCR at A) 10 days of differentiation and B) 21 days of differentiation. Levels are measured relative to WT expression at day 0. Expression levels of all mRNA osteoblast markers including transcription factors Runx2 and Osterix (Osx), and extracellular matrix proteins Collagen type 1 (Col1a1), osteopontin (OPN), alkaline phosphatase (Alp), bone sialoprotein (BSP) and osteocalcin (OCN) are reduced in Fgfr3+/− samples compared to WT. data are mean values +/- 95%CI. *p<0.05, ***p<0.0005 (2-sided, 2-sample Student's t-test).
3.5 Discussion

The purpose of this study was to investigate the role of FGFR3 in osteoblast differentiation by examining the number and proliferation of mesenchymal progenitors (SSCs) in the bone marrow of \( Fgfr3^{+/+} \) mice and to compare the ability of these progenitors to differentiate to osteoblasts using a series of in-vitro colony forming unit assays. We found that in \( Fgfr3^{+/+} \) bone marrow SSCs were scarcer and that cells within BMSC colonies derived from CFU-Fs showed reduced proliferation. Furthermore, we found that osteoprogenitors in the periosteum of healing fractures also demonstrated a reduction of proliferation in-vivo. Finally, using colony forming unit-osteoblast (CFU-O) assays and gene expression analysis we also showed that these defective SSCs (and BMSC colonies) have a reduced capacity for osteoblast differentiation. Taken together these findings offer an explanation for the in-vivo clinical findings of defective intramembranous ossification in fracture healing in mice deficient in FGFR3.

We showed, using a CFU-F assay, that \( Fgfr3^{+/+} \) bone marrow forms fewer BMSC colonies than WT bone marrow in-vitro. Seeing as each BMSC colony is derived from a single CFU-F, this finding equates to a reduction in the number of CFU-Fs in \( Fgfr3^{+/+} \) murine bone marrow (Friedenstein, 1976, 1980; Friedenstein et al., 1976; Friedenstein et al., 1978; Latsinik et al., 1986). The CFU-F concentration is currently the best experimental approximation to SSC concentration in bone marrow (Sergei A Kuznetsov et al., 2009).

Although a seemingly crude assay, the CFU-F assay has long been accepted as a recognized measure of the concentration of SSCs, or multipotent mesenchymal progenitors, in sample of bone marrow (Sergei A Kuznetsov et al., 2009). In part this is because no truly specific biochemical or morphological markers have thus far been validated and accepted to further refine the characterization of progenitors in a bone marrow sample (Bianco et al., 2001). Further limitations of the CFU-F assay are that results may show large variability between species, between replicates, and based on specific culture conditions (J. E. Aubin, 1999; Bianco et al., 2001; Sergei A Kuznetsov et al., 2009). Despite absolute numbers of progenitors therefore being difficult to quantify, the CFU-F assay does allow the opportunity for comparison between differentially manipulated samples, be it genetically modified cells, or by modification of the culture conditions. We found that despite altering the initial cell seeding density in the CFU-F assay the reduction in colonies formed using \( Fgfr3^{+/+} \) bone marrow remained proportionate with
approximately a 30% decrease in number of colonies compared to WT. This linear relationship suggests that the defect in CFE is dependent on a limited number of progenitor cells (CFU-F) in a bone marrow sample, with the ability to form BMSC colonies (J. E. Aubin, 1999; Bellows & Aubin, 1989).

To confirm the reduction in concentration of SSCs in the bone marrow of Fgfr3+/− mice we used a limiting dilution assay, which also showed the scarcity of CFU-F in Fgfr3+/− samples. Interestingly the slope of the relationship between cell densities and negative wells was not a straight line, but that of a down-sloping curve, whereby a small increase in the concentration of seeded cells dramatically increased the number of wells positive for colony formation. Using established statistical analysis we determined that the estimated slope of the line of best fit was 3.72. (Hu & Smyth, 2009) Any value greater than 1 suggests that the likelihood of a single-hit model is very low, rather the likelihood of the effect seen is due to a multi-hit model, meaning that the contribution of more than one cell type accounted for the increase in colony formation and resultant down-sloping shape of the graph. Other groups have found contrasting results, where CFU-Fs retain the ability to form colonies as an individual cell and therefore are not reliant upon the presence of multiple different cell types to form a colony (J. E. Aubin, 1999; Bellows & Aubin, 1989; Dozmorov, Eisenbraun, & Lefkovits, 2000).

CFU-F that form BMSC colonies have a variable potential to differentiate to cells of the mesenchymal lineages including osteoblasts, chondrocytes and adipocytes. In-vivo, mesenchymal progenitor cells within periosteum surrounding a fractured bone undergo intense proliferation within the first few days following fracture and, consistent with the in-vitro behaviour of CFU-F, are able to differentiate to osteoblasts and chondrocytes (Céline Colnot, 2009; Christa Maes et al., 2010). These periosteal cells have been shown to be the major source of osteoblasts and chondrocytes in a healing fracture. Our data show that mesenchymal progenitor cells in the periosteum of Fgfr3+/− mice have a reduced proliferation at PFD3. It is possible therefore that the defect in mesenchymal proliferation that is seen in the periosteum of Fgfr3+/− mice reflects the role of FGFR3 in establishing a pool of osteoprogenitors and helps to explain the finding of reduced intramembranous ossification in fracture healing that we previously identified in Fgfr3+/− mice.
Interestingly, we found that BMSCs cultured from $Fgfr3^{+/−}$ bone marrow showed a reduction in the mRNA expression of osteoblast related genes (Runx2, Col1a1, ALP and BSP) within their colonies despite no specific attempt to induce differentiation to any specific lineage. Stromal colonies are known to comprise a heterogeneous cell group with a variable capacity for further differentiation to bone, cartilage and fat (Bianco et al., 2001; Friedenstein, 1980; S. A. Kuznetsov et al., 2007). Gene expression profiling is one way that has previously been used to analyze the differentiation potential of stromal cell lines, specifically identifying the expression of osteoblast specific genes suggests the potential for osteoblastic differentiation based on a concept of osteogenic imprinting (Bosch et al., 2013; Satija, Sharma, Afrin, Tripathi, & Gangenahalli, 2013; Ulrich et al., 2013). However, previous researchers have identified that genes related to osteogenic, chondrogenic and adipogenic differentiation may be identified at varying points in normal stromal cell culture albeit with varying levels, without necessarily confirming the true in-vivo differentiation potential. These varying gene expression profiles may just reflect the inherent plasticity of the stromal cell system (Satomura, Krebsbach, Bianco, & Gehron Robey, 2000). For example Satomura identified, in a series of murine clonal stromal cell lines, that all expressed Runx2 (the master regulator of osteogenic differentiation), Col1a1 and ALP, yet not all lines proved to support osteogenesis in-vivo. BSP is, on the other hand, considered a more specific marker of osteoblast differentiation (Bianco, Rimmunici, Bonucci, Termine, & Robey, 1993), and the majority of their clonal lines that did support osteogenesis in-vivo also expressed high levels of BSP mRNA, whilst the non-osteogenic clonal stromal lines did not express BSP (Satomura et al., 2000). Whilst interpretation must therefore be made with caution concerning gene expression data in stromal cell culture, our data suggests that the BMSC colonies cultured from $Fgfr3^{+/−}$ bone marrow may have a reduced capacity for differentiating to the osteoblast lineage compared to WT, due to a reduction of the level of osteogenic imprinting of mesenchymal progenitors.

Indeed, upon directed differentiation of BMSC colonies, our data showed fewer CFU-Os and a reduction in osteoblast-related genes in $Fgfr3^{+/−}$ samples. This reduced osteogenic capacity was not merely a reflection of reduced $Fgfr3^{+/−}$ BMSC colony formation, as seen in the CFU-F assay, as the ratio of osteogenic colonies to non-osteogenic colonies was also diminished in $Fgfr3^{+/−}$ cultures. Our data confirms the effect of FGFR3 signaling on terminal osteoblast differentiation shown in a previous study, where reduced Von Kossa stained bone nodules were seen in the
osteogenic differentiation of BMSC cultures of Fgfr3\(^{-/-}\) mice (Valverde-Franco et al., 2004). In contrast to Valverde-Franco’s study we identified not only a reduction in Von Kossa-positive bone nodules but also a reduction in ALP-positive colonies, whereas her group showed an initial increase in ALP-positive colonies prior to a reduction in overall number of mineralized bone nodules. This conflicting data may represent differences in mouse strain (C3H versus C57Bl6) where differences in Fgfr3\(^{-/-}\) phenotype have been described, or variances in experimental protocol; Valverde-Franco commenced osteogenic differentiation at day 1 of culture versus our protocol of establishing BMSC colonies over 7 days prior to stimulating osteogenic differentiation. Both mouse strain and experimental conditions have previously been shown to alter colony forming and differentiation ability of BMSC cultures (J. E. Aubin, 1999; Bianco et al., 2001; Sergei A Kuznetsov et al., 2009).

Consistent with the gene expression data from undifferentiated Fgfr3\(^{+/+}\) BMSC colonies, Fgfr3\(^{+-}\) CFU-O colonies also showed a reduction in osteoblast-related genes Runx2, Osx, Colla1, Alp, Ocn, BSP and Ocn, compared to WT CFU-O colonies. Our data is in agreement with that of Su who investigated osteoblast differentiation in mice with a gain-of-function mutation of FGFR3, and found that mRNA expression of osteoblast genes Runx2, Ocn, Ocn and Colla1 were increased compared to WT (N. Su et al., 2010). In addition to the relative spatial reduction in mRNA expression of osteoblast genes in Fgfr3\(^{+-}\), we also found that osteoblast gene expression recapitulated the normal temporal relationship of osteoblast differentiation, with mRNA of extracellular matrix proteins Colla1 and Ocn peaking at an earlier time point (day 10 of differentiation) than the proteins BSP and Ocn (day 21 of differentiation). BSP and Ocn are considered the most specific of the osteoblast genes and are only expressed in terminally differentiated osteoblasts and thus our data supports that osteogenesis occurred in a developmentally appropriate sequence.

In conclusion we found that FGFR3 signaling affects SSC number and the proliferative ability of BMSCs, and may prime undifferentiated progenitor cells to preferentially differentiate to or from specific mesenchymal lineages. FGFR3 deficiency reduces the concentration of CFU-F and BMSC in addition to reducing their capacity for osteogenesis, which may be a form of upstream osteogenic imprinting. Furthermore FGFR3 deficiency inhibits osteogenesis in vitro, and taken together these findings suggest that the observation that FGFR3 acts as a bone switch in vivo may be due to both an effect on undifferentiated mesenchymal progenitors and the
ability of osteoprogenitors to differentiate down the osteoblastic pathway. Further work will be required to elucidate the mechanism by which FGFR3 controls cell fate in this manner.
CHAPTER 4 – BONE MARROW CELLS CONTROL THE MESENCHYMAL RESPONSE TO FRACTURE THROUGH FGFR3 SIGNALING
4.1 Abstract

FGFR3 affects the size and structure of healing murine fracture callus, and acts as a bone switch. Specifically, deficiency in FGFR3 signaling inhibits intramembranous ossification and enhances endochondral ossification and fracture remodeling. Cells of both the mesenchymal and haematopoietic lineage directly effect this alteration in fracture healing. Due to known interactions in fracture repair between cells of the mesenchymal and haematopoietic lineages, we hypothesized that bone marrow cells, and possibly haematopoietic cells control the mesenchymal cellular response to fracture repair through FGFR3 signaling. Using a bone marrow transplant between WT and *Fgfr3* +/- mice to investigate in-vivo fracture healing and in-vitro osteoblast differentiation we showed that transplanted bone marrow cells can control the balance of intramembranous and endochondral ossification in fracture repair via FGFR3 signaling. Engraftment of FGFR3 deficient bone marrow into WT mice recapitulated pathological fracture healing, by inhibiting intramembranous ossification and increasing endochondral ossification, seen in native *Fgfr3* +/- mice, and in addition *Fgfr3* +/- engrafted WT mice also showed defective osteoblast differentiation in-vitro. Conversely, the engraftment of WT bone marrow into *Fgfr3* +/- mice also rescued its pathological fracture phenotype and osteoblast differentiation in-vitro. Our findings add to the growing body of evidence and suggest that cells of the haematopoietic lineage may have a crucial role in controlling the mesenchymal response to fracture. We have identified that FGFR3 signaling allows haematopoietic cells to regulate fracture repair by controlling the balance between the two major pathways of bone formation. FGFR3 signaling may therefore prove to be an ideal target for novel drug development for the enhancement of fracture repair and bone regeneration.
4.2 Introduction

Bone fracture healing is a complex reparative process utilizing multiple cell types of both the mesenchymal lineage (osteoblasts and chondrocytes) and the haematopoietic lineage (osteoclasts and macrophages), that is tightly coordinated both spatially and temporarily, yet is incompletely understood (Dirckx et al., 2013; Louis C Gerstenfeld et al., 2003; Ikeda & Takeshita, 2014; Marsell & Einhorn, 2011; Vi et al., 2015; Zhou et al., 2014). Fracture healing occurs through four distinct but overlapping phases of inflammation, intramembranous ossification, endochondral ossification and remodeling (Einhorn, 1998; Louis C Gerstenfeld et al., 2003). Recent work has focused on the importance of the inflammatory phase of fracture healing and the relative contribution to the reparative process of haematopoietic cells, such as macrophages (Alexander et al., 2011; Baht et al., 2015).

There is emerging evidence that macrophages may be a key regulator of fracture healing with numerous roles including the phagocytosis of cellular debris, production of cytokines and growth factors, stimulation of angiogenesis, initiation of cellular migration and control of osteoblast differentiation and thus bone formation (Bastian et al., 2011; Sinder et al., 2015; Vi et al., 2015). Macrophages propagate the inflammatory cascade secreting IL1, IL6 and TNF-α (Ai-Aql et al., 2008; Louis C Gerstenfeld et al., 2003; Kon et al., 2001). Mice ubiquitously depleted of macrophages fail to heal fractures satisfactorily with a reduction in callus size and bone volume and demonstrate an overall slowing of the pace of fracture healing (Vi et al., 2015). Furthermore conditional depletion of macrophages before fracture, and even 3 or 7 days post-fracture also have deleterious effects in fracture healing, again with reduced callus size and reduced bone formation (Vi et al., 2015). It is not known how macrophages exert their effects on fracture healing and the concept of macrophage modulated bone repair is currently an area of intense investigation.

Other haematopoietic cells are also implicated in fracture repair. T-lymphocytes secrete interleukins that have been shown to be important in fracture repair such as IL-6, IL-17F and IL-23. Mice lacking the recombinase activating gene *Rag1* are unable to form T-lymphocytes, and have impaired fracture healing. Furthermore IL-17F secreted from T-lymphocytes has been shown to be crucial for osteoblast maturation (Nam et al., 2012).
Fibroblast growth factor receptor 3 (FGFR3) has a key role in normal skeletal development and repair. Gain-of-function mutations in FGFR3 cause numerous skeletal dysplasias including achondroplasia, thanatophoric dysplasia and hypochondroplasia and are characterized by a short-limbed phenotype (Oberklaid et al., 1979) (Rousseau et al., 1994; Shiang et al., 1994) (Maroteaux et al., 1967; Orioli et al., 1986) (Walker et al., 1971). Human disease with loss-of-function of FGFR3 (CATSHL syndrome) is an uncommon condition with a phenotype of tall stature and scoliosis (abnormal curvature of the spine), but confirms the importance of FGFR3 on skeletal development (Toydemir et al., 2006). FGFR3 has been shown to act as a negative regulator of endochondral bone growth, by inhibiting the proliferation and differentiation of growth plate chondrocytes (Colvin et al., 1996; Deng et al., 1996; Naski et al., 1996). Although most authors describe the importance of FGFR3 in chondrocyte proliferation and differentiation (Colvin et al., 1996; Deng et al., 1996; Arata Nakajima et al., 2003; Naski et al., 1996), Rundle also described the expression of FGFR3 from osteoblasts in the healing fracture callus suggesting a potential role for FGFR3 in intramembranous as well as endochondral ossification (Rundle et al., 2002). Despite earlier studies of the cranium suggesting that there were no significant sutural or cranial defects related to intramembranous ossification due to abnormalities in FGFR3 function (Colvin et al., 1996; Deng et al., 1996), more recent studies in mice and humans with FGFR3 related skeletal dysplasias showed that premature fusions of the synchondrosis were seen, as well as abnormally ossified dermatocranial bones, and alterations in osteoblast differentiation in-vivo, suggesting that FGFR3 may indeed be involved in intramembranous ossification (Di Rocco et al., 2014; Valverde-Franco et al., 2004).

The relationship between haematopoietic cells and fibroblast growth factor signaling is not well characterized to date. FGF9 has been shown to enhance macrophage differentiation, which has a role in post myocardial infarction cardiac remodeling (Singla, Singla, Abdelli, & Glass, 2015). Macrophages also have a role in promoting FGFR-driven tumour cell migration via Cxcr2 ligands (Bohrer & Schwertfeger, 2012), and have also been shown to be a source of fibroblast growth factors that play a role in wound healing (Akimoto, Ishikawa, Iijima, & Miyachi, 1999). Contradictory findings have been reported concerning the effect of FGFR3 on osteoclasts. Mice with both gain-of-function and loss-of-function FGFR3 mutations have been shown to have enhanced osteoclast formation and activity (N. Su et al., 2010; Valverde-Franco et al., 2004) Children with FGFR3 related skeletal dysplasias have an association with immune deficiency
(Patiroglu, Akar, Okdemir, & Kurtoglu, 2014), and FGFR3 mutations are also associated with myeloma (M Chesi et al., 2001; M. Chesi & Bergsagel, 2015) and thus to date the roles of FGFR3 signaling in cells of the haematopoietic lineage remain unresolved.

We previously showed that FGFR3 affects the size and structure of healing murine fracture callus, and acts as a bone switch. Specifically, deficiency in FGFR3 signaling inhibits intramembranous ossification and at the same time enhances endochondral ossification. In addition, bone fracture remodeling is also increased in $Fgfr3^{+/-}$ mice, which is effected by an increase in osteoclast number and osteoclast activity. Our findings thus demonstrate that FGFR3 signaling has wide ranging effects on both mesenchymal and haematopoietic lineage cells that control skeletal homeostasis and repair. It is possible that FGFR3 signaling from mesenchymal cells coordinates a change in activity of cells of the haematopoietic lineage during fracture repair, or alternatively the cells of the haematopoietic lineage may exert a dominant and coordinating effect of the function of mesenchymal cells in fracture repair. In light of recent studies implicating haematopoietic cells as a key regulator of fracture healing (Alexander et al., 2011; Baht et al., 2015; Nam et al., 2012; Raggatt et al., 2014; Vi et al., 2015) this poses a fascinating and crucial question to further understand the role of FGFR3 in skeletal biology and forms the basis of further work in this thesis. We therefore hypothesized that cells of the haematopoietic lineage control mesenchymal differentiation via FGFR3 signaling, which leads to alterations in intramembranous ossification, endochondral ossification and osteoblast differentiation, and thus acts a key moderator of fracture repair.
4.3 Materials and Methods

4.3.1 Mice

*Fgfr3*−/− mice were purchased from Jackson Laboratory (B6; 129S-Fgfr3tm1Dor/J, Stock #004234) and maintained at the Toronto Center for Phenogenomics (TCP). Animal protocols were approved by the animal care committee of the TCP, in accordance with the regulations and guidelines from the Canadian Council on Animal Care. Genomic DNA from skin samples of harvested mice were used to genotype animals by PCR.

4.3.2 Tibia fracture generation

Semi-stabilized tibial fractures were generated on 12-week-old male *Fgfr3*+/+ and *Fgfr3*+/− mice following a protocol previously described (Y. Chen et al., 2007). In brief, the mice were anaesthetized using inhalational general anaesthesia. The left hind limb of each mouse was surgically prepared, by shaving and cleaning with disinfectant. A small anterior midline incision was made over the knee joint and proximal tibia. A 0.7 mm pilot hole was made in the proximal tibial epiphysis just medial to the insertion of the patella tendon using a hollow needle. A 0.7mm Anticorror insect pin (Fine Science Tools, http://www.finescience.ca) was then inserted into the medullary cavity of the intact tibia and advanced to the distal tibia. A transverse fracture was then induced at the mid-shaft of the tibia using blunt scissors. The insect pin was cut 5-7mm proud of the proximal tibial epiphysis and the skin incision closed with a series of absorbable vicryl rapide sutures, and metallic wound clips. Analgesic given subcutaneously (buprenorphine, 0.1mg/kg/twice per day) was administered for 3 days after surgery. Previous data shows that a fracture generated in this manner heals through both intramembranous and endochondral ossification (Hiltunen et al., 1993; Le et al., 2001). The animals were allowed to maintain full weight bearing in their cages following surgery. At specific time points, PFD3, PFD7, PFD14 and PFD21 following fracture, samples of fractured and unfractured tibiae were harvested following euthanasia with inhaled carbon dioxide. A minimum of 3 mice per experimental condition, per time point, was used for further analysis.

4.3.3 Bone Marrow Transplant

12-week-old female *Fgfr3*+/+ and *Fgfr3*+/− mice were irradiated (900cG x 12.1 min) and tail vein-injected with bone marrow (BM) aspirated and isolated from the femora and tibiae of 12-week-
old-old male Fgfr3<sup>+/−</sup> and Fgfr3<sup>+/+</sup> mice (1 x 10<sup>6</sup> cells in 200 µl PBS) (Baht et al., 2015). Engraftment into the bone marrow was allowed to occur for 8 weeks and verified using fluorescent in situ hybridization analysis using FITC labeled Y-chromosome paint probes (CytoCell. Aquarius Murine Whole Chromosome Painting Probes, cat # AMP 0YG). Nuclei were counter-stained with 40,6-diamidino-2-phenylindole (DAPI) (1:10,000) (A. S. Lee et al., 2009). Four donor-recipient combinations were created and comprised of Fgfr3<sup>+/−</sup> BM into Fgfr3<sup>+/−</sup> (WT Control), Fgfr3<sup>+/−</sup> BM into Fgfr3<sup>+/−</sup> (Heterozygote Control), Fgfr3<sup>+/−</sup> BM into Fgfr3<sup>+/−</sup> and Fgfr3<sup>+/−</sup> BM into Fgfr3<sup>+/+</sup>. A minimum of 3 mice per donor-recipient combination was used per time point for further analysis.

4.3.4 Radiography

Tibial fracture healing was assessed radiographically at PFD14 after sacrifice using a Faxitron Cabinet X-Ray System (Faxitron X-Ray Corporation, Lincolnshire, IL, USA). Callus size was measured at the maximum width using digital measurements. The width of the tibial shaft was subtracted from entire callus width for each fracture to calculate true callus width (Adobe Photoshop, San Jose CA, USA).

4.3.5 Histology

Fractured tibiae were harvested on PFD7 and PFD14, to assess intramembranous ossification and endochondral ossification, fixed in 10% formalin following by decalcification with 20% (w/v) EDTA pH 8.0 or formic acid bone decalcifier (Decal Chemical Corp, Tallman, NY) and embedded in paraffin. Serial 5µM sections of paraffin embedded tissues were deparaffinized, and rehydrated through an alcohol gradient to water. Sections were stained with Safranin-O and counter stained with fast green/Mayer’s haematoxylin. Here red staining confirms the presence of proteoglycans, which indicates cartilaginous tissue, and green staining indicates bone (Camplejohn & Allard, 1988).

4.3.6 Histomorphometry

Callus composition was analyzed using computer–assisted histomorphometry (Adobe Photoshop, San Jose CA, USA). Cross-sections of calli were prepared for analysis using digital–
subtraction techniques to exclude native bone. The relative abundance of bone, cartilage and undifferentiated tissue were expressed as a percentage of the total callus tissue area. Three to six mice per genotype, per time point, and whole callus sections per mouse were used for analysis. Intramembranous bone analysis utilized 4 samples per callus, endochondral bone analysis utilized whole callus analysis.

### 4.3.7 Colony forming units – osteoblast (CFU-O)

Femora and Tibiae from *Fgfr3*+/− and WT mice, that 8 weeks previously had been engrafted with either a WT BMT, *Fgfr3*+/− BMT or no BMT, were isolated and debrided of soft tissue. Bone marrow was flushed into BMSC culture media containing α-modification of eagle’s medium (α-MEM) (Wisent Inc, Cat# 310-012-CL, St. Bruno, CA), 10% FBS (Gibco Life Technology, Cat# 16000-044, Burlington, ON) and 1x antibiotic and antimycotic (Wisent Inc, Cat# 450-115-EL). The cell suspensions were passed through an 18G needle and 70 μm cell strainer to dissociate clumps of cells. Single cell suspensions were plated at a density of 1x10⁶ cells/cm² surface area on 6-well plates, in BMSC culture medium for 7 days, with 50% of the media changed at day 4. At day 7 of culture, the media was changed for osteoblastic differentiation media, which consisted of BMSC culture media, as described above, with the addition of 50 μg/ml Ascorbic Acid (Sigma, Cat# A4544), 10⁻⁸M Dexamethasone (Sigma, Cat# D8893), 8mM β-Glycerol phosphate (Sigma, Cat# G9891). The osteoblastic differentiation media was subsequently changed every 2-3 days for the duration of the experiment. At day 14 or 21 cells were rinsed with PBS and fixed with 10% formalin for 30 mins and rinsed twice with water. Alkaline phosphatase staining (Fast Red TR/Naphthol AS-MX Tablets: Sigma#F4523, manufacturers protocol) and Von Kossa staining (2.5% (w/v) Silver Nitrate: Sigma #S8157, 30 minutes at room temperature on light box) were performed to assess for osteoblastic differentiation and mineralization. BMSC colonies arising from CFU-O but stained negatively for Alkaline phosphatase were analyzed further by over staining colonies with 0.5% (w/v) Crystal Violet (Sigma, Cat#: C3886) in 25% Methanol for 15 minutes at room temperature. BMSC colonies were counted manually using a microscope. CFU-Os were counted manually using a microscope. A CFU-O was defined as a discrete colony that contained 50 or more cells. CFU-Os were counted manually using a microscope. A CFU-O was defined as a discrete colony that contained 50 or more cells with the majority of cells in each colony staining for ALP or Von Kossa respectively. All quantification performed with 3 wells per mouse, and minimum of 3 mice per genotype.
4.3.8 Statistical analysis

Data are expressed as mean, +/-95% confidence intervals and were calculated for each sample. Student’s t-test was used to compare data sets. The data were considered to be statistically significant at a confidence level of 95% (p<0.05).
4.5 Results

Bone marrow transplantation (BMT) has been shown to engraft donor haematopoietic cells into irradiated host mice whereas very few, if any, mesenchymal cells engraft into the host animal (Baht et al., 2015; Shen et al., 2011). Mesenchymal cells contributing to fracture healing are therefore endogenous to the host animal after a BMT, whereas the healing response attributed to haematopoietic cells is derived from the donor animal.

![Diagram](image)

**Figure 4-1. Transplanted male BM cells engraft and survive in female BM**

A) 12-week-old female WT and Fgfr3<sup>−/−</sup> mice were irradiated (900cG) and tail vein-injected with bone marrow (BM) aspirated and isolated from the femora and tibiae of 12-week-old-old male WT and Fgfr3<sup>−/−</sup> mice. Schematic diagram demonstrating the series of bone marrow transplants (BMT) performed with both WT and Fgfr3<sup>−/−</sup> controls, and WT BM engrafted into Fgfr3<sup>−/−</sup> mice and Fgfr3<sup>−/−</sup> BM engrafted into WT mice. B) Imaging of cultured BM cells aspirated from femora and tibia of engrafted female mouse after 8 weeks. Female control did not receive BMT. Fluorescent in-situ hybridization - FITC labeling of Y-chromosome positive donor nuclei within female BM aspirate confirmed engraftment of donor bone marrow. Nuclei are stained with 4',6-diamidino-2-phenylindole (DAPI).
We therefore used BMT to investigate the role of FGFR3 signaling in cells of the haematopoietic lineage on fracture healing. We engrafted Fgfr3+/− bone marrow into WT mice (Fgfr3+/−-WT) and WT bone marrow into Fgfr3+/− mice (WT-Fgfr3+/−), using isogenetic donors and hosts as controls (WT-WT) and (Fgfr3+/−-Fgfr3+/−).

To confirm successful bone marrow transplantation and engraftment we used donor bone marrow from male mice, and used female mice as the recipients, to be able to track the survival of male engrafted cells, containing Y-chromosomes. After 8 weeks, recipient female mice were sacrificed and fresh bone marrow aspirate cultured in vitro. Fluorescent in-situ hybridization – fluorescein isothiocyanate (FITC) labeling of Y-chromosome positive donor nuclei within female bone marrow aspirate confirmed engraftment of donor bone marrow (Fig. 4-1).

With confirmation of successful BMT in recipient mice we proceeded to examine the balance of intramembranous and endochondral ossification in fracture healing in WT mice that had received Fgfr3+/− BMT, to assess whether engrafted marrow with inhibited FGFR3 signaling recapitulated the fracture healing seen in native Fgfr3+/− mice. In addition we investigated whether engrafting WT bone marrow into Fgfr3+/− mice rescued the fracture-healing defect seen in native Fgfr3+/− mice. Finally we performed colony forming unit-osteoblast assays to determine the effect of altered levels of FGFR3 in engrafted bone marrow, and therefore cells of the haematopoietic lineage on in-vitro osteogenesis.

At the very edges of fracture repair the injured subperiosteal zone gives rise to intramembranous bone formation, which can be visualized and quantified at PFD7. No cartilage template precursor is seen in this subperiosteal zone unlike in the larger central area of fracture healing which heals with endochondral ossification. Histological sections of WT mouse tibial fracture callus were stained with Safranin-O (cartilage) and Fast Green (bone) and analyzed using digital subtraction histomorphometry to isolate areas of intramembranous ossification. Here we showed a reduced ratio of bone area per callus area in WT mice that had received Fgfr3+/− bone marrow compared to control mice (Fig. 4-2). In addition these data also represented a reduction of intramembranous ossification to levels that were seen in Fgfr3+/− control mice (Chapter 2).
Figure 4.2. Engraftment of \( Fgfr3^{+/−} \) bone marrow inhibits intramembranous bone formation in fracture repair in WT mice

A) Schematic diagram of a murine tibial fracture with surrounding callus. Boxed ROI containing dark grey shaded region demonstrates peripheral subperiosteal area, where intramembranous bone formation occurs during normal fracture healing. B) Histological sections of PFD7 tibial fracture callus from WT mice engrafted with either WT or \( Fgfr3^{+/−} \) BM corresponding to boxed ROI shown in schematic diagram, stained with Safranin-O (cartilage) and Fast Green (bone). WT mice show reduced intramembranous bone formation following engraftment with \( Fgfr3^{+/−} \) BM. C) Quantification of mean intramembranous bone area per callus area and total intramembranous bone area of histological samples shown above. \( n = 3 \) mice per genotype, 4 samples per mouse, data are mean +/- 95% confidence intervals, **p<0.005 (2-sided, 2-sampled Student’s t-test)

Due to the inhibition of intramembranous ossification seen in WT mice engrafted with \( Fgfr3^{+/−} \) bone marrow, and the fact that intramembranous bone is a direct product of functional osteoblasts and their SSC progenitors in fracture repair, we next examined the osteogenic efficiency (CFE) of BMSC colonies in-vitro, using a colony forming unit-osteoblast (CFU-O) assay, which can be considered an approximation of the osteogenic potential of SSCs (Bianco et al., 2001; Sergei A Kuznetsov et al., 2009). BMSC colonies cultured from WT mice engrafted with \( Fgfr3^{+/−} \) bone marrow demonstrated a reduction in osteogenic efficiency, with a lower ratio of ALP+ staining colonies compared to the total number of BMSC colonies when cultured under osteogenic conditions, than WT control samples (Fig.4-3). In addition the reduction in osteogenic efficiency corresponded to the reduction seen in CFU-O assays in native \( Fgfr3^{+/−} \) mice (Chapter 3).
Next we investigated the effect of \( Fgfr3^{+/+} \) BMT in WT mice on endochondral ossification in fracture repair, which is best visualized radiographically and histologically at PFD14 (Fig. 4-4). Initially, radiographs of the tibial fractures were analyzed for callus size, and were found to be smaller in WT mice that had received \( Fgfr3^{+/+} \) than WT controls (Fig. 4-4A). To investigate the composition of callus at this time point, sections mouse tibial fractures callus were then examined using digital subtraction histomorphometry. Here we showed an increased ratio of bone per callus area in WT mice engrafted with \( Fgfr3^{+/+} \) bone marrow (Fig. 4-4D). Specifically the increase in bone within the \( Fgfr3^{+/+} \) engrafted WT mice was found to be in the very central most area of the fracture callus (Fig. 4-4E), which is the last area of callus to ossify, consistent with an acceleration in endochondral ossification when compared to WT control mice. No differences between groups were seen in the percentage of cartilage per callus area, or
percentage of undifferentiated tissue per callus area. Although no difference in the cartilage content was identified, the geographic distribution of cartilage differed between groups (Fig. 4-4F), with cartilage in the WT mice engrafted with \( Fgfr3^{+/--} \) bone marrow being more centrally placed, than in WT controls, where the cartilage was distributed more peripherally in the callus. A peripheral distribution of cartilage is characteristic of less mature callus than one with centrally distributed cartilage due to the dynamic nature of the healing fracture with endochondral ossification progressing from peripheral to central areas. Taken together these data show that engraftment of \( Fgfr3^{+/--} \) bone marrow into WT mice recapitulates the fracture healing phenotype of native \( Fgfr3^{+/--} \) mice, which we previously showed to be characterized by an inhibition in intramembranous ossification and an increase in endochondral ossification, and furthermore that haematopoietic cells may be responsible for the fracture phenotype seen in \( Fgfr3^{+/--} \) mice.

Considering that \( Fgfr3^{+/--} \) BMT in WT mice was able to recapitulate the pathological fracture phenotype seen in \( Fgfr3^{+/--} \) mice, we also asked whether the converse was true, that normalized FGFR3 signaling in haematopoietic cells, by way of WT BMT, could rescue the abnormal fracture phenotype in \( Fgfr3^{+/--} \) mice. Again we proceeded to examine the balance of intramembranous and endochondral ossification in fracture healing in-vivo and the osteogenic efficiency of BMSC colonies in-vitro using \( Fgfr3^{+/--} \) mice that had received WT BMT.
Figure 4-4. Engraftment of \( Fgr3^{+/−} \) bone marrow enhances endochondral ossification in fracture repair in WT mice

A) Lateral radiographs of tibial fractures at PFD14 in WT mice that received either WT or \( Fgr3^{+/−} \) BMT. Mice receiving \( Fgr3^{+/−} \) BMT showed a smaller fracture callus than those engrafted with WT BMT B) Quantification of fracture callus width from radiographs of mice as detailed in A) (n=7 mice per group). C) Schematic diagram of a murine tibial fracture with surrounding callus. Large boxed ROI containing light grey shaded region demonstrates large central area of callus, where endochondral bone formation occurs during normal fracture healing and constituted the region analyzed histologically and depicted in D). Small boxed ROI within callus represents histological region of fracture site depicted in E). D) and E) Histological sections of PFD14 tibial fracture callus at 5X and 20X respectively, from WT mice engrafted with either WT or \( Fgr3^{+/−} \) BMT, stained with Safranin-O (cartilage) and Fast Green (bone). WT mice engrafted with \( Fgr3^{+/−} \) demonstrated increased proportion of bone within callus compared to those engrafted with WT BM. In E), b depicts new woven bone, c cartilage and u undifferentiated tissue. F) Quantification of the proportion of bone, cartilage and undifferentiated tissue deposited in the fracture callus was measured using histomorphometric analysis (n=4 mice per group, whole callus analyzed in each mouse, in 2 sections). Data are mean values +/- 95%CI. **p<0.005 (2-sided, 2-sample Student’s t-test).
At PFD7 we identified an increase in bone area per callus area, corresponding to an increase in intramembranous ossification, in $Fgfr3^{+/\text{-}}$ mice engrafted with WT bone marrow, compared to $Fgfr3^{+/\text{-}}$ control mice (Fig. 4-5). In addition these data also represented a rescue of intramembranous ossification to levels seen in native WT mice (Chapter 2). Furthermore, BMSC colonies cultured from $Fgfr3^{+/\text{-}}$ mice engrafted with WT bone marrow demonstrated an increase in osteogenic efficiency, with a higher ratio of ALP$^+$ staining colonies compared to the total number of BMSC colonies, compared to $Fgfr3^{+-}$ mice that did not receive BMT (Fig. 4-6). The increase in osteogenic efficiency corresponded to a rescue of in-vitro bone forming capacity to WT levels (Chapter 3).

**Figure 4-5. Engraftment of WT bone marrow rescues intramembranous bone formation in fracture repair in $Fgfr3^{++}$ mice**

A) Schematic diagram of a murine tibial fracture with surrounding callus. Boxed ROI containing dark grey shaded region demonstrates peripheral subperiosteal area, where intramembranous bone formation occurs during normal fracture healing. B) Histological sections of PFD7 tibial fracture callus from $Fgfr3^{++}$ mice engrafted with either $Fgfr3^{++}$ or WT BM corresponding to boxed ROI shown in schematic diagram, stained with Safranin-O (cartilage) and Fast Green (bone). $Fgfr3^{++}$ mice show increased intramembranous bone formation following engraftment with WT BM. C) Quantification of mean intramembranous bone area per callus area and total intramembranous bone area of histological samples shown above. n = 3-4 mice per genotype, 4 samples per mouse, data are mean +/- 95% confidence intervals, *p<0.05 (2-sided, 2-sampled Student's $t$-test).
Figure 4-6. Engraftment of WT bone marrow rescues osteogenic efficiency of BMSC cultures in Fgfr3+/− mice
A) Representative wells from colony forming unit-osteoblast assay (CFU-O). BMSCs were aspirated from the femora and tibiae of Fgfr3+/− mice irradiated and engrafted 8 weeks previously with WT BM, adhered to tissue culture plastic and differentiated in osteogenic media. After 14 or 21 days of differentiation, cultures were washed, fixed and stained for alkaline phosphatase (ALP) and quantified for CFU-O formation, next the same wells were overstained with crystal violet (CV) for quantification of overall BMSC colony formation. Control Fgfr3+/− mice were native, and did not receive irradiation or BMT. B) Osteogenic efficiency was quantified by calculating the ratio of ALP+ colonies to total number of colonies (CV+) in Fgfr3+/− mice engrafted with WT BM. n = min 3 mice per group, 3 wells per mouse. Data are mean values +/- 95%CI. ***p<0.0005 (2-sided, 2-sample Student’s t-test).

Finally we investigated the effect of WT BMT in Fgfr3+/− mice on endochondral ossification in fracture repair (Fig. 4-7). Here we showed a reduced proportion of bone per callus area in Fgfr3+/− mice engrafted with WT bone marrow (Fig. 4-7B). With the reduction in proportion of bone we also showed a corresponding increase in the proportion of cartilage per callus area, which represents a more immature callus and thus slowing of endochondral ossification. Taken together with the data from figures 5 and 6, we showed that the pathological fracture phenotype of Fgfr3+/− mice was rescued with WT BMT, by normalizing the FGFR3 signaling in cells of the haematopoietic lineage, which lead to correction of intramembranous ossification and endochondral ossification back to normal patterns.
Figure 4-7. Engraftment of WT bone marrow rescues endochondral ossification in fracture repair in Fgfr3<sup>−/−</sup> mice

A) Schematic diagram of a murine tibial fracture with surrounding callus. Large boxed ROI containing light grey shaded region demonstrates large central area of callus, where endochondral bone formation occurs during normal fracture healing and constituted the region analyzed histologically and depicted in B). Small boxed ROI within callus represents histological region of fracture site depicted in C). B) and C) Histological sections of PFD14 tibial fracture callus at 5X and 20X respectively, from Fgfr3<sup>−/−</sup> mice after WT BMT or No BMT, stained with Safranin-O (cartilage) and Fast Green (bone). Fgfr3<sup>−/−</sup> mice engrafted with WT marrow demonstrated reduced proportion of bone and increased proportion of cartilage within callus compared to native Fgfr3<sup>−/−</sup> mice. In C), b depicts new woven bone and c cartilage. D) Quantification of the proportion of bone, cartilage and undifferentiated tissue deposited in the fracture callus was measured using histomorphometric analysis (n=3-6 mice per group, whole callus analyzed in each mouse, in 2 sections). Data are mean values +/- 95%CI. ***p<0.0005 (2-sided, 2-sample Student’s t-test).
4.6 Discussion

Fracture healing is a tightly controlled reparative process that requires the coordination of osteoblast and chondrocyte proliferation and differentiation to produce cartilage and bone that progressively heal the skeleton, via the two distinct processes of intramembranous and endochondral ossification (Ai-Aql et al., 2008; Einhorn, 1998; Louis C Gerstenfeld et al., 2003).

Bone marrow transplantation (BMT) allows investigation of the response of endogenous mesenchymal cells to engrafted haematopoietic cells with a characteristic of interest (Baht et al., 2015; Xing, Lu, Hu, Miclau, & Marcucio, 2010). We used this principle to engraft Fgfr3+/- haematopoietic cells through bone marrow transplantation into WT mice, and to engraft WT haematopoietic cells into Fgfr3+/- mice to study such the effects of FGFR3 signaling in haematopoietic cells upon fracture repair.

Our data showed that engraftment of Fgfr3+/- bone marrow into WT mice inhibited intramembranous ossification and enhanced endochondral ossification in fracture repair, recapitulating the pathological healing response that was seen in native Fgfr3+/- mice. Alternatively, the engraftment of WT bone marrow into Fgfr3+/- mice enhanced the potency of intramembranous ossification and concomitantly diminished endochondral ossification to the extent that we saw a rescue of the pathological Fgfr3+/- fracture phenotype (see Chapter 2). Our data are consistent with a previous study, which showed delayed fracture healing in a mouse with gain-of-function FGFR3 mutation (N. Su et al., 2008), in which the authors hypothesized that this finding was due to a delay in the differentiation of chondrocytes in fracture callus. Our study adds to these findings as we investigated FGFR3 signaling in fracture repair in greater detail, teased out the relative contributions of the two main bone forming pathways, and furthermore based on our data, the FGFR3 bone-switch we demonstrated in Chapter 2 appears to be controlled by cells of the haematopoietic lineage, rather than a purely autonomous mesenchymal defect.

This is important because being able to control bone formation for the purposes of skeletal regenerative therapies requires knowledge of not only the most vital signaling pathways involved, but perhaps more importantly an intimate understanding of the specific cells themselves which in effect drive the signaling pathways. Much has been published on the modification of mesenchymal cells to improve bone healing and regeneration, assuming that if
one can engineer and enhance functional mesenchymal cells that carry out the repair work then this may prove to be an efficient therapeutic intervention (Ciapetti, Ambrosio, Marletta, Baldini, & Giunti, 2006; Granchi et al., 2010; Guan et al., 2012; Kumar, Chanda, & Ponnazhagan, 2008; Muschler, Nakamoto, & Griffith, 2004). Our data, in addition to that from the emerging field of the haematopoietic control of mesenchymal reparative response (Alexander et al., 2011; Baht et al., 2015; Raggatt et al., 2014), may therefore contribute to a paradigm shift in how we approach future therapeutic enhancement of skeletal regeneration and repair, by focusing on haematopoietic cell engineering, as the drivers of mesenchymal response.

The pattern of opposing effects of FGFR3 signaling on the two main pathways of bone formation, that we previously described as a bone switch, is consistent with the pattern that we identified in fracture healing in native WT and Fgfr3+/− mice, reported in Chapter 2. Whilst our key finding in the BMT experiment was the increase in proportion of woven bone in the callus in WT mice engrafted with Fgfr3+/− bone marrow, other findings that support the enhancement of endochondral ossification were also present, despite no differences in the proportion of cartilage and the proportion of undifferentiated tissue in the calli examined. On further analysis one can see that there is a slight but non-significant decrease in both the cartilage and undifferentiated tissue proportion in the WT mice engrafted with Fgfr3+/− bone marrow when analyzed as separate tissues. If one considers the dynamic nature of tissues within the healing callus, that progressively these tissues are changing in nature from masses of proliferating chondrocytes to differentiated hypertrophic chondrocytes before they undergo apoptosis to allow bone formation, then a callus in a more immature state will contain a higher proportion of cartilage and undifferentiated tissue than a more mature callus. When we combined these “non-osseous” tissues into a single group for analysis we identified that there was a significantly lower proportion of non-osseous tissue in the WT mice engrafted with Fgfr3+/− bone marrow, which we interpreted as being representative of a more mature callus than compared to WT control mice that contained a lower proportion of bone and a higher proportion of immature non-osseous tissues. In addition the geographic distribution of cartilage within a callus changes as the callus matures. As healing progresses cartilage appears to move within the callus from a peripheral to central location. What in fact we are seeing is the proliferation and differentiation of chondrocytes occurring at different times, starting with those chondrocytes peripherally and finishing with the chondrocytes positioned centrally near the fracture site itself. Thus a fracture
callus with more central cartilage is a more mature, more healed callus compared to a callus with immature features of peripheral cartilage and a central area of undifferentiated tissues. This mature distribution of cartilage tissue is indeed what we identified in the WT mice engrafted with \( Fgfr3^{+/−} \) bone marrow, whereas the WT control callus demonstrated a more immature distribution of callus tissues. Thus taken together these findings strengthen our interpretation that a reduction in FGFR3 signaling in haematopoietic cells enhances endochondral ossification.

Bone is invariably formed by osteoblasts and therefore it may seem contradictory that bone formation can be inhibited and enhanced at the same time, within the same fracture, as we have found here. Our findings support the interpretation that lower levels of FGFR3 inhibit osteoblast differentiation, both through our assessment of intramembranous ossification in-vivo and in cell culture in vitro. The concomitant increase in endochondral ossification is therefore likely due to the effect of FGFR3 signaling on chondrocytes, with lower levels of FGFR3 signaling increasing chondrocyte proliferation and differentiation. FGFR3 has long been known as a negative regulator of chondrocyte proliferation and differentiation (Colvin et al., 1996; Deng et al., 1996; Naski et al., 1996; Valverde-Franco et al., 2004; Xiao et al., 2004). We hypothesize that the mass of rapidly accumulating hypertrophic chondrocytes, that occurs in FGFR3 enhanced endochondral ossification, likely drives an intense secondary deposition of bone, such that the increased bone formation is not an autonomous osteoblast effect rather it is stimulated by rapid differentiation and apoptosis of hypertrophic chondrocytes, releasing a mass of osteogenic signals, that are powerful enough to stimulate even defective osteoblasts to lay down copious bone. This rapid replacement of cartilage with bone in fracture repair and thus the basis of this interpretation has been well described in the literature previously (Einhorn, 1998; Ketenjian & Arsenis, 1975; Marsell & Einhorn, 2011) (L C Gerstenfeld et al., 2003).

The short-limbed phenotypes of individuals with FGFR3-related skeletal dysplasias, such as achondroplasia, are primarily explained by reduced chondrocyte proliferation and differentiation in the growth plate. Our fracture data showing enhanced endochondral ossification are consistent with the developmental findings of previous studies using the same FGFR3 deficient mouse (Deng et al., 1996), and furthermore our study demonstrates how the function of FGFR3 in fracture healing recapitulates its function in development through gross abnormalities in chondrocyte biology, and furthermore how animal models of fracture healing offer insight into developmental biology. Our findings further our understanding of FGFR3 signaling and
developmental chondrocyte biology by identifying a novel potential signaling mechanism that controls proliferation and differentiation of chondrocytes through haematopoietic cells. A limitation in our interpretation is that reduced haematopoietic FGFR3 signaling enhances chondrocyte proliferation and differentiation is based on enhanced endochondral ossification in fracture healing, and so a more detailed investigation of the specific chondrocyte response to haematopoietic cell-based FGFR3 signaling is warranted, but is outside of the scope of this thesis.

Bone marrow transplantation involves the engraftment of all of the cells aspirated from the donor bone marrow compartments to the recipient mouse, including cells of both the mesenchymal and haematopoietic lineage. It has been established that donor mesenchymal cells do not survive engraftment in the same way that haematopoietic cells do, and thus any mesenchymal response in the recipient animal is attributed to resident mesenchymal cells of the recipient animal. This has been shown elegantly by selectively depleting osteoblasts from the recipient mouse prior to BMT, leading to a complete failure of fracture healing (Baht et al., 2015). Engrafted mesenchymal cells were not seen in appreciable numbers and were unable to repair the bony defect. We are therefore confident that the activation of the FGFR3 bone switch is via haematopoietic cells, rather than transplanted mesenchymal cells.

The reported findings that tissue-resident macrophages and circulatory macrophages, have been shown to have differing abilities to regulate osteoblast differentiation and intramembranous bone formation (Chang et al., 2008; Raggatt et al., 2014) highlights a limitation of bone marrow transplantation in investigating haematopoietic control of fracture healing. For example we have not been able to determine whether it is the bone marrow engraftment of tissue-resident cells of the haematopoietic lineage, or the circulating haematopoietic cells that have the most potent effect on osteoblast differentiation, or on modifying the different modes of ossification in fracture repair. Further work is therefore required to identify specific cells of the haematopoietic lineage that use FGFR3 signaling to control mesenchymal cell differentiation in fracture repair. Based on previous publications, likely candidates for haematopoietic cells that contribute meaningfully to fracture repair are macrophages (Alexander et al., 2011; Baht et al., 2015; Raggatt et al., 2014; Vi et al., 2015) T-lymphocytes (Nam et al., 2012), and B-lymphocytes (Konnecke et al., 2014), and the identification of the cell type in question will form the basis of future work.
Limitations of bone marrow transplantation include variability in the experimental results secondary to technical factors such as potential variability in actual radiation dose received by each mouse, variability in composition of cells actually engrafted, which may lead to a mortality rate with the experimental procedure, variation in colony forming ability of in-vitro BMSC cultures, and variation in the morphology and characteristics of fracture healing. For example we saw more variation in fracture callus size and morphology in mice that have received BMT compared to non-transplanted mice from historical experiments.

Using a mouse with ubiquitous FGFR3 heterozygous knock-out limited our ability to tease out highly specific functions of FGFR3 levels related to the response of individual cell types, although we were able to obtain an informative global view of FGFR3 signaling in fracture repair. Using a BMT mitigated some of these limitations and indeed allowed us to separate the effects of FGFR3 signaling in haematopoietic cells from those in mesenchymal cells. Utilizing conditional knockouts of FGFR3 in osteoblasts, chondrocytes, osteoclasts or macrophages for example would have offered the opportunity to establish more cell-specific effects, and indeed may form the basis of future studies. It is possible that FGFR3 signaling through haematopoietic cells has a more profound effect on osteoblasts than chondrocytes, or indeed vice versa, and the known paracrine effects between these two mesenchymal cell types require that cell-specific experiments will be designed to address this issue. Previously FGFR3 signaling been shown to be to have significant paracrine effects in both skeletal and tumour biology (Giri, Ropiquet, & Ittmann, 1999; Jin et al., 2004; Mugniery et al., 2012; Turner & Grose, 2010).

In summary we have shown that haematopoietic cells control the balance of intramembranous and endochondral ossification in bone fracture repair via FGFR3 signaling. Engraftment of FGFR3 deficient bone marrow into WT mice recapitulated pathological fracture healing, by inhibiting intramembranous ossification and increasing endochondral ossification, seen in native Fgfr3−/− mice, and in addition Fgfr3−/− engrafted WT mice also showed defective osteoblast differentiation in-vitro. Conversely, the engraftment of WT bone marrow into Fgfr3−/− mice also rescued its pathological fracture phenotype and osteoblast differentiation in-vitro. Our findings add to the growing body of evidence that cells of the haematopoietic lineage have a crucial role in controlling the mesenchymal response to fracture. We have identified that FGFR3 signaling allows haematopoietic cells to regulate fracture repair by controlling the balance between the
two major pathways of bone formation. FGFR3 signaling may therefore prove to be an ideal
target for novel drug development for the enhancement of fracture repair and bone regeneration.
CHAPTER 5 - GENERAL DISCUSSION, CONCLUDING SUMMARY AND FUTURE DIRECTIONS
5.1 General Discussion

Fifty four percent of all adults in the US are affected by musculoskeletal conditions with the annual cost for treatment and lost wages totaling $874 billion (5.7%GDP) in 2011 (National Health Interview Survey (NHIS) Adult Sample, 2013). The annual cost of treating musculoskeletal injuries totals $176.1 billion, which includes the treatment of 18.3 million fractures annually. Fifty percent of women and 25% of men will suffer an osteoporosis related fracture with a 20% mortality rate within 12 months of a hip fracture (Bone and Joint Initiative USA, 2015).

Obtaining a greater understanding of skeletal biology is a fundamental strategy to develop new therapies to correct skeletal developmental diseases, to enhance bone regeneration and to enhance fracture repair. Due to the length of time even routine fractures take to repair, and the extended period of rehabilitation needed to regain full function, all fractures would likely benefit from the enhancement of healing with significant benefits to the individual, family and population at large. More potent mesenchymal cells that differentiate faster and create high quality reparative bone and cartilage tissue will therefore be crucial.

There are many genetic bone diseases, or skeletal dysplasias, that offer a window to the action of specific genes on bone and cartilage biology. These diseases demonstrate specific skeletal phenotypes in humans, such as the disproportionate short stature of achondroplasia (gain-of-function FGFR3 mutation). Approximately 20 years ago the effect of FGFR3 on cartilage biology was hypothesized based upon the phenotype seen in achondroplasia, and was later proved (Colvin et al., 1996; Deng et al., 1996; Le Merrer et al., 1994; Velinov et al., 1994). Further observations that we made concerned the ability of children with achondroplasia to make excellent regenerate intramembranous bone when undergoing limb lengthening surgery, and lead to the hypothesis that FGFR3 must in some way affect osteoblast differentiation and direct bone formation in addition to the known effects on chondrocyte biology and the growth plate. Most of the skeletal dysplasias are replicated in genetically modified mice providing excellent models with which to further test and understand the function of specific genes on, not only bone development, but also the response to injury, such as bone fracture. Thus the combination of clinical observation in humans and the availability of animal models of disease have proved to be important basis for this thesis.
Our initial hypothesis that abnormalities in FGFR3 signaling affect osteoblast differentiation, which leads to abnormal bone fracture repair, and that FGFR3 signaling in haematopoietic cells regulates osteoblast differentiation lead to the natural segmentation of this thesis into three main experimental chapters. In Chapter 2 we characterized the abnormal fracture phenotype in \( Fgfr3^{+/−} \) mice using a series of histological and radiographic techniques. For Chapter 3 we then examined the basis of abnormal ossification that we identified in fracture repair in the previous chapter using bone marrow aspirated from the same \( Fgfr3^{+/−} \) mice in in-vitro approach to investigate the specific mesenchymal progenitor and osteoblast effects of abnormal FGFR3 signaling. Finally, based on findings from both previous chapters, and work already published in the literature, Chapter 4 contains our investigation of the function of FGFR3 signaling in haematopoietic cells as a candidate regulator of mesenchymal response to fracture repair.

\( Fgfr3^{+/−} \) mice initially showed a rapid increase in the size of fracture callus at PFD7, but at PFD14 and PFD21 the callus was then smaller than WT controls. This suggests that reduced FGFR3 signaling leads to accelerated fracture healing overall. Our data are consistent with a previous study, which showed delayed fracture healing in a mouse with gain-of-function FGFR3 mutation (N. Su et al., 2008), in which the authors hypothesized that this finding was due to a delay in the differentiation of chondrocytes in fracture callus. Our data has expanded considerably upon previous findings by identifying an alteration in the balance of intramembranous and endochondral ossification, which speaks to a differential effect of FGFR signaling in both chondrocytes and osteoblasts and relates positively to our original hypothesis. We identified that intramembranous ossification is inhibited in \( Fgfr3^{+/−} \) mice, despite the initial observation that fracture repair was enhanced as a whole. Despite earlier conflicting reports concerning the effect of FGFR3 signaling on the function of osteoblasts (Colvin et al., 1996; Deng et al., 1996), our data supports more recent studies of FGFR3 related skeletal dysplasias, which have shown that abnormally ossified dermatocranial bones are related to defects of FGFR3 signaling in osteoblasts affecting intramembranous ossification (Di Rocco et al., 2014). Our findings of FGFR3 signaling regulating intramembranous ossification in fracture healing are novel, and furthermore reinforce the principles of fracture repair recapitulating the same pathways that are seen in skeletal development (Louis C Gerstenfeld et al., 2003).

Concerning our identification that endochondral ossification was enhanced in fracture healing in \( Fgfr3^{+/−} \) mice. FGFR3 has long been known as a negative regulator of chondrocyte proliferation
and differentiation, thus being a gene critical for normal skeletal development (Colvin et al., 1996; Deng et al., 1996; Naski et al., 1996; Valverde-Franco et al., 2004; Xiao et al., 2004). The short-limbed phenotypes of individuals with FGFR3-related skeletal dysplasias, such as achondroplasia, are primarily explained by reduced chondrocyte proliferation and differentiation in the growth plate (Deng et al., 1996). Our data, using a fracture repair model, are therefore consistent with the findings of previous developmental studies using the same Fgfr3 deficient mouse, leading to an increase in endochondral ossification. In addition our study demonstrates how the function of FGFR3 in fracture healing recapitulates its function in development through gross abnormalities in chondrocyte biology.

In addition we found that, despite accelerated fracture healing in Fgfr3+/− mice, the polar moment of inertia was reduced. The polar moment of inertia (PMI) is a measurement of an object's ability to resist a torsional force, and in a healing fracture is considered a measure of its integrity (Morgan et al., 2009). We were not able to determine from this study whether the reduction in PMI in Fgfr3+/− fractures was great enough to constitute insufficient stiffness for physiological purposes in our mice. Therefore whilst we have identified accelerated fracture healing in Fgfr3+/− mice in this study, we were not able to conclude if this was a true biological enhancement and advantage, but the findings certainly speaks to the concept that faster healing does not necessarily mean better healing, in terms of the quality of bone repair.

We examined the number and proliferation of mesenchymal progenitors (SSCs) in the bone marrow of Fgfr3+/− mice and compared the ability of these progenitors to differentiate to osteoblasts using a series of in-vitro colony forming unit assays. We found that in Fgfr3+/− bone marrow that SSCs were scarcer and that cells within BMSC colonies derived from CFU-Fs showed reduced proliferation.

Furthermore we found that osteoprogenitors in the periosteum of healing fractures also demonstrated a reduction of proliferation in-vivo. Upon directed differentiation of BMSC colonies, our data showed fewer CFU-O and a reduction in osteoblast-related genes in Fgfr3+/− samples. This reduced osteogenic capacity was not merely a reflection of reduced Fgfr3+/− BMSC colony formation, as seen in the CFU-F assay, as the ratio of osteogenic colonies to non-osteogenic colonies was also diminished in Fgfr3+/− cultures.
Our data confirms the effect of FGFR3 signaling on terminal osteoblast differentiation shown in a previous study, where reduced Von Kossa stained bone nodules were seen in the osteogenic differentiation of BMSC cultures of Fgfr3+/- mice (Valverde-Franco et al., 2004). In contrast to Valverde-Franco’s study we identified not only a reduction in Von Kossa-positive bone nodules but also a reduction in ALP-positive colonies, whereas her group showed an initial increase in ALP-positive colonies prior to a reduction in overall number of mineralized bone nodules. This conflicting data may represent differences in mouse strain (C3H versus C57Bl6) where differences in Fgfr3-/- phenotype have been described, or variances in experimental protocol; Valverde-Franco commenced osteogenic differentiation at day 1 of culture versus our protocol of establishing BMSC colonies over 7 days prior to stimulating osteogenic differentiation. Both mouse strain and experimental conditions have previously been shown to alter colony forming and differentiation ability of BMSC cultures (J. E. Aubin, 1999; Bianco et al., 2001; Sergei A Kuznetsov et al., 2009).

Consistent with the gene expression data from undifferentiated Fgfr3+/- BMSC colonies, Fgfr3+/- CFU-O colonies also showed a reduction in osteoblast-related genes Runx2, Osx, Col1a1, Alp, Opn, BSP and Ocn, compared to WT CFU-O colonies. Our data is in agreement with that of Su who investigated osteoblast differentiation in mice with a gain-of-function mutation of FGFR3, and found that mRNA expression of osteoblast genes Runx2, Opn, Ocn and Col1a1 were increased compared to WT (N. Su et al., 2010). In addition to the relative spatial reduction in mRNA expression of osteoblast genes in Fgfr3+/-, we also found that osteoblast gene expression recapitulated the normal temporal relationship of osteoblast differentiation, with mRNA of extra cellular matrix proteins Col1a1 and Opn peaking at an earlier time point (day 10 of differentiation) than the proteins BSP and Ocn (day 21 of differentiation). BSP and Ocn are considered the most specific of the osteoblast genes and are only expressed in terminally differentiated osteoblasts and thus our data supports that osteogenesis occurred in a developmentally appropriate sequence. Taken together our findings suggest that the observation that FGFR3 acts as a bone switch in-vivo may be due to both an effect on undifferentiated mesenchymal progenitors and the ability of osteoprogenitors to differentiate down the osteoblastic pathway.

Bone marrow transplantation (BMT) allows investigation of the response of endogenous mesenchymal cells to engrafted haematopoietic cells with a characteristic of interest (Baht et al.,
We used this principle to engraft \( Fgfr3^{+/−} \) haematopoietic cells through bone marrow transplantation into WT mice, and to engraft WT haematopoietic cells into \( Fgfr3^{+/−} \) mice to determine whether FGFR3 signaling from haematopoietic cells controlled the mesenchymal response to fracture repair.

Our data showed that engraftment of \( Fgfr3^{+/−} \) bone marrow into WT mice inhibited intramembranous ossification and enhanced endochondral ossification in fracture repair, recapitulating the pathological healing response that was seen in native \( Fgfr3^{+/−} \) mice. Alternatively, the engraftment of WT bone marrow into \( Fgfr3^{+/−} \) mice enhanced the potency of intramembranous ossification and concomitantly diminished endochondral ossification to the extent that we saw a rescue of the pathological \( Fgfr3^{+/−} \) fracture phenotype. These data therefore show that the FGFR3 bone-switch we demonstrated in Chapter 2 is likely to be controlled by cells of the haematopoietic lineage, rather than a purely autonomous mesenchymal defect. This is important because being able to control bone formation for the purposes of skeletal regenerative therapies requires knowledge of not only the most vital signaling pathways involved, but perhaps more importantly an intimate understanding of the specific cells themselves which in effect drive the signaling pathways. Much has been published on the modification of mesenchymal cells to improve bone healing and regeneration, assuming that if one can engineer and enhance functional mesenchymal cells that carry out the repair work then this may prove to be an efficient therapeutic intervention (Ciapetti et al., 2006; Granchi et al., 2010; Guan et al., 2012; Kumar et al., 2008; Muschler et al., 2004). Our data, in addition to that from the emerging field of the haematopoietic control of mesenchymal reparative response (Alexander et al., 2011; Raggatt et al., 2014; Vi et al., 2015), may therefore contribute to a paradigm shift in how we approach future therapeutic enhancement of skeletal regeneration and repair, by focusing on haematopoietic cell engineering, as the drivers of mesenchymal response.

Our results therefore broadly support the hypothesis that we initially posed, that FGFR3 does indeed affect osteoblast differentiation and thus fracture repair, and we were also able to identify an effect of FGFR3 signaling in transplanted bone marrow cells, likely an effect of haematopoietic cells, on fracture repair. Our specific aims were realistic in their scope, as we were able to build upon previous work in the literature and use a series of techniques developed in our laboratory, specifically related to fracture repair to add to the basic knowledge already known about FGFR3 signaling in mesenchymal cell biology.
The most novel findings in this thesis are those pertaining to the role of haematopoietic cells in controlling the mesenchymal response to fracture repair through FGFR3 signaling. Haematopoietic cellular control of fracture repair is an emerging area of intense investigation in the field of fracture repair, and although macrophages and lymphocytes have been broadly shown to affect osteoblast differentiation by other investigators, our work is the first to specifically identify a signaling pathway that accounts for a change in fracture phenotype, and furthermore to have the unique effects of increasing bone formation by one pathway whilst decreasing it from another pathway. In this regard our work may also be more broadly applied to developmental biology in addition to the field of fracture repair. It is possible that FGFR3 acts as a fate switch for a common osteochondroprogenitor, whereby differing levels of FGFR3 alter the balance of whether mesenchymal progenitor cells differentiate to chondrocytes or osteoblast and this may have important implications in tissue regenerative strategies in the future.

There are a number of limitations with the experimental work carried out within this thesis. We initially planned to investigate the function of FGFR3 using a gain-of-function mutation of FGFR3 in a mouse. Although such mice are available there are a number of different gain-of-function FGFR3 mice purported to represent the disease achondroplasia, however all have slightly different phenotypes and therefore may not represent the true achondroplasia phenotype (Segev et al., 2000; N. Su et al., 2010; Wang et al., 1999). For example some mouse models show only post-natal phenotypic defects, rather than embryonic and post-natal defects that one sees in the human disease (Segev et al., 2000; N. Su et al., 2010; Wang et al., 1999). We therefore elected to use a widely available knock-out FGFR3 mouse model, which would more succinctly offer the effects of FGFR3 signaling compared to a gain-of-function mutant. We found that the survival of $Fgfr3^{-/-}$ was poor, with most dying in the first few days of life, likely due to respiratory insufficiency and problems with feeding. The fact that we needed mice to survive to at least 8 weeks, and preferably 12 weeks of life for fracture precluded their use, hence the use of the $Fgfr3^{+/}$ heterozygotes. Although heterozygous knockouts often have a mild phenotype compared to their homozygous knockout counterparts, or indeed no phenotype at all, we did not find that a particular issue. This is perhaps not surprising since most FGFR3 skeletal dysplasia in humans, of which there are many, are often typically associated with heterozygous mutations, such as achondroplasia and hypochondroplasia.
Attempting to dissect the role of a specific cell type, i.e. the osteoblast, using a ubiquitously expressed mutant gene makes interpretation of results more complicated, because it is difficult to separate the effects of one cell type from another. In fracture repair multiple cell types necessarily act together and undoubtedly have a bearing on the function of each other, the main players being osteoblasts, chondrocytes, osteoclasts, macrophages, neutrophils and lymphocytes. Despite this limitation the mouse model we used had the benefit of being able to provide an broad overview of the function of FGFR3 in fracture repair, and much can be learned from looking carefully at histological specimens of complex processes such as fracture repair, as relationships between cell types and reparative tissue types can be seen, as we identified by examining the difference in intramembranous and endochondral ossification within the same fracture. There are other models that we could have used to examine in-vivo intramembranous ossification more specifically such as a calvarial defect, or an isolated stabilized cortical bone defect. Although these alternative approaches would have given us specific information about the intramembranous bone formation pathway, and thus fundamental knowledge about the behaviour of osteoblasts, these models do not represent a real-world problem, with the interaction of different tissue responses such as in a typical fracture.

Rather than use a constitutional FGFR3 knock out mouse we could have used a conditional knockout mouse model of FGFR3 for specific cell types, such as osteoblasts, but at the time of writing these models are not available to us. The downside again is that by using specific conditional knockouts we may have failed to see a broad overview of the changes that happen in fracture repair in the setting of a specific gene mutation. Therefore now that this preliminary work has been done, further work on the role of FGFR3 signaling in macrophages, lymphocytes or osteoblasts would certainly be better performed using a cell specific knock-out to further advance our knowledge.

There are currently a number of limitations of the in-vitro investigation of mesenchymal cells. Bone marrow stromal cells (BMSCs) are a heterogeneous group of cells and contain a subgroup of multipotent skeletal stem cells (SSCs) that have the ability to differentiate into osteoblasts, chondrocytes and adipocytes, which form bone, cartilage and fat respectively (Bianco et al., 2001; Friedenstein, 1980; S. A. Kuznetsov et al., 2007). The main issue is that despite this belief, there is a failure of consensus on what constitutes an SSC (alternatively named MSCs, mesenchymal stem cells, or mesenchymal stromal cells), as there are no specific markers that
clearly identify such a progenitor cell, nor any specific morphological characteristics. Due to the absence of specific characteristics of the SSC and the variable ability of identified cells to differentiate, there are currently no gold-standard assays to identify the SSC. Nevertheless, considering that there is a lack of consensus on biochemical or morphological markers for what truly constitutes a CFU-F (OR SSC), then the CFE of any given bone marrow sample is currently the best approximation to the number of SSCs in a marrow sample (Bianco et al., 2001; Sergei A Kuznetsov et al., 2009). For these reasons we utilized the colony forming unit assays as the mainstay of our investigation of the characteristics of SSCs and BMSCs from our experimental mice.

Consistent with the difficulty in identifying markers of multipotent mesenchymal progenitors in bone marrow, the identification of specific osteoprogenitors is also challenging, yet under specific in-vitro conditions cells within BMSC colonies can differentiate to osteoblast-like cells, which form bone nodules identifiable by the production of bone matrix and mineral (Bellows et al., 1998; Bhargava et al., 1988; Herbert et al., 1997; Nefussi et al., 1985). The number of bone nodules, termed colony forming units osteoblast (CFU-O), represents the number of osteoprogenitors in a bone marrow sample as each colony again forms from a single cell precursor (J E Aubin, 1998). The number of bone nodules per number of cultured cells gives a calculation of the osteogenic efficiency of a sample, and serves as measure of the bone forming capacity of the bone marrow of an individual. CFU-F and CFU-O assays therefore form an important method for systematically investigating the characteristics, and ability to differentiate, of the mesenchymal progenitor population.

Bone marrow transplantation (BMT) allows investigation of the response of endogenous mesenchymal cells to engrafted haematopoietic cells with a characteristic of interest (Baht et al., 2015; Xing et al., 2010). It has been established that donor mesenchymal cells do not survive engraftment in the same way that haematopoietic cells do, and thus any mesenchymal response in the recipient animal is attributed to resident mesenchymal cells of the recipient animal. This has been shown elegantly by selectively depleting osteoblasts from the recipient mouse prior to BMT, leading to a complete failure of fracture healing (Baht et al., 2015). Engrafted mesenchymal cells were not seen in appreciable numbers and were unable to repair the bony defect. We are therefore confident that the activation of the FGFR3 bone switch is via haematopoietic cells, rather than transplanted mesenchymal cells. There are a number of
limitations of bone marrow transplantation, which include variability in the experimental results secondary to technical factors such as potential variability in actual radiation dose received by each mouse, variability in composition of cells actually engrafted, which may lead to a mortality rate with the experimental procedure, variation in colony forming ability of in-vitro BMSC cultures, and variation in the morphology and characteristics of fracture healing. For example we saw more variation in fracture callus size and morphology in mice that have received BMT compared to non-transplanted mice from historical experiments. Despite these numerous limitations, which were offset as much as possible using control mice receiving isogenetic BMT, we identified clear differences in the fracture phenotype and clear differences in the in-vitro osteogenic efficiency of the experimental groups based upon which type of BMT they received.
5.2 Concluding Summary

FGFR3 signaling has a critical role in the function of multiple cell types in the regulation of bone fracture healing; Specifically we found that haematopoietic cells signal through FGFR3 to regulate osteoblast differentiation which control fracture repair by switching the balance of intramembranous and endochondral ossification.

We first hypothesized that FGFR3 signaling regulates osteoblast differentiation leading to an alteration in both endochondral and intramembranous ossification in fracture repair. This hypothesis was based on the observation that individuals with achondroplasia form excellent regenerate bone when undergoing limb lengthening (distraction osteogenesis), and that distraction osteogenesis primarily utilizes intramembranous ossification to regenerate bone. We showed initially that FGFR3 effectively acts as a bone switch in fracture repair, by regulating the tightly coordinated balance of intramembranous ossification and endochondral ossification, with FGFR3 deficiency inhibiting intramembranous ossification and simultaneously enhancing endochondral ossification in murine fracture repair. In addition to these primary findings, we also showed that FGFR3 signaling is important in the later phases of fracture remodeling, as inhibition of FGFR3 lead to more rapid bone remodeling. Taken together, and based upon micro-CT analysis, we showed that these alterations in the balance of bone formation and remodeling lead to a loss of structural integrity of the healing fracture callus.

Considering that one of the key steps in intramembranous ossification is the differentiation of osteoprogenitor cells to osteoblasts to form bone, we then hypothesized that deficiency in FGFR3 would decrease the number, or proliferative ability, of osteoprogenitors in bone marrow and, furthermore, that impaired FGFR3 signaling would reduce the ability of osteoprogenitors to differentiate to functional osteoblasts. We found that SSCs in Fgfr3+/− bone marrow are scarcer than in WT mice, and that cells within Fgfr3+/− BMSC colonies derived from CFU-Fs did indeed show reduced proliferation and have a reduced capacity for osteoblast differentiation. Furthermore, osteoprogenitors in the periosteum of healing fractures in Fgfr3+/− mice also demonstrate a reduction of proliferation in-vivo. FGFR3 signaling may therefore activate a form of upstream osteogenic imprinting where undifferentiated progenitor cells are primed to preferentially differentiate to or from the osteoblast lineage depending on whether FGFR3 is over or under expressed. Taking this one step further, assuming that osteoblasts and
chondrocytes share a common progenitor, FGFR3 may act as a fate switch, whereby FGFR3 deficiency inhibits osteoblast differentiation whilst simultaneously enhancing chondrocyte differentiation. Taken together, our findings and further speculation offer an explanation for the in-vivo clinical findings of defective intramembranous ossification and enhanced endochondral ossification in fracture healing in \( Fgfr3^{+/−} \) deficient mice.

Based on our initial findings that bone fracture remodeling is enhanced in \( Fgfr3^{+/−} \) mice, which is effected by an increase in osteoclast number and activity, we demonstrated that FGFR3 signaling has effects on both haematopoietic cells, as well as mesenchymal cells, in the regulation of skeletal repair. We therefore hypothesized that other cells within bone marrow, may control mesenchymal differentiation via FGFR3 signaling, which leads to the previously observed alterations in intramembranous ossification, endochondral ossification and osteoblast differentiation, and thus act a key moderator of fracture repair. Our data confirmed our hypothesis by showing that engraftment of \( Fgfr3^{+/−} \) bone marrow into WT mice recapitulated pathological fracture healing, by inhibiting intramembranous ossification and increasing endochondral ossification, seen in native \( Fgfr3^{+/−} \) mice, and in addition BMSC cultures from \( Fgfr3^{+/−} \) engrafted WT mice also showed defective osteoblast differentiation in-vitro. Conversely, the engraftment of WT bone marrow into \( Fgfr3^{+/−} \) mice also rescued its pathological fracture phenotype and in-vitro osteoblast differentiation. Thus transplanted bone marrow cells (probably haematopoietic cells) control the mesenchymal response to fracture healing via FGFR3 signaling.

The successful regeneration of high quality, functional bone tissue and the enhancement of fracture healing will depend strongly on the ability to understand and replicate the critical steps in the differentiation of mesenchymal cells that occurs under normal development and repair. Our findings add considerably to the growing body of evidence that cells of the haematopoietic lineage have a crucial role in controlling the mesenchymal response to bone fracture. We have identified that FGFR3 signaling allows haematopoietic cells to regulate fracture repair by controlling the balance between the two major pathways of bone formation, in part through the regulation of mesenchymal progenitor proliferation and osteoblast differentiation. FGFR3 signaling may therefore prove to be an ideal target for novel drug development for the enhancement of fracture repair and bone regeneration.
5.3 Future Directions

A number of experiments are ongoing, and indeed planned to further the scope of this research still within the original aim to understand further the role of FGFR3 in skeletal repair, and its effects on osteoblast differentiation. This section also includes some longer-term thoughts on where this line of research may lead.

Firstly, we aim to build on our bone marrow transplant experiments and identify whether it is truly haematopoietic cells that signal through FGFR3 to affect osteoblast differentiation. One line of investigation therefore centres on which particular cell of the haematopoietic lineage is responsible for the effects on osteoblast differentiation and in-vivo alterations in intramembranous and endochondral ossification. The most likely candidate is the macrophage followed by the T-lymphocytes and B-Lymphocytes based upon emerging evidence of their general involvement with osteoblast differentiation and specifically in fracture repair. The experimental plan will be to use both an in-vitro and in-vivo approach. Firstly we will perform a CFU-O assay on both WT and Fgfr3+/− using macrophage-conditioned media. Specifically, we will culture WT and Fgfr3+/− macrophages in vitro using M-CSF, then use fluorescence-activated cell sorting (FACS) analysis of Mac-1 and 4/80 surface antigen expression. These sorted macrophages will then be cultured with WT and Fgfr3+/− CFU-O cultures, as previously described, with the addition of FGF18 (most likely candidate ligand of FGFR3) and the effects on in-vitro osteogenesis measured.

If the secreted products from macrophages are responsible for the alteration in osteoblast differentiation we will see that when Fgfr3+/− macrophage conditioned media is used in a WT CFU-O assay that osteoblast differentiation will be inhibited and conversely when WT macrophage conditioned media is used in a Fgfr3+/− CFU-O assay that osteoblast differentiation will be rescued to wild type levels. If these in-vitro experiments prove to yield positive results and further support the macrophage as the responsible cell, then we would perform confirmatory in-vivo fracture repair experiments using conditional knockout of FGFR3 in macrophages. This would have the advantage of allowing homozygous knockout of FGFR3 in our cell of interest, and yet avoiding the considerable disadvantage of perinatal mortality associated with constitutive deletion of FGFR3 that we identified at the commencement of this research. Using a conditional knockout would, in addition, allow us to see the specific effects of the FGFR3
deficient macrophage upon normal osteoblasts, chondrocytes and this fracture repair. A recapitulation of pathological fracture healing as seen in native Fgfr\(^{3/-}\) mice would offer much stronger evidence that the macrophage is indeed the cells coordinating the abnormal bone-switch through FGFR3 signaling.

Should the in-vitro macrophage conditional media experiments fail to show a difference in osteogenic efficiency, then it is possible that either the factor controlling the change in osteoblast differentiation is not a soluble secreted factor rather a cell-cell interaction, or more likely that the macrophage is not the cell responsible for the regulation of osteoblast differentiation. Experiments would then be repeated using media conditioned by other cells of the haematopoietic lineage such as T- and B-lymphocytes, and again, conditional knockout FGFR3 mice would offer a confirmatory in-vivo approach.

Other than the cell of interest, it is possible that whichever cell is responsible for regulating osteoblast differentiation and fracture healing through FGFR3 signaling does so through a secreted factor. We would take a candidate approach, and have already identified three candidate proteins that may be responsible. Based on a report of human chondrocyte gene expression profiling in individuals with FGFR3-related chondrodysplasias a number of candidate proteins were identified including POSTN, TNFAIP6 and HSST2 (Schibler et al., 2009). Of these, periostin (POSTN) is potentially the most interesting as it is involved with the adhesion and migration of cells, increases bone formation, is a cell cycle regulator. In addition, based on unpublished data from our laboratory, periostin is expressed in macrophages amongst other cell types.

To test these candidate proteins as actuators of the FGR3 bone-switch, we would use qPCR to look for changes in mRNA expression in CFU-O assays, attempt to modify osteoblast differentiation in CFU-O assays with the addition of recombinant candidate protein or neutralizing antibody, and use immunohistochemistry to detect the presence of these candidate proteins and their distribution in histological sections of fracture healing from both Fgfr\(^{3/-}\) and WT mice. Finally in-vivo we would administer recombinant protein directly to the fracture site to attempt to rescue normal fracture repair in native Fgfr\(^{3/-}\).

Looking longer term, I believe that there will be a move towards systemic therapies for the enhancement of fracture repair and bone regeneration using genetically modified cells that are
targeted to the area of regeneration. This type of therapy would be a more sophisticated and I believe more controllable approach than some of the current attempts to enhance fracture repair and bone regeneration by direct injection of the fracture site with a drug of choice. It would allow consistent doses of cell or drug delivery and offer a way of targeting multiple signaling mechanisms, or allowing the release of a cocktail of therapeutic proteins. By using a cellular therapy allows the intrinsic machinery of the cell to act as a control centre for the bone enhancement.

A crucial advance in this line of research will be to move experimentation from mouse to human biology, which is a necessary step closer to therapeutic intervention in human subjects. With the advances in human biology taking place such as induced pluripotent stem cell (iPSC) technology and the promise of gene editing through the CRISPR/CAS9 system, a potential avenue for our line of research will begin. This could take two forms. Firstly we would use cells from individuals with genetic diseases for the purpose of discovery, by investigating the function of that particular gene, and second approach would be to use this knowledge of gene function to provide genetically manipulated cellular therapies for normal individuals. These two distinct programs would work something like this,

We would reprogram somatic cells from individuals with achondroplasia (or any other skeletal dysplasias of interest) into iPSCs. The achondroplastic iPSCs can then be gene edited, to excise the over active FGFR3 gene, and to replace the defective gene with a normal functioning FGFR3 gene, so as to derive a patient specific control cell line. In parallel we will need to develop a precise differentiation protocol for the human iPSCs to be able to replicate every important developmental step to allow their differentiation into functional osteoblasts (or chondrocytes, macrophages etc). The differentiation of mutant and control osteoblasts will be investigated in-vitro using a combination of colony forming units, gene expression analysis, flow cytometry to understand the effects of the FGFR3 on osteoblast differentiation..

The second approach would be to again use iPSCs but derived from normal individuals and gene-edit them to express a particular modification of a gene of interest. For instance, should we prove that FGFR3 is indeed better off over-expressed in macrophages in the setting of distraction osteogenesis to enhance intramembranous ossification, then patient specific iPSC-derived macrophages which over express FGFR3 can be generated. These therapeutic
macrophages would then be infused back into the patient undergoing limb lengthening surgery to speed up and improve the bone regenerative process.

It is clear that there are a number of interesting and worthwhile avenues of investigation that lead on from the work contained within this thesis, from low-risk natural follow-on experiments that will establish a more detailed mechanism for our observations thus far, to more high-risk and cutting edge approaches, which will further the knowledge and application of musculoskeletal regeneration. It is rather timely that the advances in knowledge and techniques for investigating human cell biology offer such exciting opportunities to translate fundamental basic scientific findings into useful therapeutic application.
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