Age Related Tissue Fibrosis During Fracture Repair Is Mediated by Wnt/β-catenin Signaling

by

David Craig Silkstone

A thesis submitted in conformity with the requirements for the degree of Masters of Science
Institute of Medical Sciences
University of Toronto

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University of Toronto
2010

Abstract

The regenerative potential of tissue injury declines with age. Recently, a significant role for Wnt/β-catenin signaling has been shown in tissue specific stem cell aging, leading to increased tissue fibrosis. Wnt/β-catenin signaling regulates the differentiation of multipotent mesenchymal stem cells into osteoblasts during fracture repair. We investigated the potential role of dysregulated Wnt/β-catenin signaling in delayed fracture union and tissue fibrosis in the elderly. Old mice displayed increased total β-catenin protein levels at 4 and 7 days post-fracture and tissue fibrosis at 14 and 21 days post-fracture compared to young mice. Furthermore, treatment with a pharmalogical agent decreased total β-catenin protein levels in the fracture callus at 4 days post-fracture and prevented tissue fibrosis at 21 days post-fracture. Our data suggests that dysregulated Wnt/β-catenin signaling in the elderly contributes to delayed fracture repair and tissue fibrosis and offers a potential therapeutic strategy to improve fracture outcome in the elderly.
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<th>Full Form</th>
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<tr>
<td>Actα2</td>
<td>Smooth Muscle α-Actin</td>
</tr>
<tr>
<td>ALP</td>
<td>Alkaline Phosphotase</td>
</tr>
<tr>
<td>BMM</td>
<td>Derived Monocyte or Macrophage Precursor Cell</td>
</tr>
<tr>
<td>BMD</td>
<td>Bone Mineral Density</td>
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<td>BMP</td>
<td>Bone Morphogenetic Protein</td>
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<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
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<tr>
<td>ColI</td>
<td>Collagen type I</td>
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<tr>
<td>ColII</td>
<td>Collagen type II</td>
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<tr>
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<td>Collagen type III</td>
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<td>Collagen type X</td>
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<tr>
<td>DHH</td>
<td>Desert Hedgehog</td>
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<tr>
<td>DSH</td>
<td>Dishevelled</td>
</tr>
<tr>
<td>eNOS</td>
<td>Nitric Oxide Synthase</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast Growth Factor</td>
</tr>
<tr>
<td>FN1</td>
<td>Fibronectin 1</td>
</tr>
<tr>
<td>FoxO</td>
<td>Forkhead Box O</td>
</tr>
<tr>
<td>Fzd</td>
<td>Frizzled</td>
</tr>
<tr>
<td>Fzd1</td>
<td>Frizzled1</td>
</tr>
<tr>
<td>Gjα1</td>
<td>Gap Junction α-1 Protein</td>
</tr>
<tr>
<td>Hh</td>
<td>Hedgehog</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and Eosin</td>
</tr>
<tr>
<td>HSC</td>
<td>Haematopoietic Stem Cell</td>
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<tr>
<td>IHH</td>
<td>Indian Hedgehog</td>
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<tr>
<td>IFNγ</td>
<td>Interferon Gamma</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>LEF</td>
<td>Lymphoid Enhancer Factor</td>
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<td>MMSC</td>
<td>Multipotent Mesenchymal Stem Cells</td>
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<tr>
<td>Myh3</td>
<td>Myosin Heavy Chain 3</td>
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<tr>
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<td>Osteocalcin</td>
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<tr>
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<td>Osteoprotegrin</td>
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<tr>
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<td>Osterix</td>
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<tr>
<td>PCNA</td>
<td>Proliferating Cell Nuclear Antigen</td>
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<td>PCP</td>
<td>Non-canonical polarity pathway</td>
</tr>
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<td>PDGF</td>
<td>Platelet Derived Growth Factor</td>
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<tr>
<td>PECAM</td>
<td>Platelet Endothelial Cell Adhesion Molecule</td>
</tr>
<tr>
<td>PTCH1</td>
<td>Patched 1</td>
</tr>
<tr>
<td>RANKL</td>
<td>Receptor Activator NFκB Ligand</td>
</tr>
<tr>
<td>RunX2</td>
<td>Runx-related Transcription Factor 2</td>
</tr>
<tr>
<td>SC</td>
<td>Satellite Cell</td>
</tr>
<tr>
<td>SMO</td>
<td>Smoothened</td>
</tr>
<tr>
<td>SO</td>
<td>Safranin O</td>
</tr>
<tr>
<td>TCF</td>
<td>T-cell Factor</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Name</td>
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<tr>
<td>TGF-β</td>
<td>Transforming Growth Factor β</td>
</tr>
<tr>
<td>TNC</td>
<td>Tenascin</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor Necrosis Factor α</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
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Chapter 1

1 Introduction

Fracture repair is a regenerative process of tightly coordinated biological events that partially recapitulate normal bone development. Approximately one-third of all individuals will experience a fracture during their lifetime, and in most cases the reparative process results in successful fracture union (Praemer et al., 1992). However, there is a failure of normal healing in 5% of fractures, leading to non-union and requiring additional surgery (Figure 1.1) (Komatsu and Warden, 2010). One of the largest contributing factors to fracture risk and non-union is age, with 25% of fracture patients requiring extensive surgery and care (Einhorn, 1998). As such, a pharmacological approach that enhances fracture repair would avoid the need for additional surgery, improve patient outcome and decrease the economic burden on healthcare systems. Understanding the cell types and signaling pathways that direct fracture repair could provide insight into possible therapeutic targets and thereby help to achieve this aim.

1.1 Embryonic Bone Development

The gene expression profile and histological appearance of fracture repair is similar to the multiple phases of embryonic bone development (Ferguson et al., 1999). The embryonic skeleton develops through two different ossification processes. Intramembrous ossification is the process by which bones develop from the direct differentiation of mesenchymal stem cells (MSCs) into osteoblasts, which synthesize and mineralize the newly formed bone rich in type I collagen and hydroxyapatite crystals (comprised of calcium and phosphate) (Kronenberg, 2003; Olsen et al., 2000). Long bones develop through a process known as endochondral ossification, involving both chondrocytes and osteoblasts (Figure 1.2). Mesenchymal stem cells condense and differentiate into chondrocytes, which secrete a cartilage matrix rich in type II collagen (Hall and Miyake, 2000). Cells adjacent to the mesenchymal condensations elongate and form the perichondrium, forming a layer of dense connective tissue that surrounds the cartilage (Caplan and Pechak, 1987). The cartilage template provides the framework for the developing long bone to assume its shape. Long bone consists of the epiphyses and metaphyses at each end as well as
Figure 1.1
X-ray analysis of a non-union tibia fracture in a 65 year old male patient.
the diaphysis, the shaft in between. As the cartilage template lengthens towards the epiphyses, the central chondrocytes become hypertrophic, expressing collagen type X (ColX) (Erlebacher et al., 1995). Hypertrophic chondrocytes secrete various factors that initiate angiogenesis and perichondral cells adjacent to the cartilage template differentiate into osteoblasts and form the bone collar (Alini et al., 1996). The cartilage template surrounding the hypertrophic chondrocytes becomes mineralized by the deposition of hydroxyapatite crystals. Following calcification, proteinaceous components of the cartilage template such as collagen type II (ColII) and aggrecan are degraded by various proteinases (Cawston and Wilson, 2006). To prepare the developing long bone for angiogenesis, the calcified cartilage template is degraded by osteoclasts, which release H⁺ through a proton pump, acidifying the matrix into Ca²⁺ and H₃PO₄. Blood vessel and perivascular invasion of the cartilage template begins following the acidification and degradation of the calcified cartilage template and perivascular cells differentiate into osteoblasts and form the bone matrix rich in Coll (Chilosi et al.) and hydroxyapatite crystals (Zelzer et al., 2004). Long bones continue to elongate through the growth plate, which consists of chondrocytes and continues until skeletal maturity.

The regenerative capacity of adult bone largely depends on the re-initiation of molecular pathways that also direct chondrogenesis and osteogenesis during embryonic bone development (Gerstenfeld et al., 2003b; Sandberg et al., 1993; Vortkamp et al., 1998). The skeletal and connective tissues are composed of various cell types such as osteoblasts, chondrocytes, myoblasts and adipocytes, all of which are believed to originate from multilineage mesenchymal stem cells (MMSC) (Grigoriadis et al., 1988; Pittenger et al., 1999; Rogers et al., 1995; Yamaguchi and Kahn, 1991). Although osteoblasts form bone, the levels of bone mass are dependent on the balance between bone formation by osteoblasts and bone resorption by osteoclasts. Osteoclasts are multinucleated cells that resorb bone (Teitelbaum, 2000). As osteoclasts resorb bone, osteoblasts fill the cavity with bone. The balance between bone formation and bone resorbing is necessary for a healthy skeleton.

Regulation of osteoblast differentiation is mediated by many factors such as transcription factors, local factors and cell signals. These include runt-related transcription factor-2, bone morphogenetic proteins, Indian hedgehog signaling and Wnt/β-catenin signaling.
1.1.1 Runt-related Transcription Factor

Multiple transcription factors play a role in osteoblast and chondrocyte differentiation, but early osteoblast differentiation and terminal differentiation require Runt-related transcription factor 2 (RunX2). Overexpression of RunX2 in C3H10T1/2 cell and skin fibroblasts induced osteoblast related gene expression and positive alkaline phosphatase (ALP) staining (Ducy et al., 1997). ALP is a membrane-bound metalloenzyme, which catalyzes the hydrolysis of phosphonates at an alkaline pH, and includes the non-specific liver/bone/kidney ALP isoforms (Miao and Scutt, 2002). In vitro assays have demonstrated that ALP activity is absent during the proliferation phase of osteoblast maturation, but expressed during the differentiation phase (Nefussi et al., 1997). ALP staining is the most frequent biochemical marker to detect osteoblast differentiation and bone formation. (Nefussi et al., 1997) Antisense oligonucleotides for RunX2 inhibited osteoblast differentiation of rat primary osteoblasts, suggesting that RunX2 plays a crucial role in osteoblast differentiation (Banerjee et al., 1997; Otto et al., 1997). To determine the precise role of RunX2, a RunX2<sup>-/-</sup> was generated by deleting exon 1 of RunX2, which contains the runt-domain, but RunX2<sup>-/-</sup> mice die shortly after birth due to respiratory insufficiency (Komori et al., 1997). Von Kossa staining indicated that only parts of the tibia and vertebrate were slightly calcified and no calcification was detected in the skull, mandible or femur compared to wild-type controls, which displayed extensive calcification (Komori et al., 1997). Furthermore, many mRNAs related to bone matrix formation such as osteocalcin (OC), osteopontin (OP), and Coll contain RunX2 binding sites in their promoters (Ducy et al., 1997). Analysis of the RunX2<sup>-/-</sup> mouse indicated that mRNA expression levels of OP, Coll and OC were extremely reduced or not detectable, confirming arrest of osteoblast differentiation and bone formation (Komori et al., 1997). These results conclude that RunX2 is essential during the early phases of osteoblast differentiation, from the progenitor cell to an immature osteoblast. The immature osteoblast can then be further differentiated by growth factors. Various cell and animal models have shown that the potent local growth factor bone morphogenetic proteins (BMPs) increase RunX2 expression, and are one of the most potent osteoinductors (Grigoriadis et al., 1988; Lee et al., 1999).

1.1.2 Bone Morphogenetic Protein

BMPs were not discovered through analysis of embryonic bone development, but Urist reported that implantation of decalcified bone into muscle tissue resulted in new ectopic bone formation.
(Urist, 2002). The protein in the decalcified bone that was osteoinductive was identified as BMPs. Following the purification of BMP as single protein, it was found to belong to the transforming growth factor β (TGFβ) gene superfamily, which signal through Smad transcription factors. BMPs have a diverse role during bone development since various BMP’s are expressed in different locations with a spatial level of expression.
Figure 1.2
Endochondral bone formation of the developing limb. (a) Mesenchyme cells condense. (b) Condensed mesenchyme cells differentiate to chondrocytes and form the cartilage template, which is rich in ColII. (c) Chondrocytes at the center of the developing limb stop differentiating into chondrocytes and proliferate into hypertrophic chondrocytes expressing ColX. Perichondrium cells adjacent to the hypertrophic chondrocytes differentiate into osteoblasts and begin the formation of the mineralized matrix and bone collar. Hypertrophic chondrocytes then undergo apoptosis and the cartilage template is calcified. (d) The calcified cartilage surrounding the hypertrophic chondrocytes is acidified and degraded and following vascular invasion, perivascular cells differentiate into osteoblasts and form bone. (e) Chondrocytes continue to proliferate, become hypertrophic and cartilage is replaced with mineralized matrix, lengthening the bone. Osteoblasts and mineralized matrix of the bone collar will become the cortical bone and mineralized matrix and osteoblasts of the primary spongiosa will become the trabecular bone. (f) The secondary ossification center (SOC) forms after multiple rounds of chondrocyte hypertrophy, osteoblast differentiation, mineralized matrix formation and vascular invasion. The growth plate forms as the SOC as chondrocytes arrange themselves in columns. Adapted from (Kronenberg, 2003).
Gene expression studies and in-situ hybridization localized BMP-5 to areas of cartilage development and chondrocyte differentiation, BMP-2 and BMP-7 in the developing mesenchyme and cartilaginous analage, and BMP-4 in the mesoderm (King et al., 1994; Lyons et al., 1995; Winnier et al., 1995). To elucidate the role of each BMP during development, various BMP knockout mice have been generated. However, mouse models have failed to provide information on BMPs in skeletogenesis as mice die early during gastrulation. BMP-2−/− and BMP-4−/− mice die between 6.5 and 9.5 days p.c. and fail to enter gastrulation or develop a mesoderm (Winnier et al., 1995). BMP-7−/− mutated mice die shortly after birth due to renal failure; however there were only minor defects in the developing ribs and cartilage (Dudley et al., 1995). Due to the limited information gathered from BMP knockout studies in-vivo, the osteoinductive effects of BMPs were mainly discovered in osteoblast lineage cells and in-vitro experiments.

Treatment of the multipotent mesenchymal cell line C3H10T1/2 with BMP-2 and BMP-7 promoted osteoblast differentiation, induced osteoblast-related markers and demonstrated positive ALP activity. Untreated samples had extremely low levels of osteoblast differentiation markers (Wang et al., 1993). Furthermore, transfection of C3H10T1/2 cells with cDNAs encoding BMP-2 and BMP-4 induced both osteoblast and chondrocyte differentiation (Ahrens et al., 1993). In addition, the osteoblast induction potential of BMPs have been studied in multiple cell lines. BMP-2, 6 and 7 induced osteoblast differentiation in the ROB-C26 cell lines (Gitelman et al., 1995; Kobayashi et al., 1999; Tamaki et al., 1998). Yamaguchi and colleagues further demonstrated that BMP2, 4 and 7 induced osteoblast differentiation in both bone marrow stromal cell lines ST-2 and MC3T3-G2/PA6 (Yamaguchi et al., 1996). To determine the molecular mechanism of BMP induced osteoblast differentiation, downstream targets of BMP’s were identified as osteoblast-related transcription factors such as OSX and RunX2 (Lee et al., 2000; Nakashima et al., 2002). In RunX2−/− deficient mice, BMPs are unable to induce bone mineralization in calvarial cells, and blocking BMP-2 and BMP-4 signal transduction disrupts the ability of RunX2 to induce osteoblast differentiation, demonstrating that BMP signaling is upstream of RunX2 (Komori et al., 1997; Phimphilai et al., 2006). Independently, IL-1β and tumor necrosis factor-alpha (TNF-α) have no affect on ALP activity in the MC3T3-E1 cell line. However, IL-1β can synergistically increase BMP-2 induced ALP activity and TNF-α inhibited BMP-2 induced ALP activity (Nakase et al., 1997). In addition, over-expression of Noggin, a BMP antagonist, under the Col1α-Cre line caused a significant decrease in osteoclasts, resulting
in increased bone mass with decreased bone quality (Okamoto et al., 2006). This illustrates that BMP signaling is dynamic, co-operating with multiple cell signals and growth factors.

1.1.3 Indian Hedgehog Signaling

During normal bone development, Hedgehog (Hh) signaling is involved in bone formation, and it is likely that similar processes occur in post-natal bone repair (Ferguson et al., 1999; Vortkamp et al., 1998). Hh signaling was discovered by Christiane Nüsslein-Volhard and Eric Wieschaus as a gene that controls Drosophila embryonic cuticle patterning (Nüsslein-Volhard and Wieschaus, 1980). Hh signaling has an important role in Drosophila segmented polarity, but has been shown to have an important role in mammalian development, stem cell self-renewal and cancer growth (Beachy et al., 2004; Ingham and McMahon, 2001). As reviewed by Hammerschmidt and colleagues, there are three Hh ligands in vertebrates: Sonic (Shh), Indian (Ihh) and Desert (Dhh) (Hammerschmidt et al., 1997). Dhh is required for spermatogenesis, and Shh and Ihh have similar roles in development (Fan and Tessier-Lavigne, 1994; Lupo et al., 2006; McMahon et al., 2003). Shh regulates cell proliferation and plays a vital role in the survival of neural progenitors by inhibiting Gli3 (Litingtung and Chiang, 2000).

Hedgehog proteins are synthesized as approximately 45-kDa precursors, are cleaved by the C-terminal domain and post-translationally modified with the addition of cholesterol and a palmitoyl group (Hall et al., 1995; Porter et al., 1996a; Porter et al., 1996b). All three ligands are able to bind to the Patched (PTCH1) receptor, a seven-span transmembrane protein that acts as a pathway inhibitor, blocking Hh signaling in the absence of a Hh ligand (Chen et al., 2002; Marigo et al., 1996a). Ptch1 prevents Smoothened (SMO) downstream activation of Hh signaling by promoting SMO endocytosis and degradation (Denef et al., 2000). In the presence of a Hh ligand, SMO is phosphorylated by the GPCF kinase GRK2, relieving SMO inhibition and positively activating Hh signaling (Denef et al., 2000). Smo accumulation results in the upregulation of the Gli family of zinc-finger transcription factors, more specifically Gli1 and Gli2 (Ogden et al., 2006). In mammals, there are three Gli transcription factors: Gli1, Gli2 and Gli3, which act as activators or repressors (Ingham and McMahon, 2001). Cloning and gene expression patterns in the chick embryo identified Gli1 and Gli3 as direct targets of Shh (Marigo et al., 1996b). Gli transcriptional activation is mainly from Gli2 and repression from Gli3 (Jiang and Hui, 2008; Mo et al., 1997). Target genes of Hh signaling include proteins implicated in the
Hh pathway, such as Gli1 and Ptc1. Ihh is primarily expressed by prehypertrophic chondrocytes and Ihh signals to both immature chondrocytes and surrounding cells.

Hh signaling is required for endochondral bone formation during embryonic development. Genetic manipulation of Hh signaling during embryonic bone development revealed that Ihh regulates proliferation, differentiation and bone formation (Long et al., 2001; Rodda and McMahon, 2006; St-Jacques et al., 1999). Ihh−/− null mice display normal general patterning of the skeleton; however, long bones were one-third the length compared to their wild-type littermates, and calcification was delayed by four days. To determine the role of Ihh on chondrocyte proliferation, Bromodeoxyuridine (BrdU) incorporation from 12.5 and 14.5 dpc revealed a 50% drop in BrdU-positive nuclei in the Ihh−/− compared to the Ihh+/+ mouse, and in situ hybridization of ColX displayed reduced chondrocyte hypertrophy (St-Jacques et al., 1999). Furthermore, analysis of the expression levels and localization of RunX2 was completely absent from the periostium or perichondral tissue, trabecular and cortical bone. BMP3 levels were non-detectible in the Ihh−/− mutants, concluding that Ihh signaling is essential for osteoblast development and cortical bone formation (St-Jacques et al., 1999). To understand the role of Ihh signaling specifically on chondrocytes, a chondrocyte specific Col2-Cre mouse was used to over express or delete Ihh and Smo. Deletion of Smo had a similar reduction in chondrocyte proliferation and reduced growth of long bones as the Ihh−/− mutants, but chondrocyte differentiation was normal as shown by ColX expression levels (Long et al., 2001). Overexpression of Ihh and Smo increased chondrocyte differentiation and BrdU labelling by 60% at E16.5. Both gain- and loss-of-function approaches demonstrate a direct requirement pf Ihh for chondrocyte proliferation in development (Long et al., 2001). Although ubiquitous deletion of Ihh demonstrated that it is indispensable for embryonic bone development, it was unknown if the role of Ihh on osteoblast development is a direct or secondary effect to chondrocyte proliferation.

Utilizing the osteoblast-specific Col1a-Cre mouse, Smo was deleted to interrupt Hh signaling (Long et al., 2004). Morphological analysis with Alizarin red and Alcain blue revealed an absent bone collar in the Smo−/− mice. Cells from the periochondrium failed to undergo osteoblast differentiation, which was confirmed by the absence Col1, Runx2 and a decrease in Bmp2 expression. It was shown that the undifferentiated osteoblasts assume a chondrocyte morphology and expressed Col2 (Long et al., 2004). Downstream of Smo, the Gli family of...
proteins have direct roles in skeletal development, which was identified through molecular analysis of human Greig cephalopolysyndactyly syndrome (GCPS) (Hui and Joyner, 1993). Mutant mice deficient in Gli2 and Gli3 display a diverse defect in the skeleton development (Mo et al., 1997).

To understand the molecular mechanisms of how Hh signaling regulates osteoblast and chondrocyte differentiation, in vitro models have demonstrated that Hh signaling cooperatively works with various other osteogenic mechanisms to promote osteoblast differentiation. C3H10T1/2 cells treated with recombinant Shh (rShh) stimulated osteoblast differentiation and increased both ALP and BMP-2 activity (Yamaguchi et al., 2000). Furthermore, there was a synergistic increase in ALP staining and OC gene expression with the addition of both Shh and BMP-2 (Nakamura et al., 1997). To understand how Ihh and the Gli family of proteins regulate osteoblastogenesis, Shimoyama and colleagues investigated the relationship between Gli2 and Gli3 with RunX2 (Shimoyama et al., 2007). Gli2 and Gli3 were overexpressed in C3H10T1/2 cells with an adenovirus vector. Overexpression of Gli2 induced ALP activity and OC expression, and this effect was enhanced with Ihh treatment. However, overexpression of Gli3 did not increase ALP activity or osteocalcin expression and suppressed Ihh-induced ALP activity (Shimoyama et al., 2007). In addition, overexpression of Gli2 increased RunX2 expression, which acts downstream of BMP induced osteoblastogenesis (Lee et al., 2000; Shimoyama et al., 2007; Yamaguchi et al., 1996). In vitro analysis demonstrates that Hh signaling is involved in both immature and mature development in the osteoblast lineage.

In vitro models of Shh- or Ihh overexpressing chicken fibroblasts induced endochondral bone formation when injected into intraperitoneal sites of WT mice (Kinto et al., 1997). Ihh produced by post-natal chondrocytes is essential for maintaining the growth plate and trabecular bone, as Col2-Cre-ER-Ihh<sup>−/−</sup> mice have decreased trabecular bone and display dwarfism (Maeda et al., 2007). To address the role of Hh signaling in osteoblasts during bone homeostasis, Hh signaling was activated or blocked in mature osteoblasts using the human Osteocalcin-Cre (HOC-Cre), which expresses Cre recombinase in mature osteoblasts and osteocytes (Zhang et al., 2002). In adult mice, activating Hh signaling in mature osteoblasts caused osteopena, with an approximate 85% decrease in bone mineral density, 95% decrease in trabecular bone volume and decreased body size (Mak et al., 2008). Expression of proliferating cell nuclear antigen (PCNA), Terminal deoxynucleotidyl transferase (TUNEL) assay, calcein green labeling and tartate acid
phosphatase (TRAP) staining confirmed that the increased Hh signaling increased osteoblast proliferation and survival; however, there was a dramatic increase in receptor activator for nuclear factor κB ligand (RANKL) causing increased osteoclastogenesis and bone resorption. Removing Smo and inhibiting Hh signaling in 2 month old mice resulted in no differences in bone morphology. In 1 year old mice, trabecular bone volume was increased by approximately 50%, and RANKL and PTHrP expression was decreased (Mak et al., 2008). These results demonstrate that inhibiting Hh signaling in post-natal bone homeostasis could improve bone density and decrease the risk of fractures, showing a diverse role in Hh signaling from embryonic development and bone homeostasis.

Hh and Wnt/β-catenin signaling are both involved in embryonic skeletal development and osteogenesis. Utilizing a mature osteoblast promoter, Osx1-GFP:Cre;B-cateninΔcn mouse displayed decreased mineralized bone from E16.5-E18.5 and TCF/LEF transcription targets were absent (Rodda and McMahon, 2006). Furthermore, the development of an Osx1-GFP:Cre;SmoΔcn had no changes in chondrogenic and osteogenic markers and bone mineralization was normal for both groups, indicating that Hh signaling is not essential for the terminal differentiation and maturation of osteoblasts. These two mouse models demonstrate that Wnt/β-catenin signaling is required during osteoblast specification and further differentiation, whereas Hh signaling is required in skeletal progenitors and does not have an essential role in matrix-producing osteoblasts. (Rodda and McMahon, 2006). This was further confirmed using the C3H10T1/2 cells, which demonstrated that Hh-induced osteogenesis requires Wnt signaling and that Hh signaling works upstream of Wnt/β-catenin signaling (Hu et al., 2005).

1.1.4 Wnt/B-catenin Signaling

The Wnt/β-catenin pathway regulates multiple biological events during embryonic development and fracture repair, such as osteoblast differentiation and bone formation (Brault et al., 2001; Chen et al., 2007; Clevers, 2006; Kato et al., 2002).

The canonical Wnt/β-catenin signaling pathway is initiated by the interaction of Wnt ligands with their cognate receptor complex. The term Wnt was coined from the orthologous genes wingless, identified in Drosophila melanogaster, and the int-1 gene found in mouse musculus. Wingless is required for organizing the nervous system and wing during development (Miller and
Moon, 1996). The int-1 gene in mouse musculus was identified as the site of insertion of the mouse mammary tumor virus (Tsukamoto et al., 1988). Transcriptional activation of int-1 results in mammary gland hyperplasia and adenocarcinomas (Tsukamoto et al., 1988).

Genome sequencing has identified 19 secreted Wnt proteins in the mammalian species. Wnt molecules such as Wnt1, Wnt3a, Wnt4, Wnt7a and Wnt11 inhibit the differentiation of MSCs into chondrocytes (Church et al., 2002; Hartmann and Tabin, 2000). Furthermore, Wnt molecules have opposing effects on chondrocyte maturation. Overexpressing Wnt5a or Wnt5b delays maturation of hypertrophic chondrocytes, whereas overexpressing Wnt4 and Wnt8 expedites their maturation (Church et al., 2002; Yang et al., 2003). Wnt ligands can activate two other pathways upon receptor activation: the noncanonical polarity pathway (PCP), and the Wnt/Ca\(^{2+}\) pathway (Katoh, 2005). The receptor complex consists of a seven-transmembrane-domain receptor of the Frizzled (Fz) family of receptors, and an LDL-related protein 5 and 6 (LRP5/6) co-receptor. Canonical Wnt signaling is activated when Wnt ligands bind to both LRP5/6 and Fz (Clevers, 2006; Dale, 1998). There are numerous Wnt ligands, some of which activate the canonical or non-canonical signaling pathways, or even inhibit Wnt signaling (Hartmann and Tabin, 2000).

In the presence of a Wnt ligands binding to LRP5/6 and Fz, Axin mobilizes to the cell membrane and interacts with LRP5, and the LRP5 sequences responsible for interacting with Axin are required for LEF-1 mediated β-catenin signaling (Figure 1.3) (Mao et al., 2001). The binding of Axin to LRP-5 prevents the formation of the Axin-APC-GSK-3β destruction complex (Mao et al., 2001). β-catenin is not phosphorylated and not targeted for ubiquitin-mediated proteosomal degradation (Kitagawa et al., 1999). Furthermore, Dishevelled becomes phosphorylated, which sequesters the action of Axin and ultimately inhibits the kinase activity of the destruction complex (Hartmann and Tabin, 2000). Cytoplasmic accumulation of β-catenin results in the translocation of β-catenin to the nucleus. Import into the nucleus requires ARM repeats 10-12 and the C-terminus tail of β-catenin (Koike et al., 2004). Furthermore, β-catenin binds directly
with the phenylaline-glycine (FG) repeats on the NPC (nuclear pore complex) components, suggesting a bidirectional movement of β-catenin through the nucleus (Fagotto et al., 1998). Once in the nucleus, β-catenin interacts with TCF (T-cell Factor) / LEF (Lymphoid Enhancer Factor) transcription factors (Clevers, 2006).

Both humans and mice have four TCF/LEF genes and include TCF-1, TCF-3, TCF-4 and LEF-1 (Roose and Clevers, 1999). TCF/LEF transcription factors contain a high mobility group (HMG) domain which interacts with the minor groove of DNA and inducing an approximate bend of 130° (Giese et al., 1992). TCF factors can bind to DNA, but they require an interaction with β-catenin to mediate transcription of Wnt target genes (Molenaar et al., 1996). Canonical activated genes include c-myc, n-mycl, Clycin D1, TCF1, Axin-2, BMP-4, VEGF, RunX2, etc. (http://www.stanford.edu/~rnusse/pathways/targets.html).

In the absence of activating Wnt ligands, the cytoplasmic phosphoprotein Dishevelled (Hadshiew et al.) is not phosphorylated and is unable to inhibit the multiprotein complex composed of GSK-3β, APC, Axin and other co-accessory proteins (Church et al., 2002; Clevers, 2006). β-catenin becomes phosphorylated and is targeted for ubiquitination and degradation by proteasomal machinery.
Figure 1.3
Canonical Wnt/β-catenin signaling pathway. (a) In the absence of a Wnt ligand, β-catenin is phosphorylated by CK1γ at S45. β-catenin is then further phosphorylated at S33, S37 and T41 by GSK-3β and the multiprotein complex. Phosphorylated β-catenin is then targeted for proteosomal degradation and is unable to translocate to the nucleus and activate TCF/LEF transcription factors. (b) In the presence of a Wnt ligand binding to LRP5/6 and Fz, Dsh and the cytoplasmic tail of LRP5/6 become phosphorylated. The multiprotein complex is unable to phosphorylate β-catenin for proteosomal degradation. β-catenin is able to translocate to the nucleus, interact with TCF/LEF transcription factors and activate Wnt target genes. Adapted from (Silkstone et al., 2008).
The N-terminus of β-catenin has four serine/threonine phosphorylation sites located at S33, S37, T41 and S45, and is initially phosphorylated by CK1γ at S45 (Amit et al., 2002; Behrens et al., 1993; van Noort et al., 2002). Once β-catenin is phosphorylated by CK1, APC and Axin form a complex with β-catenin, and Axin acts as a scaffold binding to APC, GSK-3β and β-catenin (Hart et al., 1998). APC presents β-catenin to GSK-3β allowing for more efficient phosphorylation. GSK-3β binds to the destruction complex and phosphorylates β-catenin at S33, S37 and T41 (Hino et al., 2005). The phosphorylated Ser/Thr residues on β-catenin are recognized by β-TrCP, a component the Skp1/Cullin/F-box protein (SCF) ubiquitin ligase complex (Kitagawa et al., 1999). β-TrCP targets β-catenin for proteosomal degradation by the 26S proteosome (Orford et al., 1997). Once degraded, β-catenin is unable to translocate to the nucleus and activate TCF/LEF transcription factors. Furthermore, the Groucho (Grg) proteins are a family of transcription repressors that condense the chromatin by histone deacetylases, making TCF target genes inaccessible (Brantjes et al., 2001).

The role of Wnt/β-catenin signaling in bone development and homeostasis was identified when gain-of-function mutations in the human LRP5 was identified in Osteoporosis-Pseudoglioma Syndrome (OPPG) patients (Gong et al., 2001). Since then, various mouse models have been generated to understand the role of Wnt/β-catenin signaling in bone development. LRP5−/− mice display reduced bone mass, osteoblasts, and increased skeletal fragility (Gong et al., 2001; Kato et al., 2002). The LRP6−/− mouse is embryonically lethal, suggesting that LRP5 may have a more prominent role in post-natal bone homeostasis (Tamai et al., 2000). At the receptor level, Wnt-1 inhibited chondrogenesis and increased osteoblast differentiation and maturation in vivo and in vitro (Rudnicki and Brown, 1997). Upon overexpressing Wnt14a under the Col1α-1 promoter, β-catenin, and the increased levels of Wnt14 blocked chondrocyte differentiation and increased endochondral bone formation (Guo et al., 2004). Furthermore, loss-of-function mutations of Wnt5a, Wnt7a or LRP-6 in mice lead to abnormal development or lack of skeletal elements (Parr and McMahon, 1995; Tufan and Tuan, 2001).

To determine the role of β-catenin during bone development, β-catenin was deleted using the Cre-loxP approach; however, mice die of gastrulation defects at E8.5 before skeletal development (Haegel et al., 1995). To overcome this problem, the Prxl-Cre line conditionally deleted β-catenin (β-catΔPrx−/−) in limb and head mesenchyme (Figure 1.4) (Hill et al., 2005). In
the mutant mice, no cortical or trabecular bone formed, and cells lacking β-catenin differentiated into chondrocytes, as there was a decrease in Coll expression and increase in ColII expression. In situ hybridization demonstrated that several genes involved in the early phases of both chonrogenesis and osteogenesis were unaffected in the mutant mouse. However, in the mutant mice, osteoblastogenesis was terminated as both Osx or Oc were undetectable. To further demonstrate that osteoblastogenesis in the developing limb is dependent on β-catenin, an in vitro bone mineralization assay revealed decreased Alzhiren Red staining in the mutant mice, and this phenotype could not be rescued with the addition of recombinant Shh or BMP2 (Figure 1.4) (Hill et al., 2005). Under the Dermol-Cre line, which directs Cre expression in mesenchymal precursors of both chondrocytes and osteoblasts, β-catenin was conditionally deleted (Hu et al., 2005; Yu et al., 2003). Von Kossa staining displayed no endochondral or intramembrinous ossification, and in situ hybridization demonstrated that early osteoblast related genes such as Colla1, ALP and RunX2 had decreased expression levels, but mature osteoblast genes such as Osx were undetectable. Utilizing an osteoblast specific promoter, Colla1-Cre activation of β-catenin resulted in higher bone mass, while deletion resulted in osteopenia, inhibiting osteogenesis and increasing chondrogenesis (Glass et al., 2005). These results demonstrate that β-catenin is indispensable for osteoblast differentiation and regulates multiple pathways involved in osteoblast differentiation.

In vitro analysis has demonstrated a diverse role of Wnt/β-catenin signaling in chondrogenesis and osteoblastogenesis. Wnt/β-catenin signaling inhibits multipotent mesenchymal stem cells from differentiating into adipocytes, and commits progenitor cells to the osteoblast lineage (Ross et al., 2000). Wnt/β-catenin signaling has shown to have opposite osteogenic affects on undifferentiated and differentiated mesenchymal cells. Wnt3a or constitutively activated β-catenin inhibits the osteogenic differentiation of undifferentiated mesenchymal cells and juvenile calvarial cells in a dose-dependent fashion. Conversely, Wnt3a induces osteogenesis in mature calvarial osteoblasts in a dose dependent manner, demonstrating that Wnt/β-catenin signaling has a diverse role on different cell types (Quarto et al., 2010). Utilizing the C3H10T1/2 cell line, ectopic expression of stabilized β-catenin or activation of endogenous β-catenin with LiCl induces expression of ALP and Coll. However, unlike BMP2 induced osteoblastogenesis, stabilized β-catenin does not induce the expression of mature osteoblast markers such as OSX, suggesting that β-catenin is insufficient by itself to induce osteoblast maturation (Mbalaviele et
al., 2005). However, with the addition of BMP-2 to the β-catenin stabilized C3H10T1/2 cell line, early osteoblast markers were significantly up-regulated and mature osteoblast markers such as OSX were observable (Bain et al., 2003; Mbalaviele et al., 2005). These results suggest that β-catenin commits MSC’s to the osteoblast lineage, but is unable to fully differentiate osteoblasts without synergistically combining with other osteoinductive molecules such as BMP-2. In addition, deleting β-catenin by Cre-adenovirus in isolated calvarium cells abolished bone nodule formation compared to the control, which stained positive with von Kossa (Day et al., 2005).
Figure 1.4

β-catenin mutant mice lack mature osteoblasts and demonstrate defects in bone mineralization and femora cultures. (A-F) In situ hybridization of wild type and mutant β-catenin ΔPrx/- femurs at E16.5 (A-B, D-E) and WT femurs at E16.5 (C-F). Gene expression of RunX2 (A,D), Osx (B,E), and Col1α1 (C,F) demonstrate that mutant mice fail to develop mature osteoblasts and osteoblastogenesis is blocked in the final stages. (G-J) Alizarin red staining indicates decreased bone mineralization in β-catenin mutant β-catenin ΔPrx/- femurs compared to WT at 21 days post-differentiation in the presence or absence of recombinant Shh and BMP2. Adapted from (Hill et al., 2005).
Observing that \textit{LRP5} mutations cause decreased bone mass, a role for Wnt/\(\beta\)-catenin signaling has been identified in bone homeostasis. To increase bone mass, therapeutic drugs are available to alter Wnt/\(\beta\)-catenin signaling. LiCl increases active \(\beta\)-catenin by inhibiting GSK-3\(\beta\), the kinase that phosphorylates \(\beta\)-catenin targeting it for ubiquitination and degradation (Phiel and Klein, 2001; Zhang et al., 2003a). Furthermore, it was shown LiCl treatment in C57BL/6 mice leads to significant increase in bone mass and bone volume (Clement-Lacroix et al., 2005). LiCl was then administered to \textit{LRP5}\(^{-/-}\) mice, and bone volume, trabecular thickness and osteoblast number increased. Furthermore, LiCl and Wnt3a were shown to increase both ALP activity and \textit{Coll} expression in \textit{LRP5}\(^{+/+}\) and \textit{LRP5}\(^{-/-}\) progenitor cells, demonstrating the LiCl can increase osteoblast differentiation in a Wnt-canonical independent manner (Clement-Lacroix et al., 2005). The number of adipocytes per bone marrow area in \textit{LRP5}\(^{-/-}\) mice was higher than \textit{LRP5}\(^{+/+}\), but treatment with LiCl decreased the number of adipocytes in \textit{LRP5}\(^{-/-}\) mice and increased osteoblast numbers. Although LiCl works through an LRP5 independent manner, expression levels of Wnt target genes such as \textit{Ahr}, \textit{Nkd2} and \textit{Axin2} were increased in both \textit{LRP5}\(^{+/+}\) and \textit{LRP5}\(^{-/-}\) mice treated with LiCl (Clement-Lacroix et al., 2005; Jackson et al., 2005).

Although Wnt/\(\beta\)-catenin signaling increases bone mass, OPG is a direct target gene of Wnt/\(\beta\)-catenin signaling and regulates osteoclast mediated bone resorption (Jackson et al., 2005). Deletion of \(\beta\)-catenin under the \textit{Coll1a1-Cre} system decreased OPG and increased osteoclastogenesis as indicated by increased TRAP staining and \textit{RANK} gene expression, although \textit{RANKL} remained normal (Glass et al., 2005). Deleting \(\beta\)-catenin in more mature osteoblast by \textit{OC-Cre} increased \textit{RANKL} levels and decreased OPG (Holmen et al., 2005). These experiments demonstrate a diverse role for Wnt/\(\beta\)-catenin signaling regulating osteoclast differentiation. In the \textit{LRP5}\(^{+/+}\) and \textit{LRP5}\(^{-/-}\) LiCl treated mice, there were no differences in osteoclast numbers as indicated by TRAP staining and no changes in \textit{OPG} gene expression (Clement-Lacroix et al., 2005). Recently, BMP-2 was shown to increase Wnt/\(\beta\)-catenin induction of OPG expression, and Wnt/\(\beta\)-catenin induced OPG promoter activity (Sato et al., 2009). Collectively, these findings suggest that Wnt/\(\beta\)-catenin signaling in bone may be regulated by crosstalk between various bone regulatory molecules involved in bone formation and bone homeostasis.
1.2 Fracture Repair

Fracture repair is defined as three distinct phases: the inflammatory phase, the repair phase and the remodeling phase (Figure 1.5). Immediately following injury, hematoma formation, platelet degranulation and inflammation are initiated. Inflammation is a physiological response that initiates the fracture repair process and macrophages, inflammatory cells and PMSC contribute during this stage (Kon et al., 2001). Following the inflammatory phase of fracture healing, the repair process is initiated which recapitulates cell signaling pathways present in embryonic bone development and osteoblast differentiation. The repair phase is followed by the remodeling phase, involving the delicate balance between osteoblasts and osteoclasts.

Initially, MMSC differentiate into osteochondral progenitor cells, which can further differentiate into either mature chondrocytes or osteoblasts. MMSC’s from the bone marrow, periosteum, circulation and damaged muscle contribute to the repair process. Permanently labeling myogenic cells using \textit{MyoD-Cre}+/--;\textit{Z/AP}+/− mice demonstrated that approximately 30-50% of cells contributing to the early phases of fracture repair originated from myogenic lineage (Liu et al., 2009). Furthermore, using an \textit{Osterix-Cre} expressing LacZ reporter mice, pulse chased studies demonstrated that vascular invasion is a source of osteoblast precursors during fracture repair (Maes et al., 2010). Fracture repair can then be divided histologically into direct (primary) or indirect (secondary) repair (Einhorn, 1998). Direct fracture repair involves rigid internal fixation and decreased intrafragmentary strain (McKibbin, 1978). The majority of fractures heal through indirect (secondary) healing. During indirect fracture repair, fracture repair can then progress through either intramembranous or endochondral ossification (Ai-Aql et al., 2008; McKibbin, 1978; Miclau et al., 2005; Yoo and Johnstone, 1998). Intramembranous ossification entails the direct formation of bone without a cartilage intermediate (Brighton and Hunt, 1991). PPMS’s differentiate to an osteoblastic phenotype and contribute to the formation of the “hard callus”, which is rich in ColI and hydroxyapatite crystals (Crisan et al., 2008; Granero-Molto et al., 2008; Gulotta et al., 2009; Kronenberg, 2003; Waese et al., 2008; Yoo and Johnstone, 1998; Young, 1962). Osteoblasts that remain within callus become osteocytes, and the callus is remodeled by the interaction of osteoblasts and osteoclasts. In endochondral ossification, MMSC’s undergo chondrogenesis, which are responsible for the mineralization of the cartilage template or “soft callus”, as observed in fetal bone development (Kronenberg, 2003; Probst and
Spiegel, 1997). Recapitulating embryonic bone development, chondrocytes undergo hypertrophy and the cartilage matrix is calcified, acidified and degraded. Following vascular invasion, MMSC’s differentiate to osteoblasts and synthesize bone rich in ColI and hydroxyapatite crystals and begin bridging the fracture gap. The “hard callus” is remodeled by osteoclasts and osteoblasts until the broken bone regains its structural and mechanical properties.
Figure 1.5
Cell types and signals during fracture repair. During the inflammatory phase, inflammation and hematoma formation occur. Following hematoma formation, MMSCs differentiate into an osteochondral progenitor cell. Under specific cell signaling, the osteochondral progenitor cell will differentiate into either an osteoblast in the presence of β-catenin or a chondrocyte in the absence of β-catenin. Chondrocytes synthesize cartilage rich in Coll and osteoblasts synthesize woven bone rich in Coll. Following cartilage and bone formation, osteoclasts and osteoblasts remodel the fracture callus until weight-bearing bone is synthesized and the mechanical properties are retained. Adapted from (Silkstone et al., 2008).
Bone remodeling is the dynamic interaction between osteoblasts and osteoclasts. Osteoclasts are large multi-nucleated cells that originate from the haematopoietic stem cell (HSC) lineage and are formed by precursors from the monocyte-macrophage lineage (Suda et al., 1999). Osteoclasts decalcify and degrade the bone matrix by both acid decalcification and proteolytic degradation (Boyle et al., 2003).

1.2.1 Inflammatory Phase

The inflammatory phase initiates the fracture repair cascade, but its role in embryonic skeletogenesis is still unknown. During the inflammatory phase of fracture repair, levels of several inflammatory cytokines such as interleukin (IL)-1, IL-6, IL-11, IL18 and tumor necrosis factor-alpha (TNFα) are significantly elevated and are responsible for recruiting inflammatory cells, such as T and B cells (Gerstenfeld et al., 2003a; Lieberman et al., 2002).

The molecular interplay between osteoblast and the immune system began by studying the specialized calcified matrix that osteoblasts produce, creating a microenvironment. This specific microenvironment, which osteoblasts contribute to is defined at the HSC “niche”, the in vivo microenvironment where HSCs reside, awaiting for their cell fate. It has been observed that HSCs injected into the tail vein of mice migrate to the bone marrow cavity (Adams et al., 2006). In a conditional BMP receptor type 1A (BMPRIA) null mouse model, there was an increase of spindle-shaped N-cadherin+CD45-osteoblasts cells (SNO) and HSCs (Zhang et al., 2003b). The adhesion between the SNO and HSCs was mediated by N-cadherin and β-catenin, indicating that the SNO lining the bone surface contribute to the HSC niche and is maintained by β-catenin. Furthermore, osteoblast-specific activation of the PTH/PTHrP receptors stimulated osteoblast cells, increased Notch signaling and increased the number of HSC’s (Calvi et al., 2003). In addition, the number of HSC’s was increased in animals treated with PTH, indicating a regulatory role of osteoblasts in maintaining the HSC niche. Xie and colleagues utilized an ex vivo imaging system to detect the functional hematopoietic stem cell niche (Xie et al., 2009b). Co-staining of bone sections with Osx and N-cadherin showed that N-cadherin was expressed in both Osx+ and Osx− cells, and the Osx− cells localized themselves adjacent to the Osx+ cells, suggesting that these were a pre-osteoblast population. In 59% of the cases, transplanted GFP+HSC interacted with the N-cadherin+ OSX+ cells (Xie et al., 2009b). In response to bone marrow damage by irradiation, GFP+HSC homed to the bone marrow and underwent transient
expansion, suggesting that a damaged bone marrow microenvironment promotes HSC expansion.

In addition to the role of osteoblasts maintaining the bone microenvironment, patients with human immunodeficiency virus (Yan et al.), and clinically apparent acquired immune deficiency syndrome (AIDS) have decreased bone mineral density (BMD) and increase delayed and non-union of fractures (Bruera et al., 2003; Richardson et al., 2008; Singh and Moyle, 2006). This suggests that fracture healing not only depends on the normal function of the immune system, but there is an association between dysfunctional lymphocytes and compromised fracture union.

1.2.1.1 Tumor Necrosis Factor-Alpha

TNF-α is expressed in a biphasic manner during fracture repair, peaking at 24 hours post-fracture before returning to baseline levels at 72 hours and increasing at 3 weeks post-fracture and returning to baseline levels at 4.5 weeks, demonstrating a diverse role during the inflammatory and remodeling phase of fracture repair (Gerstenfeld et al., 2003b; Kon et al., 2001). TNF-α induces cell survival by NFκβ or API transcription factors, and prevents cell death mediated apoptosis (Nagata, 1997). Using calvaria precursor cells and BMSCs, TNF-α inhibited the differentiation of the calvaria precursors cells into mature osteoblasts as shown by reduced ALP, mineral nodules and decreased OC secretion (Balga et al., 2006; Gilbert et al., 2000). Furthermore, administration of TNF-α in vitro to osteoblasts and fibroblasts inhibits Coll synthesis but treatment to hypertrophic chondrocytes expressing ColX induced apoptosis (Aizawa et al., 2001; Centrelia et al., 1988; Greenwel et al., 2000). TNF-α stimulates osteoclastogenesis from HSC and modulates the expression of RANKL by osteoblasts (Abu-Amer et al., 2004; Balga et al., 2006). To determine the role of TNF-α in fracture repair, Gerstenfield and colleagues generated a TNF-α receptor (p55+/p75−) deficient mouse (Gerstenfeld et al., 2003a). Contrary to in vitro data, histological analysis of the fracture healing by SO staining indicated a delay in endochondral tissue formation. This was shown by a delay in extracellular matrix markers, such as ColIII and ColX. Furthermore, chondrocyte apoptosis, as indicated by gene expression of Fas, Caspase 8, TNF-related apoptosis-inducing ligand and TUNEL assay, demonstrated that apoptosis was delayed by 7-10 days and that TNF-α signaling mediates apoptosis from hypertrophic chondrocytes (Simonet et al.) (Gerstenfeld et al., 2003a). As expected, mutant mice displayed decreased expression of RANKL and M-CSF, and decreased
TRAP staining, indicating a 7 day delay in bone remodeling. These results suggest that TNF-α plays a role in the recruitment of progenitor cells, chondrocyte apoptosis and bone remodeling during fracture repair (Gerstenfeld et al., 2003a). The negative effects of TNF-α in vitro do not correspond to the fracture phenotype in vivo, suggesting that TNF-α may have an alternative role in fracture repair instead of being osteoinductive.

### 1.2.1.2 Cyclooxygenase 1 and 2

The cyclooxygenase pathway, mainly cyclooxygenase 1 (COX1) and cyclooxygenase 2 (COX2) are lipid mediators involved in prostaglandin synthesis and are activated in many inflammatory reactions, pain and involved in normal physiological functions (Smith et al., 1998). Inflammatory cells such as macrophages, granulocytes and T cells synthesize both Cox1 and Cox2 (Iniguez et al., 1999; Smith et al., 1998). In murine fracture models, Cox2−/− mice show delayed endochondral bone formation, proliferating endothelial cell adhesion molecule (PECAM1) and VEGF staining indicated a severe angiogenesis deficiency and fracture healing was delayed by 14 days; however, these effects were rescued with the administration of an EP4 agonist (Xie et al., 2009a). Although current anti-inflammatory treatments include non-steroidal anti-inflammatory agents, which block Cox-2 synthesis, mouse studies demonstrate an essential role for this pathway in fracture repair.

### 1.2.1.3 Interferon Gamma

IFN-γ levels increase immediately following injury and are elevated throughout the differentiation and repair process before returning to baseline levels during the remodeling phase (Mountziaris and Mikos, 2008b). Interestingly, IFN-γ inhibited 3H-thymidine incorporation into human osteoblasts but was able to increase ALP activity and had no effect on osteocalcin secretion (Gowen et al., 1988). Similar to TNF-α, IFN-γ inhibited collagen mRNA synthesis in rat calvarial cells (Smith et al., 1987). In addition, previous work has identified that T cells promoted osteoclastogenesis in vitro by increasing RANKL expression (Horwood et al., 1999). However, IFN-γ-producing T cells co-cultured with bone-marrow derived monoctye or macrophage precursor cells (BMM) inhibited osteoclastogenesis (Takayanagi et al., 2000). The inhibitory effects of the IFN-γ-producing T cells were cancelled when co-cultured with IFN-γR−/− BMM (Takayanagi et al., 2000). Furthermore, low levels of IFN-γ inhibited RANK-L and M-CSF osteoclastogenesis in vitro. These results suggested that IFN-γ inhibits RANK-L induced
osteoclastogenesis (Takayanagi et al., 2000). Systemic administration leads to increased bone loss, and IFN-\(\gamma\)R\(^+\) are resistant to normal and ovariectomy-induced bone loss (Cenci et al., 2003). In osteopoetrosis patients, administration of IFN-\(\gamma\) stimulated bone resorption (Key et al., 1992). Together these findings suggest IFN-\(\gamma\) plays a diverse role during fracture repair, particularly during the inflammatory and remodeling phase and although IFN-\(\gamma\) demonstrates anti-osteoclastogenesis properties in vitro, in vivo results are conflicting.

1.2.1.4 Interleukins

Multiple cytokines of the interleukin family have been identified in the fracture callus during the inflammatory phase and remodeling phase. These include IL-1, IL-4, IL-5, IL-6, IL-12 and IL-18 (Mountziaris and Mikos, 2008a). Similar to TNF-\(\alpha\), gene expression studies have identified a biphasic pattern in IL-1 expression during fracture repair with peak expression at 24 h and 28 days post-fracture (Kon et al., 2001). IL-1 induced osteoblast differentiation, increased ALP activity, but inhibited DNA synthesis and cell growth in the MC3T3-E1 cell line (Hanazawa et al., 1986). However, OC secretion in osteoblastic cell line derived from ROS17/2.8 rats was decreased and both collagen and mineralized matrix were decreased (Stashenko et al., 1987; Taichman and Hauschka, 1992). During the remodeling phase, IL-1 promotes tissue degradation, osteoclastogenesis and bone remodeling (Kon et al., 2001). Following an increase in IL-1 secretion, osteoblasts responded by secreting IL-6. IL-6 increases during the inflammatory phase and declines to baseline levels one-week post-fracture (Gerstenfeld et al., 2003b). Contrary to most inflammatory cytokines, IL-6 is made by T-cells, macrophages, osteoblasts, fibroblasts and endothelial cells and the precise role of IL-6 in fracture repair is unknown (Haynes et al., 1997; Littlewood et al., 1991). The opposite effects of interleukins both in vitro and in vivo have made it difficult to assess their role in fracture repair.

1.2.1.5 Chemokines

During the inflammatory phase of fracture repair, the majority of T cells express CD45RO, chemokine CXCR3 and adhesion molecules CD11a and CD49d (Butcher and Picker, 1996; Taichman and Emerson, 1998). CXCL9, CXCL10, CXCL11 and CXCL12 bind to the CXCR3 activates signaling pathways involved in chemotaxis, adhesion and proliferation (Bonacchi et al., 2001). In rheumatoid arthritis patients, the increased levels of CXCL10 correlate with increased number of T lymphocytes recruited to the site of injury (Patel et al., 2001). Osteoblasts and other
mesenchymal stem cells have been shown to release CXCR3 ligands in inflammatory conditions, as IFN-γ has been shown to induce the release of CXCL9, CXCL10 and CXCL11 by osteoblasts (Lisignoli et al., 2004). It has been shown using blocking antibodies that osteoblasts and T lymphocytes adhesion is mediated by CD54/CD11a and CD106/CD49b, and under inflammatory conditions OBS promote the proliferation of T-lymphocytes and induce cytokine and chemokine synthesis of T-lymphocytes (Tanaka et al., 1998; Tanaka et al., 1995). The relationship between T cells and osteoblasts has not been investigated in fracture repair, and how this relationship affects chemotaxis of other cells types is unknown.

1.2.2 Repair Phase

1.2.2.1 Transforming Growth Factor-Beta Superfamily

In addition to the release of multiple inflammatory cytokines, various growth factors are present within the fracture callus 24h-72h post-fracture as reviewed by Tsiridis and colleagues (Tsiridis et al., 2007). The most important growth factors identified in fracture repair belong to the TGF-β superfamily and include BMPs, fibroblast growth factors (FGF), and platelet derived growth factor (PDGF) (Bolander, 1992; Dimitriou et al., 2005; Einhorn et al., 1995). Profiling of mRNA expression from TGF-β superfamily members showed a temporal level of expression throughout the fracture healing process (Cho et al., 2002). BMP-2 is present from day 1-21, BMP-4 from 6h-day 5, BMP5-6 from day 3-21, TGFβ1 from day 1-21, TGFβ2 from day 3-14 and PDGF from 6h-day 3 post-fracture (Bostrom et al., 1995; Cho et al., 2002). The sequential expression of each member suggests a distinct and unique role throughout the fracture repair process.

1.2.2.2 Bone Morphogenetic Proteins

Bone morphogenetic proteins are osteoinductive. A comparison of 14 different BMP proteins discovered that BMP-2, BMP-4, BMP-6 and BMP-9 may be the most osteoinductive (Cheng et al., 2003). In a fracture repair model, localization of BMP-2 and BMP-4 in fracture calluses discovered strong expression levels in the location of osteoblasts and newly synthesized bone (Bostrom et al., 1995). Utilizing a BMP-14−/− (GDF-5) fractured femur model, histological analysis demonstrated a delay in fracture repair, with bone fragments and defect still present in the fracture callus at 42 days post-fracture (Chhabra et al., 2005). At 14 days, the callus of the control group contained a trabecular network bone, where as the experimental group contained
cartilage and fibrous tissue. Furthermore, the callus size was significantly (p<0.05) smaller at 14 days. Although BMP-14 is not the most osteoinductive BMP member, it demonstrates a crucial role for BMP signaling in bone development during fracture repair.

Multiple clinical trials have demonstrated the efficacy of rhBMPs and their ability to improve fracture repair. The efficacy of rhBMP-2 has been demonstrated in mouse, rat, rabbit, sheep, dog and human (Bostrom and Camacho, 1998; Gerhart et al., 1993; Muschler et al., 1994; Yasko et al., 1992). In a rabbit osteotomy model, rabbits were treated with 200 mg of rhBMP-2 delivered in a collagen sponge and had accelerated fracture repair histologically and radiographically. Bone mineral density and stress to failure at 2, 3, 4 and 6 weeks post-fracture were statistically increased (Bostrom and Camacho, 1998). In sheep, compared to the unbroken bone, BMP-2 treated fractures had a mean bending strength of 91% compared to the control group of 11% (Gerhart et al., 1993). Most importantly, a prospective controlled and randomized study of 400 human tibia fractures treated with 1.5 mg/mL (total dose of 12 mg) of rhBMP-2 demonstrated a 44% reduction in the risk of fracture failure and a significant increase in fracture union (p<0.0022) (Govender et al., 2002). Currently, BMPs are offered in the clinical setting to treat non-union fractures.

1.2.2.3 Transforming Growth Factor-Beta

TGF-β includes five isoforms (TGF-β1-5) and has been shown to be involved in cell growth and differentiation. During fracture repair, it is presumed that TGF-β is released following blood clot formation by platelets (Robey et al., 1987). Immunohistochemistry of TGF-β1 expression during tibia fracture repair revealed intense staining at day 7 surrounding chondrocytes and cartilage and at day 14 during endochondral ossification (Bourque et al., 1993; Joyce et al., 1990).

The efficacy of TGF-β in fracture repair has been studied in various models, such as mid-shaft fractures, critical size defects and subperiosteal injection into calveria (Lieberman et al., 2002). Using 30 tibia fractured rats, 1 or 10 ug of TGF-β was delivered continuously from a mini-pump (Lind et al., 1993). The treated group had no significant difference (p>0.05) is bone mineral density or cortical bone thickness. At day 7 and 14, there was a significant increase in the size of the fractures callus but healing was not accelerated (Lind et al., 1993). Although multiple studies with continuous treatment of TGF-β1 during fracture repair demonstrate significant improvement, a single dose of TGF-β1 had no histological, radiographic or biomechanical
differences compared to the control group (Critchlow et al., 1995; Lind et al., 1993; Nielsen et al., 1994). The single dose injections performed by Critchlow and colleagues had no clinical effect, suggesting that TGF-β1 needs to be continuously administered for a clinical effect. This continuous treatment regime has made TGF-β1 an unattractive candidate for non-union fractures.

1.2.2.4 Indian Hedgehog Signaling

Ihh signaling is known to be essential for embryonic bone development, osteoblast and chondrocyte differentiation and is osteoinductive (Kinto et al., 1997; McMahon et al., 2003; Nakamura et al., 1997). This suggests that regenerative capabilities of bone recapitulate the cell signals involved in embryonic bone development (Ferguson et al., 1999; Gerhart et al., 1993).

Gene expression studies were performed on WT tibia fractures to determine the location and expression patterns of Ihh. Ihh transcripts were undetectable in tibia fractures at 3 days post-fracture but were detectable at 6 days post-fracture (Ferguson et al., 1999). The expression location of Ihh is similar to that of Col2, suggesting that Ihh signaling plays a role in the formation of the soft callus during fracture repair. At 10 days post-fracture, the highest levels of Ihh expression were detectable, and at 14 days post-, Ihh expression was localized in areas of OC expression, suggesting that Ihh may play a role in differentiating osteoblasts and the formation of the hard callus (Ferguson et al., 1999).

The long expression pattern of Ihh in fracture repair demonstrates its diverse role in chondrogenesis and osteoblastogenesis. Due to the importance of Ihh signaling in bone development and tissue regeneration, Hh agonists have been developed. Hh agonists such as Purmorphamine could be a therapeutic option for non-union fractures (Sinha and Chen, 2006).

1.2.2.5 Wnt/β-catenin Pathway

Since the molecular mechanisms and signaling pathways that regulate fracture repair are not well understood, gene profiling studies were performed on WT mouse tibia fractures (Hadjiargyrou et al., 2002). One pathway with significant up-and-down regulation was the Wnt/β-catenin pathway. Although gene-profiling arrays identified Wnt4, Wnt5a, Wnt5b, Wnt10b, Wnt11, Wnt13 and β-catenin in fracture repair, transcriptional activation of TCF/LEF target gene in the fracture callus of a TCF/LEF reporter mouse provided histological evidence (Hadjiargyrou et al.,
2002; Zhong et al., 2006). Furthermore, using a TOPgal reporter mouse, Kim and colleagues observed increased reporter activity at the site of injury (Kim et al., 2007). Chen and colleagues demonstrated an increase in total β-catenin protein levels from 4 to 14 days post-fracture and was confirmed with an increase in transcriptional activity in a β-catenin-dependent TCF/LEF LacZ reporter mouse (Chen et al., 2007).

To determine the precise role of β-catenin, a loss- and gain-of-function approach was utilized. Catnb\textsuperscript{tm2kem} mice possess loxP sites located in introns 1 and 6 of β-catenin, and when treated with Cre recombinase results in a null allele (Brault et al., 2001). To stabilize β-catenin, Catnb\textsuperscript{lox(ex3)}\textsuperscript{m} mice possess loxP sites located in exon3. As hypothesized, the Catnb\textsuperscript{tm2kem} Ad-Cre treated mouse displayed a non-union 21 days post-fracture; however, the stabilization of β-Catenin in the Catnb\textsuperscript{lox(ex3)} treated mice displayed a non-union and undifferentiated mesenchymal-like tissue in the fracture callus (Figure 1.6) (Chen et al., 2007). Next, to determine the role of β-catenin in osteoblasts, mice expressed conditional alleles was crossed with the α1(I)-Cre mice, resulting in a a1(I)-Catnb\textsuperscript{null} and a1(I)-Catnb\textsuperscript{stab} (Chen et al., 2007; Dacquin et al., 2002). One week post-fracture, the a1(I)-Catnb\textsuperscript{null} mice displayed a fracture callus that was mainly composed of cartilage while the a1(I)-Catnb\textsuperscript{stab} mouse displayed no chondrogenesis, illustrating the essential role for β-catenin in osteoblast differentiation. In addition, at 3 weeks post-fracture, the a1(I)-Catnb\textsuperscript{null} mouse showed small amounts of calcified callus but no bridging of the fracture gap; however, there was an increase in Sox9 expression compared to the a1(I)-Catnb\textsuperscript{stab}. Upon radiographic examination, the a1(I)-Catnb\textsuperscript{stab} mice had increased bone density, enhanced fracture healing and increased RunX2 expression, which supported the concept that early osteoblast lineage cells lacking β-catenin develop a chondrocyte phenotype and the stabilization of β-catenin in early osteoblast lineage cells promotes osteogenesis. However, ubiquitous β-catenin stabilization or deletion results in non-union fracture, reduced osteogenesis and mesenchymal like cells present in the fracture callus.

Understanding the clinical relevance of fracture repair, Chen and colleagues attempted to modulate β-catenin with the use of LiCl, a clinically approved drug that activates β-catenin signaling by inhibiting GSK3β (Chen and Alman, 2009; Chen et al., 2007; Phiel and Klein, 2001; Zhang et al., 2003a). Two groups of wildtype mice were utilized: one group received LiCl treatment two weeks prior to the fracture, and one group received LiCl 4 days post-fracture. Early treatment of LiCl reduced the amount of calcified bone in the fracture, while late LiCl
treatment enhanced fracture healing and increased bone density and bone volume. Histological examination of the early LiCl treated group revealed undifferentiated mesenchymal cells at the fracture callus, similar to the $\text{Catnb}^{\text{lox(ex3)}}$ and $\text{Catnb}^{\text{tm2kem}}$ mice (Figure 1.7). These results support the concept that β-catenin signaling functions differently during different stages of fracture repair, and dysregulated β-catenin signaling impairs fracture repair (Chen et al., 2007).

To further activate Wnt/β-catenin signaling, Minear and colleagues utilized an Axin2(LacZ/LacZ) mouse line, in which the cellular response to Wnt is increased and found that bone healing was accelerated (Minear et al., 2010). In addition, purified Wnt3a was packaged into a liposomal vehicle and administered following a skeletal defect and faster bone regeneration was observed due to an acceleration of skeletal progenitors into osteoblasts. In addition to activity Wnt/β-catenin signaling utilizing Wnt3a or LiCl, Krause and colleagues utilized the PPARγ inhibitor GW9662 (GW), which increased nuclear localization of β-catenin in hMSC’s (Krause et al., 2010). hMSC’s were cultured in osteogenic base media with and without GW, and injected into a calvarial defect in nude mice, histological evidence demonstrated increased angiogenesis and osteogenesis in the GW treated group and x-rays confirmed increased healing following 28 and 42 days (Krause et al., 2010). However, inhibiting GSK3β with 6-bromo-indirubin-3'-oxime (BIO) in hMSC’s did not increase calvarial healing following a defect. In an $\text{LRP5}^{+/+}$ and $\text{LRP}^{-/-}$ mouse model, x-rays, pQCT and biomechanical analysis demonstrated delayed fracture healing in the $\text{LRP}^{-/-}$ mouse compared to the $\text{LRP5}^{+/+}$ (Komatsu et al., 2010) (Komatsu et al., 2010).

In addition to traditional treatment options for non-union fractures such as BMP therapy, modulation of the Wnt/β-catenin pathway with LiCl, Wnt3a or alternative therapies have the clinical potential to treat non-union fractures (Komatsu and Warden, 2010) (Secreto et al., 2009). In a preclinical trial, treatment with antibodies against Dkk1 and the positive Wnt modulator R-Spondin1 (RSpo1) were able to repair bone lesions in patients with multiple myeloma and rheumatoid arthritis (Zhao et al., 2009).
Figure 1.6
Fracture repair analysis of Catnb<sup>tm2kem</sup> (β-catenin null), Catnb<sup>lox(ex3)</sup> (β-catenin stabilized) and control mice 21-days post-fracture. (A) Faxitron analysis of Catnb<sup>tm2kem</sup> mice demonstrating a non-union fracture. (B) Faxitron analysis of Catnb<sup>lox(ex3)</sup> mice demonstrating a non-union fracture. (E) Faxitron analysis of a control mice demonstrating full fracture union. (D) HE staining of Catnb<sup>tm2kem</sup> mice demonstrating mesenchymal like tissue at the fracture callus. (E) HE staining of Catnb<sup>lox(ex3)</sup> mice demonstrating mesenchymal like tissue at the fracture callus, similar to the Catnb<sup>tm2kem</sup> mice. (F) HE stain of control mice demonstrating no mesenchymal like tissue at the fracture callus and normal fracture histology. Adapted from (Chen et al., 2007).
1.2.3 Remodeling Phase

Following the repair phase, the absorption of bone by osteoclasts and the formation of bone by osteoblasts results in a fully remodeled fracture callus that histologically and mechanically are approximately identical to an unfractured bone. Osteoclast differentiation is regulated by receptor activator NFκB ligand (RANKL) and monocyte colony stimulating factor (M-CSF) (Asagiri and Takayanagi, 2007). RANKL is secreted by osteoblasts in response to 1,25-dihydroxyvitamin$_3$ (1,25(OH)$_2$D$_3$), PGE2 and PTH (Suda et al., 1999). Osteoblast-secreted osteoprotergerin (OPG) acts as a decoy ligand inhibiting the interaction between RANK and RANKL and preventing osteoclastogenesis (Lacey et al., 1998; Simonet et al., 1997). Osteoclastogenesis can be inhibited by osteoblasts by inhibiting the binding of RANKL to RANK with the decoy receptor osteoprotegerin (OPG) (Simonet et al., 1997). Overexpression of OPG in transgenic mice causes increased bone density and a decrease in osteoclast numbers. Furthermore, administration of recombinant OPG protects against ovariectomy-associated bone loss in rats, and OPG has been shown to be a potent inhibitor of osteoclast-mediated bone resorption (Bucay et al., 1998; Simonet et al., 1997).
Lithium chloride increased β-catenin protein levels and regulates fracture repair. (A-D) Radiographic analysis of mice receiving early LiCl treatment (A) and late LiCl treatment (B), or demonstrates that late LiCl treatment enhances fracture healing and early LiCl treatment impairs fracture healing. HE staining of early LiCl treatment (C) and late LiCl treatment (D). Early LiCl treatment results in immature mesenchymal tissue at the fracture site and late LiCl treatment results in increased amounts of bone. Adapted from (Chen et al., 2007).
1.3 Aging and Tissue Regeneration

In most adult tissue, typical adult MMSC’s are able to self-renew in vivo to repair tissue and maintain tissue homeostasis. With aging, there is a physiological change that is characterized by a decline in tissue function and tissue regeneration (Rando, 2006). This change in MMSC function could be due to an inherent defect of aging progenitor cells, changes in the stem cell niche or systemic circulation.

One of the most recognizable signs of aging is chronic inflammation. Chronic inflammation is an underlying cause of multiple age related diseases such as atherosclerosis, arthritis, diabetes and osteoporosis. The mechanism of the age-related inflammatory process has been linked to deregulation of the immune system or changes in redox status with age (Chung et al., 2009). Osteoporosis is an age-associated decline in bone mass, bone mineral density and impaired mobility (McCormick, 2007). An imbalance of bone homeostasis between osteoblasts and osteoclasts results in hyper-bone resorption and osteoporosis. Age-associated changes in T-cell function have been associated with increases in IL-1, IL-4, IL-5, IL6, TNF-α, COX2 and iNOS (Yun and Lee, 2004). These cytokines have been shown to induce osteoclast differentiation by increasing RANKL and NF-κβ signaling (Yamashita et al., 2007). Decreased bone matrix affects the bone microenvironment and the HSC niche with age. Furthermore, NF-κβ signaling pathway can be activated by oxidative stimuli. While changes in cell signaling due to chronic inflammation develop with age, progenitor cell function and gene expression have been shown to change as well.

In aging studies, a number of differences have been identified in old and young HSC. Multiple studies have identified that the number of cells with an HSC phenotype increased five fold in old C57BL/6 mice (Morrison et al., 1996). A microarray study of 14,000 genes of isolated HSC found 1,500 genes that were age-induced and 1,300 that were age-repressed (Chambers et al., 2007). A large proportion of up-regulated genes involved in inflammation and included TLR4, NF-κβ, PGE2 and Cox2. Furthermore, there was a decline in the repopulation ability of aged HSC compared to young HSC in bone marrow transplants of irradiated mice (Chambers et al., 2007). Understanding how aging affects progenitors cells at the genetic and cell signaling level could provide therapeutic targets for age related diseases.
One area of interest is changes in the stem cell niche. The stem cell niche was initially described as a specialized microenvironment regulating cell interaction and behavior, such as osteoblasts in the hematopoietic stem cell niche (Schofield, 1978). Within the bone microenvironment, it has been shown that osteoblasts secrete various factors such as matrix sialoprotein and osteopontin, which bind to CD44 and α4β1 integrin to inhibit hematopoietic stem cells from proliferating and differentiation (Nilsson et al., 2005). Multiple studies have examined the age dependent changes in the stem cell niche and how these changes regulate stem cell signaling.

One characteristic change in the microenvironment during aging is decreased myofibers and muscle mass (Booth et al., 1994). Changes in the myofibers have been shown to directly affect satellite cells (SC) and SC signaling. Conboy and colleagues observed that loss of Notch signaling impaired tissue regeneration in old mice and resulted in tissue fibrosis (Conboy et al., 2005). To alter the system environment of the old mice, a parabiotic pairing was performed with a young mouse. Exposure of old SC with young SC increased Notch ligand δ, increased Notch signaling, restored muscle regeneration and decreased tissue fibrosis (Conboy et al., 2005). Further analysis of the extracellular matrix of aged mice identified that there was an intrinsic overexpression of collagen-related genes, most notably ColIII. Although the upregulation of ColIII in aged mice could increase tissue fibrosis, there is a possibility that degradation of the fibrotic tissue by MMP’s may be impaired (Goldspink et al., 1994). Furthermore, there is age-induced defects of vascularization in old muscle. In old muscle, there is a significant decrease in vascular endothelial growth factor (VEGF) and endothelial nitric oxide synthase (eNOS), which are both important in endothelial proliferation and migration (Brandes et al., 2005).

1.3.1 Skeletal Muscle Repair and Wnt/β-catenin Signaling

Aging not only has dramatic affects on fracture repair but also on skeletal muscle and the regenerative potential of skeletal muscle. In muscle regeneration, older mice display an age-related increase in fibrosis and have increased gene expression of ColIII and fibronectin genes (Goldspink et al., 1994). In addition, aging has been shown to increase pulmonary fibrosis and renal fibrosis (Chilosi et al., 2003; Cruz et al., 2000). The molecular mechanisms associated with age-induced tissue fibrosis include the initial inhibition of the Notch signaling pathway. Following injury, satellite cells from young mice upregulate Delta01 while old satellite cells failed to do so (Conboy et al., 2003). Using FLOW analysis of CD34+/M-cad+/Delta+/Numb-,
only 25% of the old muscle cells were activated. Interestingly, inhibition of Notch signaling in young muscle cells resulted in a phenotype similar to untreated old muscle cells and activation of Notch signaling in old muscle cells restored muscle proliferation and myogenic differentiation (Conboy et al., 2003). These experiments demonstrate deregulation in cell signaling in old muscle cell signaling and modulation could rescue the age-induced tissue fibrosis.

Observing that muscle stem cells have decreased myogenic capabilities and convert towards a fibrogenic lineage in old animals, the systemic environment was analyzed. When aged mice were exposed to young systemic environment, there was a marked reduction in the myogenic-to-fibrogenic conversion from 17% to 10%, while the opposite was observed for the young mice increasing from 1 to 9% (Brack et al., 2007). Performing a myogenic-to-fibrogenic assay in vitro, serum from old mice increased the fibrosis of young cells, and young serum increased the myogenic capabilities of the old cells as demonstrated by MyoD and Pax7 staining. Using a tamoxifen inducible Pax7.Cre-ER.Rosa26 mouse for myogenic lineage studies, roughly 10% of the β-gal positive cells were ER-TR7+, which is specific for fibroblasts, confirming that the systemic aged environment promotes a myogenic-to-fibrogenic conversion (Brack et al., 2007).

To determine a mechanism for the systemic changes in old mice, Brack and colleagues investigated the Wnt pathway, which had previously been shown to lead to a fibrogenic conversion of endothelial cells to fibrogenic cells (Chilosi et al., 2003). In uninjured muscle, Axin2 transcripts were approximately 2.5 times higher in old muscle compared to young. TOPGAL reporter mice showed an increase in β-gal activity in cells during aging and FACS sorting demonstrated decrease GS3Kβ* (Figure 1.9) (Brack et al., 2007). Exposure of young progenitors to aged serum increased β-gal activity, while these observations were reversed by a soluble Wnt inhibitor, Frizzled-related protein 3 (sFRP3), suggesting that the increase in β-gal activity was Wnt ligand dependent. Administration of activating ligand Wnt3a into young muscle 1 day following injury increased tissue fibrosis and displayed a phenotype similar to old injured muscle. Finally, treatment of old injured muscle with Wnt inhibitors, either DKK1 or sFRP3, in old injured muscle enhanced regeneration, reduced fibrosis and restored a phenotype similar to the young mice.
1.3.2 Fracture Repair in Elderly

One population that has significant difficulties with non-union fractures and other regenerative processes is the elderly. Although there is much known about fracture repair, the explanation for delayed and non-union fractures in the elderly population is poor.

Physiological changes in the skeleton, such as osteoporosis and osteoarthritis, can affect bone repair. Numerous studies have provided evidence for age-related changes in mouse, rat and human fracture repair (Desai et al., 2003; Ekeland et al., 1982; Meyer et al., 2001; Nieminen et al., 1981; Nilsson and Edwards, 1969). Young 6-week-old rats are able to bridge the fracture gap in 4 weeks while adult 26-week-old rats require 10 weeks and old 52-week-old rats require more than 26 weeks to successfully bridge the fracture gap, demonstrating a relationship between increased age and fracture union time (Meyer et al., 2001).

The cellular differences in age-related fracture repair begins 3 and 7 days post-fracture during chondrocyte differentiation (Figure 1.8). At 3 days post-fracture, only young mice expressed ColII and stained positively for SO, where as middle-aged and old mice didn’t express ColIII until 5 days post-fracture. In addition, only young mice expressed ColX, a marker for hypertrophic chondrocytes (Lu et al., 2005). Differentiation of osteoblasts is delayed with increased age. At 3 and 5 days post-fracture, young mice expressed OC and newly formed bone was observed in the periostium; however, only limited amounts of OC was expressed in the middle-aged and elderly mice (Lu et al., 2005). As both ColX and OC were delayed in middle-aged and elderly mice, chondrocyte maturation and endochondral ossification were also delayed. At 21 days post-fracture, the cartilage in young mice had been absorbed but old mice displayed cartilage remnants at 28 days post-fracture (Lu et al., 2005). One of the most dramatic changes in fracture phenotype between young and elderly mice is the thickness of trabecular bone volume. Young mice had a trabecular volume of 0.270mm$^3$ and old mice had 0.007mm$^3$. In addition, overall PECAM staining was decreased in elderly mice, indicating that vascular invasion of the hypertrophic cartilage in the elderly mice was compromised. The effects of age on vascularization during fracture repair were further investigated by analyzing the expression of molecules that regulate vascular invasion such as Hif-1α, VEGF, MMP9 and 13 (Lu et al., 2008). Both immunohistochemistry and in-situ hybridization at 3, 5 and 7 days post-fracture...
demonstrated that VEGF and Hif-1α expression was strong at 3 days post-fracture in the young mice, while old mice displayed only weak expression at 5 days and normal expression at 7 days.

To further elucidate differences in fracture repair between the young and elderly, a microarray was performed on stabilized femoral fractures in rats. Of the 8700 genes on each array, 3300 were present and only 180 genes were upregulated in elderly rats (p<0.01), suggesting that young, adult and old rats displayed similar changes in mRNA expression of skeletal genes (Meyer and Meyer, 2007; Meyer et al., 2006). The earliest change in mRNA expression at 1 week post-fracture was an up-regulation of seven genes that expressed exclusively in myofibroblasts. The expression levels of α-SMA mRNA were twice as high in old rats at 1 week post-fracture as compared to young rats, and the increase in expression did not subside as it did in the young (Meyer and Meyer, 2007). In addition, seven transcripts involved in the inhibition of calcification were up-regulated in older rats at 1, 2, 4 and 6 weeks post-fracture, a time period essential for endochondral ossification to occur (Meyer and Meyer, 2007). IGF-BP6 and TGF-β related genes were also upregulated in older mice. Analysis of genes involved in endochondral ossification such as Ihh and BMP-2 had a slight decrease in mRNA expression levels from 4-7 days post-fracture, but levels off at 3 weeks post-fracture, which is consistent with previous expression studies (Lu et al., 2005; Meyer et al., 2006).
Figure 1.8
Delayed cellular changes in fracture repair in elderly rats seven days post-fracture compared to juvenile rats. (A,C) Delayed proteoglycan and cartilage synthesis in (C) elderly rats compared to (A) juvenile rats as indicated by SO staining. (B,D) Within the chondrocyte domain, (D) ColX staining indicated delayed hypertrophic chondrocytes within the chondrocyte domain compared to (B) juvenile rats. Adapted from (Lu et al., 2005).
1.3.3  β-catenin Blocking Drug

Hypertrophic wounds, neoplasms such as aggressive fibromatosis, and tissue fibrosis are characterized by excessive proliferation of mesenchymal fibroblast-like cells. In lung repair, fibroproliferation is associated with increased β-catenin levels and aggressive fibromatosis tumors are associated with stabilization of β-catenin (Douglas et al., 2006; Lattes, 1980). A pharmacological agent with the ability to reduce β-catenin protein levels could reduce the fibrosis associated with tissue injury and neoplasms.

Utilizing the MicroSource Spectrum Collection, our lab performed a high-throughput drug screen consisting of approximately 2,000 compounds. With over two-thirds of the compounds approved for clinical use, the 2,000 compounds consisted roughly of 50% drug components, 30% natural components and 20% bioactive components (http://www.msdiscovery.com, MicroSource Discovery Systems, Inc.). The screening criteria involved compounds that decreased cell viability of aggressive fibromatosis tumors but not normal fibroblasts. The assay used was the Sulforhodamine B (SRB) assay which detects changes in cell survival by measuring cell density as reflected by total cell protein content (Vichai and Kirtikara, 2006).

This screening process identified nefopam. Nefopam (5-methyl-1-phenyl-1,3,4,6-tetrahydro-2,5-benzoazocine) is a post-operative analgesic used in European countries for the relief of moderate to severe pain. Clinically, it is used as a non-opioid and has been proven to be safe with minimal side effects, which include nausea, dizziness and sweating. Furthermore, in contrast to opioid analgesics, it has no depressant action on the central nervous system (Pillans and Woods, 1995; Tigerstedt et al., 1979).

The precise mechanism and off target effects of nefopam are still unknown, but it has been shown to modulate serotonin receptors, G-protein coupled receptors and voltage sensitive calcium channels (Girard et al., 2009; Novelli et al., 2005). Nefopam has also been shown to have an important role in modulating alpha(2A)-adrenoceptors in theremoregulation in an animal model (Hocker et al., 2010).
1.4 **Rationale**

Fracture repair is a regenerative process of coordinated biological events that partially recapitulate normal bone development. Pathways recapitulated include RunX2, Ihh, BMP and Wnt/β-catenin signaling. Gene profiling studies have identified an increase in Wnt ligand transcription during tibia fractures and TCF/LEF reporter mice have provided histological evidence for the activation of Wnt/β-catenin signaling in fracture repair. Genetic alteration of β-catenin under a ubiquitous promoter results in a non-union fracture and tissue fibrosis; however, stabilization under the osteoblast specific ColI-cre improved fracture repair. Treatment of tibia fracture with LiCl 2 weeks before a fracture results in delayed union and tissue fibrosis, but treatment 7 days post-fracture increases fracture repair, indicating that Wnt/β-catenin signaling needs to be precisely controlled. In an aging animal model, Wnt/β-catenin signaling has been associated with tissue fibrosis during muscle injury, and this phenotype can be rescued by inhibiting Wnt/β-catenin signaling at the receptor level.

With aging, changes in intrinsic cell signaling, cell microenvironment and systemic circulation alter the regenerative ability of an organism. Delayed fracture union in old animal models and humans is well established; however, an underlying molecular mechanism for delayed fracture union is not fully understood.

1.5 **Hypothesis**

We hypothesize that age induced tissue fibrosis and delayed tibia fractures in old mice are caused by an upregulation of Wnt/β-catenin signaling at 3 and 7 days post-fracture compared to young mice and modulation of this pathway with a pharmalogical agent during the early phases of fracture repair can decrease total β-catenin protein levels and result in a normal fracture phenotype.

1.6 **Objectives**

1) A microarray analysis of adult (26 weeks) and old (56 weeks) *Rattus Norvegicus* tibia fractures at 3 and 7 days post-fracture and analyze changes in genes associated with Wnt/β-catenin signaling, Wnt target genes and genes associated with tissue fibrosis.
2) Determine if total β-catenin protein levels are increased in 16 month old mice compared to 3 month old mice at 3 and 7 days post-fracture.

3) Determine if TCF/LEF mediated transcription is increased during fracture repair in 16 month old mice compared to 3 month old mice by utilizing a β-catenin-dependent TCF/LEF reporter mouse.

4) Confirm delayed fracture union in 16 month old mice at 7, 14 and 21 days post-fracture by faxitron analysis and H&E and SO staining.

5) Determine if tissue fibrosis is present at the fracture site in 16 month old mice at 7, 14 and 21 days post-fracture by H&E and SO staining.

6) Confirm decreased ALP activity and Coll gene expression in 16 month old BMSC 10 and 20 days post-differentiation.

7) Determine total β-catenin protein levels and β-catenin-dependent TCF/LEF transcription from BMSC 10 and 21 days post-differentiation.

8) Determine if nefopam treatment from 0-4 days post-fracture decreased total β-catenin protein levels in the fracture callus of 16 month old mice 4 days post-fracture compared to a control group.

9) Determine if nefopam treatment from 0-4 days post-fracture improves fracture union in 16 month old mice at 21 days post-fracture by faxitron analysis, H&E and SO staining.

10) Determine if tissue fibrosis was reduced and fracture repair nefopam treatment from 0-4 days post-fracture in 16 month old mice 21 days post-fracture by H&E and SO staining.
Chapter 2

2 Materials and Methods

2.1 Microarray Analysis

Previously published microarray datasets of adult 26 week (young) and 56 week (old) Rattus Norvegicus tibia fractures from profile GSE594 on the NCBI Gene Expression Omnibus (GEO) were analyzed (Meyer and Meyer, 2007; Meyer et al., 2006). Briefly, left femoral mid-shaft transverse fractures were induced followed by intramedullary rod fixation with a Bonnarens and Einhorn device. Triplicate biological samples were collected from one third of the femoral length and cDNA was prepared and hybridized to the Affymetrix U34A microarray, which probes for more than 8700 rat genes. Microarray data from unbroken, 3 days and 7 days post-fracture were analyzed using GeneSpring (V.10). Signal intensities were normalized to the 50th percentile and mean signal values of each gene in the fractured samples were compared to the mean signal values of the same gene in the unbroken control sample, and a list of genes showing a 2-fold change was generated. The list of genes for the young and old rats were analyzed at 3 and 7 days post-fracture by parametric two way ANOVA (young 3 days vs. old 3 days, young 7 days vs. old 7 days), assuming normal distribution and variance between groups and Benjamini and Hochberg post-hoc tests. A sublist of genes showing significant changes in expression (p < 0.05) was generated. The list of genes with a significant, 2-fold change in expression was cross-referenced with known genes involved in the Wnt/β-catenin pathway, Wnt target genes and genes involved in tissue fibrosis.

2.2 Mouse Model and Fracture Model

All animal procedures were reviewed and approved by the animal care committee of the Hospital for Sick Children in Toronto. Male CD1 mice age 3 month and 16 month were utilized for the study. On the dorsal side of the thigh, a small incision was made to the knee. A longitudinal incision was made in the patellar tendon and a hole was drilled above the tibia. An Anticorro insect pine (Fine Science tools, http://www.finescience.com) was placed in the marrow pin to stabilize the tibia. A fracture was then created by cutting the mid-shaft of the tibia. The incision was sutured and following recovery from anesthesia, the animals were allowed free, unrestricted weight bearing movement. At various time points (7, 14 and 21 days) post-fracture, mice were
sacrificed and fracture calluses were harvested for further analysis. Five animals at each time point for each experimental condition were studied.

To determine which cells have dysregulated TCF mediated transcription during fracture repair in old mice, male 3 month and 16 month TCF-reporter mouse were utilized. TCF-reporter mice contain a LacZ gene downstream of a c-fos minimal promoter with three consensus TCF-binding motifs.

### 2.3 Drug Treatments in Mice

Mice were treated with 0.6 M LiCl (Amini Nik et al.), resulting in a final concentration of 0.02 M or approximately 200mg/kg per day. The treatment resulted in the blood plasma levels of LiCl similar to those treated with bipolar disorders (Clement-Lacroix et al., 2005). LiCl was administered from either 0-4 days post-fracture or 0-14 days post-fracture.

Saline and nefopam were administered at 30mg/kg body weight via intraperitoneal injections daily from day 0 to day 4 post fracture.

### 2.4 Faxitron Analysis

To evaluate fracture healing, fractured tibias were examined using the Faxitron MX20 –ray system for radiographic analysis (Faxitron X-ray Corporation, http://www.faxitron.com/).

### 2.5 Histological Analysis

Fractured tibias from CD-1 males mice were harvested and fixed in 4% Zn^{2+} formalin for 4 days and decalcified in 1% formic acid for 72 hours, replacing the formic acid every 24 hours. Samples were then washed in PBS for 30 minutes and transferred to a tissue cassette (Fisher Brand) and placed in 70% ethanol for 24 hours at RT. Samples were then dehydrated by washing the samples in 80% ethanol twice for 15 minutes, 95% ethanol twice for 15 minutes and 100% ethanol four times for 30 minutes. Samples were then washed in 1:1 ethanol: xylene mixture for 15 minutes and embedded in a paraffin wax. 10 µm sections were cut, mounted on slides and dried.

Standard immunohistochemistry protocols were utilized according to manufacturer’s instructions for Safranin O (Sigma) and Mayers Hematoxylin and Eosin (Electron Microscopy Sciences).
For LacZ staining of TCF/LEF reporter mice, cells were fixed as previously described and the stained for β-galactosidase activity (Chen et al., 2007). Samples were then decalcified, dehydrated, embedded and sectioned as previously described. Sections were then counterstained with Nuclear Fast Red according to manufactures protocol (Vector Laboratories).

### 2.6 Bone Marrow Stromal Cell Osteoblast Differentiation Assay and ALP Staining

BMSC were isolated from 3 month and 16 month old mice. Mice were sacrificed and tibias, femurs and humorous were harvested and cleaned of any attached tissue. The growth plates of the bones were removed, and bone marrow was flushed out of the bone marrow cavity with a 25-gauge syringe filled with αMEM (Wisent Inc). Bone marrow cells were then passed through a 180-gauge needle and filtered over a 70 µm nitrocellulose filter. Filtered cells were centrifuged at 800 rcf for 10 minutes, resuspended in αMEM with 10% FBS (Gibco) supplemented with 5% penicillin and streptomycin (Wisent Inc.) at a final concentration of 5.0x10^6 cells/ml. Cells were plated in a 35 mm 6 well plate at a density of 1.0x10^7 cells/well. After 4 days, half the medium was removed and replaced with fresh medium. After 7 days, the medium was exchanged with osteoblast differentiation medium supplemented with 50 µg/ml of asorbic acid (Sigma), 10^-8 M dexamethasone (Sigma) and 8 mM β-glycerolphosphate (Sigma). Medium was exchanged every 48 hours. At 10 and 20 days, cells were washed three times with PBS, fixed in 4% Zn^{2+} formalin for 5 minutes and washed with ddH_2O three times. Cells were then stained for alkaline phosphatase activity with SIGMAFAST® FastRed TR/Napthol AS-MX Tablets according to manufacturers instructions and counterstained with Mayers Hematoxylin (Sigma).

### 2.7 Protein Extraction and Immunoblot

Samples were washed twice with PBS and lysed with Reporter Gene Assay Lysis Buffer (Robey et al.). Lysates were centrifuged at 16,000 x g for 5 minutes to remove cell debris and quantified using the Bicinchoninic Acid (BCA) Protein Assay (Pierce). Equal amounts of total protein were separated by electrophoresis through an SDS-polyacrylamide gel, transferred to a nitrocellulose membrane (Amersham), and immunoblotted overnight at 4 °C with primary antibodies against total β-catenin (mouse monoclonal, Upstate Biotechnology) and GAPDH (mouse monoclonal, Calbiochem). Horseradish Peroxidase (HRP)-tagged secondary antibodies and Enhanced ChemiLuminescence (Amersham) were used to detect hybridization.
Densitometry was performed using the AlphaEaseFC software (Alpha Innotech). Western blotting was performed in triplicates to ensure reproducibility.

2.8 Beta-galactosidase Assay

Beta-galactosidase activity in BMSC’s was quantified by measuring ONPG incorporation. BMSC cells were scraped after being in osteoblast differentiation medium for 10 days and placed in a 1.5 mL eppendorf tube and centrifuged at 6,000 rpm for 10 minutes. The supernatant was removed and cells were suspended in Z buffer and OD$_{600}$ reading was taken of the suspended cells. Cells were diluted in Z buffer and permeabilized with 100 µL of chloroform and 50 µL of 0.1% SDS. Samples were vortexed for 30 seconds and incubated for 5 minutes in a 28°C waterbath. 0.2 mL of o-nitrophenyl-β-D-galactosidase (ONPG; 4 mg/mL) was added to each sample. Samples were placed in a 28°C water bath until a sufficient yellow colour developed. Once a sufficient yellow colour was observed, 0.5 mL of 1 M Na$_2$CO$_3$ was added to stop the reaction. Samples were vortexed, centrifuged at 16,000 rpm for 5 minutes. The supernatant was removed and an OD$_{420}$ and OD$_{550}$ reading was taken for each samples. Values were normalized by protein concentration and expressed as the percentage change in OD$_{420}$ relative to control.

2.9 RNA Preparation and Real-time Polymerase Chain Reaction

RNA was isolated from BMSC’s in osteoblast differentiation medium at 10 and 20 days by the TRIzol method (Invitrogen). In a 15mm well, cells were incubated with 1mL of TRIzol (Invitrogen) for 5 minutes and scrapped and pipetted into a 1.5 mL eppendorf tube. 200µL of chloroform were added to the mixtures, vortexed for 15 seconds and centrifuged at 12,000g for 15 minutes at 4°C. The aqueous layer was transferred to a 1.5mL eppendorf tube and 500µL of 100% isopropyl alcohol was added. The tube was vortexed for 5 seconds and the mixture incubated at room temperature for 10 minutes. The sample was then centrifuged at 12,000g for 10 minutes, the supernatant was removed and the RNA was washed with 75% ethanol. For the synthesis of cDNA, 1 µg of RNA was used for reverse transcription. Superscript First strand synthesis (Invitrogen) kit was used according to manufacturers specifications.

*ALP* gene expression was measured using TaqMan probe Mm00479448_m1 (Applied Biosystems) and *Col1* gene expression was measured using TaqMan probe Mm00489940_m1 (Applied Biosystems).
2.10 Statistical Analysis

Young and old samples were prepared in biological triplicates and compared (3 day young vs. 3 day old) using a two-tailed Student $t$ test. A two tailed Student $t$ test was utilized to test if the population represented by the sample (old mice) will differ from the general population (young mice) in an unpredicted direction. Furthermore, the population variances were unknown in the various experiments and the variances did not fit a standard normal distribution. A null and alternative hypothesis were generated with alpha=0.05 (95% confidence) and the null hypothesis was rejected if $p<0.05$ and the alternative hypothesis was accepted.
Chapter 3

3 Results

3.1 MicroArray Analysis of Wnt/β-catenin Pathway

To determine if there were changes in gene expression in the Wnt/β-catenin pathway and Wnt target genes, microarray data from adult and old Rattus Norvegicus tibia fractures were analyzed. Significant (p<0.05) genes were then cross-referenced with known genes in the Wnt/β-catenin pathway and Wnt target genes (Table 3.1). Furthermore, genes were analyzed that are expressed during tissue fibrosis.

<table>
<thead>
<tr>
<th>Gene</th>
<th>3 Day Young</th>
<th>3 Day Old</th>
<th>7 Day Young</th>
<th>7 Day Old</th>
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<td>3.06</td>
<td>7.42</td>
<td>3.53</td>
<td>15.69</td>
</tr>
<tr>
<td>Fzd1</td>
<td>3.75</td>
<td>6.01</td>
<td>2.04</td>
<td>5.95</td>
</tr>
<tr>
<td>Fzd2</td>
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<td>2.93</td>
<td>10.88</td>
<td>2.37</td>
<td>8.51</td>
</tr>
<tr>
<td>Tnc</td>
<td>2.84</td>
<td>14.74</td>
<td>2.62</td>
<td>16.72</td>
</tr>
<tr>
<td>Spp1</td>
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<td>9.25</td>
<td>1.92</td>
<td>8.86</td>
</tr>
<tr>
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<td>15.83</td>
<td>1.88</td>
<td>19.00</td>
</tr>
<tr>
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<td>3.31</td>
<td>2.28</td>
<td>5.36</td>
</tr>
<tr>
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<td>-1.09</td>
<td>-1.02</td>
<td>2.30</td>
<td>13.11</td>
</tr>
</tbody>
</table>

Table 3.1
Gene list of significant (p<0.05) fold changes identified in the Wnt/β-catenin pathway during fracture repair in adult and old rats. Unbroken samples were used as a control for each time point.

Old broken bones had a significant increase in Wnt/β-catenin target genes and genes associated with tissue fibrosis compared to adult. Fibronectin 1 (Fn1), which is an extracellular matrix glycoprotein that binds to integrins, collagen and fibrin had a fold change increase of 4.36 and 12.17 in the old at 3 and 7 days compared to the young, respectively. Furthermore, tenascin (Tnc), which is also an extracellular glycoprotein, showed had a fold chance increase of 11.9 and 14.1 at 3 and 7 days compared to the young, respectively. Frizzled 1 (Fzd1), a transmembrane
receptor had a fold change decrease of 0.06 and increase of 3.91 in the old at 3 and 7 days compared to the young, respectively. *Gap junction α-1 protein (Gja1)* had fold change increase of 7.95 and 6.14 in the old at 3 and 7 days compared to the young, respectively. Secreted *phosphoprotein 1, or osteopontin (SPP1)* had a fold change increase of 7.21 and 6.94.

Analysis of genes involved in tissue fibrosis included *ColIII, Acta2* and *myosin heavy chain 3 (Myh3)*. *ColIII, Acta2* and *Myh3* had a fold change increase of 13.87, 0.87 and 0.07, respectively, 3 days post-fracture in the old rats compared to the young. At 7 days post-fracture in the old, *ColIII, Acta2* and *Myh3* had a fold change increase of 17.12, 3.08 and 10.81, respectively. This indicates that genes involved in fibrosis are increased in the old rat fractures compared to the young.

### 3.2 β-catenin Signaling is Increased in Old Mice During Fracture Repair

To determine the β-catenin protein levels in 3 month and 16 month old mouse fractures, a β-catenin immunoblot was performed. Total β-catenin protein levels in 3 month and 16 month old fractures demonstrated a significant increase (p<0.05) in total β-catenin protein levels in 16 month old mice compared to the 3 month old mice at 4 and 7 day post fracture (Figure 3.1). To determine the transcriptional activity of β-catenin, 3 month and 16 month old TCF/LEF reporter mice were fractured and harvested 7 days post-fracture. Upon activation of β-catenin-mediated signaling, LacZ is produced and can be visualized by performing X-gal staining. X-gal staining indicated a complete tibia fracture in both the 3 and 16 month old mice (Figure 3.2A and 3.2B). Furthermore, there was increased β-catenin–dependent TCF/LEF transcription activity in 16 month old mice compared to 3 month old mice (Figure 3.2C and 3.2D).

### 3.3 Old Mice Display Delayed Fracture Healing and Tissue Fibrosis

To evaluate fracture healing, Faxitron analysis of 3 month and 16 month old tibia fractures was performed at 7, 14 and 21 days post-fracture (Figure 3.3). Faxitron analysis displayed complete tibia fractures at 7 days post-fracture in both the 3 and 16 month old mice. At 14 days, 3 month mice displayed radiographic evidence of cortical bridging and a small fracture callus, and the 16 month old mice displayed a large fracture callus, non-union and no bridging of the cortical bone.
as suggested by radiographic evidence. At 21 days post-fracture, 3 month old mice displayed complete radiographic union of the fracture and no fracture callus, while 16 month old mice displayed a large fracture callus.
Figure 3.1
Total β-catenin protein levels are increased at 4 and 7 days post-fracture in 16 month old mice. (A) Total β-catenin immunoblot of 3 and 16 month old mice fractures at 4 and 7 days post-fracture shows increased total β-catenin protein levels in 16 month old mice at both time points. (B) Densitometry analysis total β-catenin immunoblots from 3 and 16 month old mice fractures at 4 and 7 days post-fracture shows a significant (p<0.05, n=3) increase in total β-catenin protein levels at 4 and 7 days post-fracture. A two-tailed t-test compared β-catenin protein levels between young and old mice fractures at the same time point. (C) Graphical representation of total β-catenin protein levels at the fracture site throughout the fracture healing process in 3 and 16 month old mice.
β-catenin mediated TCF/LEF transcription is activated in 3 month and 16 old mice during fracture repair 7 days post-fracture. (A-B) TCF/LEF reporter mice demonstrate a complete tibia fracture at 7 days post-fracture in both young and old mice. (C,D) LacZ staining of TCF-reporter mice 7 days post fracture in 3 month old mice (C) and old mice (D) demonstrates increased β-catenin mediated TCF/LEF transcription in the 16 month old mice compared to the 3 month old mice. Scale bars represent 1 mm and .2 mm.
Figure 3.3
Delayed fracture union in 16 month old compared to 3 month old mice. (A-F) Radiographic analysis of 3 month (A, C, E) and 16 month old mice (B, D, F) 7, 14 and 21 days fracture. At 7 days post-fracture (A,B), both 3 and 16 month old mice have complete fracture of the tibia. At 14 days post-fracture (C,D), 3 month old mice demonstrate cortical bone bridging and small fracture callus compared to 16 month old mice. At 21 days post-fracture (E-F), 3 month old mice demonstrate complete union and 16 month old mice demonstrate a large fracture callus.
To determine the presence of proteoglycans and histological analysis of the fractures, SO and H&E staining was performed (Figure 3.4, 3.5 and 3.6). At 7 days post-fracture, SO staining indicated that 3 month old had a complete tibia fracture and the presence of proteoglycans (red) and the formation of the cartilage template as shown in endochondral ossification (Figure 3.4A and 3.4B). 16 month old mice did show a complete tibia fracture at 7 days post-facture, but SO staining did not show the presence of proteoglycans or the initiation of a cartilage template, indicating a delay in fracture repair (Figure 3.4E and 3.4F). HE staining at 7 days in both the 3 month and 16 month old mice did not indicate the presence of tissue fibrosis (Figure 3.4C, 3.4D, 3.4G and 3.4H). At 14 days post-fracture, SO staining demonstrated that 3 month old mice had reabsorbed the cartilage template and had been replaced with woven bone (Figure 3.5A). Cortical bone bridging was evident with decreased fracture callus and trabecular bone (Figure 3.5B). H&E staining did not detect tissue fibrosis (Figure 3.5C and 3.5D). SO staining of 16 month old mice indicated the presence of proteoglycans and a cartilage template, trabecular bone and an unremodelled fracture callus (Figure 3.5E and 3.5F). H&E staining detected the presence of tissue fibrosis in the 16 month old mice (Figure 3.5G and 3.5H). At 21 days post-fracture, SO staining indicated that 3 month old mice had complete fracture union and complete bone remodeling of the fracture callus (Figure 3.6A and 3.6B). HE staining did not indicate the presence of tissue fibrosis (Figure 3.6C and 3.6D). SO staining in 16 month old mice indicated delayed proteoglycan resoprtion, increased trabecular bone and non-union of cortical bone (Figure 3.6E and 3.6F). HE staining indicated the presence of tissue fibrosis in the fracture callus (Figure 3.6G and 3.6H).

3.4 Osteoblast Differentiation Assay in Young and Old Mice

To determine if there were changes in β-catenin protein levels during osteoblast differentiation, an osteoblast differentiation assay was performed on 3 month and 16 month old CD-1 mice BMSC (Figure 3.7). At 20 days, 16 month old mice had decreased ALP staining and a two tailed t-test detected a significant decrease (p<0.05) in Col1 gene expression. 3 month old mice had a relative expression of 1.14 (s.d 0.09, n=3) at 10 days and 2.27 (s.d 0.089, n=3) at 20 days. 16 month old mice had a relative expression of 0.56 (s.d 0.018, n=3) at 20 days and 0.833 (s.d 0.06, n=3) at 20 days. To detect changes in total β-catenin, a two tailed t-test of 3 and 16 month old mice comparing the densitometry analysis of total β-catenin immunoblots at 10 and 20 days
indicated no significant difference (p>0.05, n=3) between 3 month and 16 month old mice (Figure 3.8A). To determine if there were changes in TCF/LEF-dependent transcription during osteoblast differentiation, an osteoblast differentiation assay of 3 month and 16 month old TCF/LEF reporter mice was performed (Figure 3.8B). 3 month old mice had an average relative substrate absorbance of 1.23 (s.d 0.06, n=3) at 10 days and 1.51 (s.d 0.08, n=3) at 20 days. 16 month old TCF/LEF reporter mice had an average relative substrate absorbance of 1.38 (s.d 0.036, n=3) at 10 days and 1.57 (s.d 0.08, n=3) at 20 days. A two-tailed t-test comparing the young and old BMSC mice at 10 and 20 days, respectively, indicated no significant difference (p<0.05) between the relative substrate absorbance.
Figure 3.4
Complete tibia fracture and 3 and 16 month old mice 7 days post-fracture. (A-B) SO staining of 3 month old mice indicating a complete tibia fracture and the presence of proteoglycans (red). (C- D) H&E staining of 3 month old mice do not indicate the presence of tissue fibrosis. (E-F) SO staining of 16 month old mice indicating a complete tibia fracture and minor proteoglycan staining. (G-H) HE staining of 16 month old mice do not indicate the presence of tissue fibrosis. Scale bars represent 1 mm and 0.1 mm.
Figure 3.5
Tissue fibrosis and delayed cartilage remodelling in 16 month old mice 14 days post-fracture. (A, B) SO staining of 3 month old mice with little proteoglycan staining and bridging of the cortical bone. (C D) H&E staining of 3 month old mice does not indicate the presence of tissue fibrosis. (E,F) SO staining of 16 month old mice demonstrating delayed proteoglycan synthesis and remodeling and no bridging of the fracture callus. (G,H) HE staining of 16 month old mice indicating the presence of tissue fibrosis (arrows). Scale bars represent 1 mm and 0.1 mm.
Figure 3.6
Tissue fibrosis and delayed fracture union in 16 month old mice 21 days post-fracture. (A-B) Safarin O staining of 3 month old mice indicating fracture union and remodeled fracture callus. (C-D) H&E staining of 3 month old mice does not indicate the presence of tissue fibrosis. (E-F) SO staining of 16 month old mice demonstrating delayed proteoglycan remodeling, a large fracture callus and increased trabecular bone. (G-H) HE staining of 16 month old mice indicating the presence of tissue fibrosis (arrows). Scale bars represent 1 mm represent 0.1 mm.
Figure 3.7
16 month old mice have decreased ALP capability and decreased Coll expression. (A) Osteoblast differentiation assay from 3 and 16 month old BMSC from CD-1 mice 20 days post-differentiation. 16 month old mice show decreased ALP staining and decreased staining intensity. (B) RT-PCR of Coll gene expression from an osteoblast differentiation assay from 3 and 16 month old BMSC from CD-1 10 and 20 days post-differentiation. A two tailed t-test demonstrated that old mice have a significant (p<0.05, n=3) decrease in Coll gene expression at both 10 and 20 days post-differentiation compared to the young.
No change in total β-catenin protein levels or β-catenin dependent TCF/LEF transcriptional activity during an osteoblast differentiation assay in 3 and 16 month old mice. (A) Total β-catenin protein levels from an osteoblast differentiation assay of BMSC from 3 and 16 month old TCF-reporter mice 10 and 20 days post-differentiation. There is no change in total β-catenin protein levels between 3 and 16 month old mice. (B) β-galactosidase assay of cell lysate harvested from an osteoblast differentiation assay of BMSC from 3 and 16 month old TCF-reporter mice 10 and 20 days post-differentiation. There is no significant (p>0.05, n=3) difference in β-catenin dependent TCF/LEF transcriptional activity.
3.5 Nefopam Decreases Total β-catenin Protein Levels in Fracture Repair

Our lab identified a pharmacological agent with the ability to reduce β-catenin protein levels in mesenchymal cells. To determine if nefopam could reduce β-catenin levels during fracture repair, 16 month old CD-1 mice were treated with nefopam from 0-4 days post-fracture (Figure 3.9). To increase β-catenin protein levels, LiCl was administered from 0-4 days post-fracture. As a control, 16 month old mice were administered saline from 0-4 days post-fracture. A two tailed \(t\)-test of densitometry analysis of the β-catenin immunoblot demonstrated a significant decrease (\(p<0.05, n=3\)) in β-catenin protein levels in mice treated with nefopam compared to saline and LiCl treated groups. A two tailed \(t\)-test of densitometry analysis of the β-catenin immunoblot demonstrated no significant increase (\(p>0.05, n=3\)) in β-catenin protein levels in mice treated with LiCl compared to the saline.

3.6 Nefopam Reduced Tissue Fibrosis and Improved Fracture Healing in Old Mice

To determine if nefopam could reverse the phenotype of non-union fracture repair in 16 month old mice, nefopam was administered from 0-4 days post-fracture. Radiographic analysis of 16 month old mice treated with saline showed no cortical union of the fracture at 21 days and increased fracture callus size, indicating delayed fracture healing (Figure 3.10A). Treatment with LiCl resulted in a large fracture callus and cortical bone union (Figure 3.10C). Treatment with nefopam resulted in a decreased fracture callus size and increased cortical bone bridging (Figure 3.10E). Radiographic analysis of 3 month old mice 21 days post-fracture treated with nefopam from 0-4 days post fracture displayed bridging of the cortical bone and complete fracture union (Figure 3.10G).

To further investigate the effects of nefopam on fracture repair in 16 old mice, histological analysis was performed at 21 days post-fracture to determine if nefopam could reverse the aged-induced tissue fibrosis (Figure 3.11, 3.12 and 3.13). SO staining of saline treated mice demonstrated delayed proteoglycan synthesis, proteoglycan absorption and no union of the fracture callus (Figure 3.11). Furthermore, H&E staining indicated tissue fibrosis in the fracture callus as indicated by the arrows. These results are similar to the untreated 16 month old mice histology at 21 days post-fracture (Figure 3.6E-3.6H). SO staining of LiCl treated mice
demonstrated no proteoglycan staining and union of cortical bone (Figure 3.12). However, H&E staining indicated the presence of tissue fibrosis in the fracture callus. SO staining of nefopam treated mice showed little trabecular bone, remodelled callus and bridging of the cortical bone (Figure 3.13). H&E staining did not reveal tissue fibrosis at the fracture site.
Nefopam decreases total β-catenin protein levels in fracture calluses (A) Total β-catenin protein levels from 3 and 16 month old mice treated with saline, LiCl and nefopam for four days following a tibia fracture. Total β-catenin protein levels are increased in the LiCl group and decreased in the nefopam group. (B) A two tailed t-test from densitometry analysis of total β-catenin immunoblots of biological replicates from 16 month old mice treated with saline, LiCl and nefopam. Compared to the saline group, LiCl showed no significant (p>0.05, n=3) difference in total β-catenin protein levels although an upwards trend is observed. Compared to the saline and LiCl group, nefopam showed a significant decrease in total β-catenin protein levels (p<0.05, n=3).
Figure 3.10
Radiographic evidence demonstrating improved fracture healing in nefopam treated mice. (A-F) Radiographic analysis of 16 month old mice treated with saline (A,B), LiCl (C,D) and nefopam (E,F) 21 days post-fracture. Saline treated mice have a large fracture callus and no bridging of the cortical bone. LiCl treated mice have a larger callus compared to the NaCl. Nefopam mice have a small fracture callus and bridging of the cortical bone is evident. (G,H) Radiographic analysis of 3 month old mice treated with nefopam 21 days post-fracture. 3 month old mice demonstrated bridging of cortical bone and complete fracture union. (n=1).
Figure 3.11

Delayed fracture healing and increased tissue fibrosis in 16 month old mice treated with saline. SO staining of 16 month old mice 21 days post-fracture demonstrates delayed proteoglycan synthesis and non-union of cortical bone. H&E staining of 16 month old mice 21 days post-fracture demonstrates tissue fibrosis (arrows) at the fracture site. Scale bars represent 1 mm, 0.2 mm and 0.1 mm.
Figure 3.12
Increased tissue fibrosis and bone in 16 month old mice treated with LiCL. SO staining of 16 month old mice 21 days post-fracture does not detect the presence of proteoglycans, indicates an unremodelled callus and increased bone mass. H&E staining of 16 month old mice 21 days post-fracture demonstrates tissue fibrosis (arrows) at the fracture site. Scale bars represent 1 mm, 0.2 mm and 0.1 mm.
Figure 3.13
Improved fracture healing in 16 month old mice treated with nefopam. SO staining of 16 month old mice treated with nefopam for 4 days following a tibia fracture shows complete union of the fracture, increased trabecular bone and small fracture callus. H&E staining demonstrates no tissue fibrosis at the fracture site. Scale bars represent 1 mm, 0.2 mm and 0.1 mm.
Chapter 4

4 Discussion, Conclusion and Future Experiments

In this study, we have demonstrated that total β-catenin protein levels are significantly increased at 4 and 7 days post-fracture in 16 month old mice compared to 3 month old mice, and there is increased β-catenin dependent TCF/LEF transcriptional activity at 7 days post-fracture in 16 month old mice. The increase in total β-catenin protein levels were associated with tissue fibrosis in the fracture site at 14 and 21 days post-fracture in the 16 month old mice and delayed fracture union. Furthermore, inhibition of β-catenin in old mice with the β-catenin blocking agent nefopam reversed the aged induced tissue fibrosis and restored the fracture callus to a phenotype similar to that of 3 month old mice. Unpublished data from our lab demonstrated that nefopam was able to affectively decrease total β-catenin levels in primary cells derived from aggressive fibromatosis tumors, which are characterized by elevated β-catenin levels. Together, these results demonstrate that nefopam decreases β-catenin levels in the fracture callus, prevents age-induced tissue fibrosis and increases the rate of fracture repair in old mice.

4.1 Discussion

4.1.1 Deregulated β-catenin in Old Fracture Repair

β-catenin has been shown to be essential for osteoblast differentiation, embryonic bone development, fracture repair and β-catenin protein stabilization is osteoinductive (Brault et al., 2001; Chen and Alman, 2009; Chen et al., 2007; Day et al., 2005; Haegel et al., 1995; Mbalaviele et al., 2005). In fracture repair studies, radiographic evidence demonstrated non-union at 21 days in fractured Catnb<sup>−/−</sup> (β-catenin null) and Catnb<sup>+/+</sup> (β-catenin stabilized) mice (Chen et al., 2007). In vitro evidence also supports that β-catenin needs to be precisely regulated during osteoblast differentiation. Exposure of human MSCs with canonical activating ligand Wnt3a prior to treatment with osteoblast differentiation media inhibited osteogenesis (Boland et al., 2004). However, deletion of β-catenin under the <i>Prx-1-Cre</i> system demonstrated that BMSC failed to differentiate into osteoblasts and stabilization of β-catenin prevented the differentiation of chondrocytes (Hill et al., 2005). Administration of LiCl at different time points further demonstrates the importance of β-catenin levels in the differentiation of progenitor cells to osteoblasts and chondrocytes (Chen et al., 2007). LiCl is an inhibitor of GSK3β, a primary
regulator of β-catenin in the canonical Wnt signaling pathway. When LiCl was given two weeks before a mid-shaft tibia fracture, which resulted in increased total β-catenin levels, radiographic evidence demonstrated non-union and histological examination of the fracture site showed tissue undifferentiated like tissue. However, when LiCl was given 4 days after fracture, radiographic and histological evidence demonstrated accelerated fracture repair and increased bone density and bone volume (Figure 1.6) (Chen et al., 2007).

In this study, we observed the presence of tissue fibrosis in the fracture callus of 16 month old mice at 14 and 21 days post fracture. The presence of tissue fibrosis was also observed in saline and LiCl treated 16 month old mice. This phenotype is similar to the undifferentiated like tissue observed in the fracture callus of β-catenin stabilized mice described in Chen and colleagues study (Chen et al., 2007). This suggests that tissue fibrosis during fracture repair in old mice may be attributed to increased levels of β-catenin. We also demonstrated that TCF/LEF transcriptional activity is not active in 3 month old mice until cells expressed markers of osteoblast of chondrocytes; however, 16 month old mice exhibited an increase in total β-catenin levels and β-catenin dependent TCF/LEF activity before cells exhibit this phenotype. This was observed in Catnblox(ex3) mice, where Cre mediated conditional stabilization of β-catenin repressed the differentiation of progenitor cells to the osteoblast lineage and maintained them in an undifferentiated proliferative state, resulting in tissue fibrosis. The early up-regulation of β-catenin and TCF/LEF mediated transcription in old mice may have resulted in the differentiation of progenitor cells to a fibroblast-like cell instead of an osteoblast (Figure 4.1). This affect was enhanced with the treatment of LiCl. Treatment with a β-catenin blocking agent, nefopam, reversed these effects as demonstrated by decreased β-catenin protein levels during the early phases of fracture repair, which allowed for cells to differentiate and proliferate to an osteochondral phenotype as opposed to a fibroblast-like cell (Figure 4.2). Following MMSC differentiation, increased levels of β-catenin allowed for the terminal differentiation of osteoblasts and normal fracture repair (Figure 3.14)
β-catenin mediated fibrosis during fracture repair. During hematoma formation and the early phase of fracture repair (days 0-4) in old mice, increased β-catenin protein levels contribute to tissue fibrosis by preventing MMSCs from differentiating into osteochondral progenitor cells and into a fibroblast-like cell. Adapted from (Silkstone et al., 2008).
β-catenin blocking agent prevents tissue fibrosis during fracture repair in old mice. Administration of the β-catenin blocking agent nefopam prevents fibrosis by inhibiting the differentiation of MMSCs into fibroblast-like cells. MMSCs differentiate into osteochondral progenitor cells and fracture repair continues. Adapted from (Silkstone et al., 2008)
4.1.2 Increased Mechanical Strain and Motion

To downregulate β-catenin in 16 month old mice, nefopam was administered from 0-4 days post-fracture. Nefopam is a clinically approved potent non-opiate analgesic with an unknown mechanism of action (Du Manoir et al., 2003; Girard et al., 2009; Pillans and Woods, 1995). More specifically, in a randomized prospective study on the analgesic effects of nefopam following orthopedic surgery, post-operative pain was reduced in the nefopam group compared to the placebo (Du Manoir et al., 2003). As the mice have free mobility and movement following the tibia fracture, the analgesic affects of nefopam could increase movement, mechanical stress and strain on the fracture callus. Increased mechanical stress and force have been shown to accelerate fracture repair in animal models (Carter et al., 1988; Wohl et al., 2009; Yukata et al., 2009). Initial studies demonstrated that weight-bearing rats had accelerated fracture healing and increased osteogenesis (Sarmiento et al., 1977). Furthermore, increased mechanical stress and strain have been shown to increase woven bone by 80% compared to the control group (Uthgenannt et al., 2007). To determine how the molecular mechanisms of mechanical loading affect bone formation, Wohol and colleagues utilized a fatigue-induced stress fracture (Wohl et al., 2009). Following one hour of mechanical loading, IL-6, BMP-2, VEGF and PECAM1 were upregulated and osteogenic genes Osx, RunX2, BSP, Osc were upregulated from 1-3 days compared to the unloaded group. The temporal expression of these genes is comparable to that observed in unloaded fracture repair studies. The improved fracture repair observed in the 16 month old mice treated with nefopam may be due to the analgesic affect, increasing mechanical strain and stress. The increased mechanical stress and strain could up-regulate genes observed during the inflammatory and repair phases of fracture repair, resulting in increased fracture union in the treated mice. The decrease in β-catenin from days 0-4 could be due to the up-regulation of other signaling molecules during the inflammatory phase.

4.1.3 Transforming Growth Factor-Beta Tissue Fibrosis

Aging has been associated with increased expression levels of TGF-β (Ruiz-Torres et al., 1998). TGF-β ligands act through Smad transcription factors and regulate gene expression. TGF-β has been shown to inhibit GS3K-phosphorylation of β-catenin, resulting in increased total β-catenin in fibroblasts (Amini Nik et al., 2007). An Affymetrix murine U74 Genechip analysis of myogenic progenitors isolated from both 3 and 24 month old mice demonstrated an increase in
TGF-β transcription and a significant increase in TGF-β regulated genes (Beggs et al., 2004). In addition, TNF-α was shown to increase TGF-β levels more than 5.4 fold in old rat transformed thyroid cells, while in young cells there was no detectable differences (Du Manoir et al., 2003; Pekary et al., 1995).

TGF-β has been linked to tissue fibrosis in multiple tissues, including kidney, lung and arteries (Branton and Kopp, 1999; Gramley et al., 2009; Petersen et al., 2008; Seeland et al., 2002; Tamaki and Okuda, 2003). Progressive kidney fibrosis precedes end stage kidney failure in up to a third of patients with diabetes mellitus (Petersen et al., 2008). During disease progression, TGF-β was shown to in increase fibrosis, deposition of the extracellular matrix and epithelial-mesenchymal transition. Oral administration of GW788388, a TGF-β receptor type I and type II inhibitor, reduced TGF-β Smad activation and gene expression. Furthermore, renal fibrosis and mRNA levels of extracellular matrix deposition in the kidney were reduced (Petersen et al., 2008). Yang and colleagues naturally inhibited TGF-β signaling using Safflower extract and observed decreased kidney fibrosis and observed lower levels of tissue collagen (Yang et al., 2008b). In a hepatic fibrosis animal model, a retinoic acid derivative reduced and reversed hepatic fibrosis by reducing hydroxyproline, α-SMA and ColII (Yang et al., 2008a).

Increased levels of TGF-β has been associated with aging and tissue fibrosis in injured animal models. Increased systemic levels of TGF-β in old mice may explain differences in β-catenin levels observed in our 3 and 16 month old mice. The increased levels of β-catenin and TGF-β could both be contributing to aged induced tissue fibrosis during fracture repair.

4.1.4 β-catenin Regulation of Oxidative Stress

One hallmark physiological change with age is oxidative stress, resulting in the development of age-related diseases (Yu and Yang, 1996). The “oxidative stress hypothesis” states that oxidative damage from reactive oxygen species (ROS) such as H₂O₂•O₂ increases with age (Cho et al., 2003; Chung et al., 2009; Davies and Goldberg, 1987). In response to ROS molecules, the FoxO family of ubiquitous transcription factors consisting of Fox01, Fox03a, Fox4 and Fox6, are transcribed. The FoxO family of transcription factors play a role mammalian cell survival by arresting cell cycle arrest (Medema et al., 2000; Tran et al., 2002).
It has been shown that β-catenin levels increase with age in a comparative study of calvaria cells isolated from 4 to 31 month old mice (Almeida et al., 2007). Interestingly, mRNA levels of β-catenin-TCF/LEF-mediated transcription target genes such as Axin2 and OPG were significantly decreased while, Forkhead Box O (FoxO)-mediated target genes such as Gadd45 were significantly increased with age (Almeida et al., 2007).

In aging, it has been shown that H₂O₂ oxidative stress suppresses TCF/LEF-mediated transcription and stimulates FoxO-mediated transcription (Almeida et al., 2007). Interaction between β-catenin and FoxO transcription factors have been shown to occur through the armadillo repeats of 1-8 of β-catenin directly with the C-terminal domain of FoxO1 and FoxO3a. Furthermore, β-catenin is required for FoxO-mediated transcription (Essers et al., 2005). This suggests that in aging and in response to ROS, accumulated β-catenin in the nucleus preferentially interacts with FoxO and activates FoxO-mediated transcription rather than TCF/LEF-transcription. In fractured rats, ROS and malondialdehyde (MDA), which is an indirect measure of ROS, are increased at 5 and 10 days post-fracture (Yeler et al., 2005). Furthermore, ROS inhibits osteoblast differentiation and bone nodule formation, which are mediated by TCF/LEF transcription. This could be due to the possibility that nuclear β-catenin from TCF/LEF-mediated transcription is directed to FoxO-mediated transcription (Almeida et al., 2007). As such, accumulated β-catenin diverted from TCF/LEF-mediated transcription to FoxO-mediated transcription in old mice results in decreased osteoblastogenesis and age induced osteoporosis. Interestingly, β-catenin returns from interacting with FoxO transcription factors to mediate TCF/LEF transcription of downstream Wnt target genes, which has been shown in in muscle fibrosis (Brack et al., 2007; Chen et al., 2007). During fracture repair in old mice, accumulated β-catenin mediating FoxO transcription due to increased ROS is directed to TCF/LEF mediated transcription immediately following tissue injury. This accounts for increased β-catenin levels in the fracture callus, causing differentiation of MMSC’s to a fibroblast-like cells causing tissue fibrosis. However, treatment with β-catenin blocking agent, nefopam, which resulted in a significant decrease in β-catenin inhibited the tissue fibrosis.

4.1.5 Decreased Osteoblast Potential in Old Fractures

Stem cells are regulated by cell-autonomous regulation, cell signaling, local and stem-cell niche and systemic regulation (Rando, 2006). The declining ability of age induced stem cell repair
could be due to alterations in the stem cell environment or niche. Mammalian aging is associated with reduced regenerative potential. One hallmark phenotype of aging is decreased wall width, bone and osteoblasts, resulting in age-associated osteoporosis and decreased bone formation (Bielby et al., 2007). Although the definition of MSCs has been controversial, two properties of MMSCs are the capacity for clonal self-renewal and multilineage differentiation into the five mesenchymal lineages, such as myogenic, adipogenic, chondrogenic, osteogenic and fibroblastic (Pittenger et al., 1999). However, these two properties have been difficult to assay.

Sarugaser and colleagues isolated human umbilical cord perivascular cells HUCPVSs that are CD44+CD73+CD90+CD105+CD106+CD45-CD34- and have single-cell-derived (SCD) clonal population (Sarugaser et al., 2009). The HUCPVS populations maintained multipotent capacity and differentiated into all five mesenchymal cell lineages. The HUCPV were able to increase femur BMD by approximately 40% in two weeks after transplantation into the NOD-SCID mice, which displayed age induced osteoporosis (Sarugaser et al., 2009). Analysis of the lineage potential of parent and daughter clones of HUCPVC populations revealed that daughter clones could maintain their self renewing capabilities or were unable to differentiate into more than one lineage. Sarugaster and colleagues postulated a hierarchical structure of MSC differentiation, whereby MSCs lose their differentiation potential until complete restriction is reached and by default differentiates into a fibroblast (Smith et al., 2004).

The differentiation capacity of MSCs into either an adiposite, chondrocyte or osteoblast phenotype has been shown to deteriorate with age (Muraglia et al., 2000). Furthermore, various studies have shown that both human, mouse and rat BMSC lose osteogenic, adipogenic and chondrogenic potential with age (Kretlow et al., 2008; Zheng et al., 2007; Zhou et al., 2008). During fracture repair in the old mice, MSCs may loose their differentiating capacity to differentiate into either a chondrocyte or osteoblast. Instead, MSCs in old mice may have lost differentiation capacities and differentiate to the default lineage of a fibroblast. Fibroblast differentiation from MSCs requires an immediate increase in β-catenin, which is observed in old mice. Together, differentiation by default into a fibroblast along with increased β-catenin levels could explain the tissue fibrosis observed in fractured old mice.
4.1.6 Therapeutic Modulation to Improve Fracture Repair

The clinical and economic affects of non-union fractures have spurred research to discovery therapies to improve fracture outcome. These therapies include bone tissue engineering that utilizes nanotechnology and 3D scaffolds with or without growth factors (Harvey et al., 2010; Stylios et al., 2007). Growth factors and transplantation of various cell types including pluripotent progenitor cells or differentiated chondrocytes and osteoblasts are being investigated in non-union fractures (Clines, 2010; Kallai et al., 2010; Sundelacruz and Kaplan, 2009). Further applications include genetically engineering various cell types with adenovirus vectors expressing osteogenic genes such as BMP2, BMP4 and FGF-2 (Granero-Molto et al., 2009; Kallai et al., 2010).

Our findings raise the possibility that correctly modulating β-catenin in both young and old patients could be used to enhance fracture repair in a clinical environment. Yen and colleagues demonstrated that administration of LiCl 4 days following a fracture improved fracture repair (Chen et al., 2007). Our findings demonstrated that administration of nefopam from days 0-4 following a fracture in old mice reduced tissue fibrosis and improved fracture repair. Lithium chloride is a pharmalogical agent approved to treat bipolar patients and its ability to improve bone repair can be tested clinically. In addition, in older patients, nefopam can be administered immediately following a fracture and followed up with LiCl treatment. Preliminary results in our lab have demonstrated that administration of nefopam from days 0-4 followed by administration of LiCl from days 5-14 demonstrated the greatest improvements in fracture repair in old mice.

4.1.7 Data Anomalies and Experimental Design

In this study, we utilized mice at 3 and 16 months of age. The 16 month mice weighed approximately 50% more, demonstrated increased adipose tissue and were housed in mouse cages individually. Further physiological changes in the 16 month old mice included age-induced osteoporosis. Due to the brittleness of the bones, creating the mid-shaft tibia fracture in the 16 month old mice occasionally resulted in minor bone fragments instead of a clean break, which was observed in the 3 month old mice. These conditions decreased the movement of the 16 month old mice and could contribute to the delayed fracture healing.
The difference in delayed fracture healing in the 16 month old mice at 21 days post-fracture in the untreated (Figure 2.6 J-L) and saline treated (Figure 2.11) could be due to the increased manipulation of the saline treated mice. The increased number of injections and extra strain on the fractured tibia could contribute to the increased delaying fracture healing (Figure 2.11).

Although the cells isolated from the 3 and 16 month old mice were BMSC, there was no significant difference in \( \beta \)-catenin protein levels or TCF/LEF mediated transcription. Almeida and colleagues analyzed \( \beta \)-catenin protein levels and TCF target genes immediately following harvest, as opposed to our procedure which analyzed \( \beta \)-catenin protein levels and TCF/LEF mediated transcription following osteoblast differentiation (Almeida et al., 2007).

### 4.2 Conclusion

In this study, we found that 16 month old mice have delayed chondrogenesis, osteoblastogenesis, and fracture healing compared to 3 month old mice. Fractured 16 month old mice displayed tissue fibrosis at 14 and 21 days post-fracture. Furthermore, \( \beta \)-catenin protein levels were elevated at 4 and 7 days post-fracture, accompanied by an increase in \( \beta \)-catenin dependent TCF/LEF-mediated transcription at 7 post-fracture in 16 month old mice compared to 3 month old mice. Although osteoblast differentiation assay demonstrated decreased osteogenic capacities of 16 month old BMSC compared to 3 month old BMSC, there was no significant difference in \( \beta \)-catenin protein levels or TCF/LEF mediated transcription. In 16 month old mice, administration of nefopam from 0-4 days post-fracture significantly decreased \( \beta \)-catenin levels. The decrease in \( \beta \)-catenin levels rescued the age-induced tissue fibrosis of the fracture callus, increased cortical bone union and accelerated fracture healing. Together, this suggests that elevated \( \beta \)-catenin levels in old mice could lead to differentiation of progenitor cells to a fibroblast-like cell rather than to an osteoblast or chondrocyte cell fate, as observed by lack of union and tissue fibrosis.

### 4.3 Future Experiments

#### 4.3.1 Molecular Mechanism of Nefopam

To further analyze the molecular mechanism of nefopam and its effects on \( \beta \)-catenin, in vitro analysis can be performed utilizing cell lines and controlling Wnt/\( \beta \)-catenin signaling at different levels. This could include targeting LRP5/6, GSK-3\( \beta \), and Axin using a genetic approach or
siRNA. Furthermore, mouse lines can be utilized using the Cre-loxP approach to disrupt the Wnt/β-catenin signaling pathway and fracture repair studies can be performed following nefopam treatment to further deduce the molecular mechanism.

4.3.2 Nefopam Treatment on β-catenin Null and Stabilized Mice

To further determine if tissue fibrosis observed in 16 month old mice is β-catenin mediated, from 0-4 days post-fracture nefopam will be administered to null (Catnb\textsuperscript{tm2kem}) and stabilized (Catnb\textsuperscript{lox(ex3)}) mice. β-catenin protein levels will be analyzed in the fracture callus at 4 and 7 days post-fracture, and histology sections will be prepared at 21 days post-fracture to determine tissue fibrosis. To deduce the molecular mechanism of nefopam during osteoblast differentiation, BMSC from Catnb\textsuperscript{tm2kem} and Catnb\textsuperscript{lox(ex3)} mice can be isolated and an osteoblast differentiation assay can be performed in the presence of nefopam. ALP and Aliziran Red staining will detect osteoblast differentiation and bone mineral formation.

4.3.3 Young and Old Bone Marrow Transplant

Literature has demonstrated that aging affects the bone microenvironment, resulting in decreased bone density or age-induced osteoporosis. These changes in the bone microenvironment or niche may alter the differentiation ability of progenitor cells to chondrocytes and osteoblasts, or alter bone mineral formation of osteoblasts. A bone marrow transplant between young and old mice will be performed to alter the bone microenvironment and progenitor cell signaling during fracture. To kill pre-existing cells, young and old mice will be irradiated. To differentiate between young and old cells, yellow fluorescent protein (YFP) will be expressed in all young cells. Furthermore, these YFP mice will be crossed with the TCF/LEF LacZ reporter mice to determine changes in TCF/LEF transcription in young mice. The bone marrow transplants will include transplanting young BMC’s into old marrow cavity, old BMC’s into young, old BMC’s into old (control) and young BMC’s into young (control).

The quality of fracture repair will be assessed with, x-rays, histology, μ-CT and mechanical testing. A β-catenin western blot will reveal changes in β-catenin protein levels in the four bone marrow transplant groups during fracture repair.

In addition, to determine if changes in the bone marrow microenvironment affect osteoblast differentiation, transplanted BMSC’ can be harvested and an osteoblast differentiation can be
performed. Osteoblast differentiation and bone mineral formation can be detected with ALP and Alizarin Red staining.

### 4.3.4 Changes in Bone Microenvironment or Circulating Factors

Aging causes changes in the bone marrow environment, progenitor cell fate and circulating systemic factors. A parabiosis experiment between young mice and old mice will alter the circulating factors. Combining the circulation of a young and old mice will determine if the circulatory factors in the young mice can rescue the age-induced tissue fibrosis and delay in fracture repair in the old mice. A young TCF/LEF LacZ reporter mice can be utilized to determine changes in TCF/LEF mediated transcription during fracture repair in the parabiosis experiment. To examine the quality of fracture repair and determine if age induced changes in the bone micro-environment affect fracture repair following the bone marrow transplants, x-rays, histology, μ-CT and mechanical testing will be performed. A β-catenin western blot will reveal changes in β-catenin protein levels.

To determine if cell secreted factors affect osteoblast differentiation and bone mineralization, an osteoblast differentiation assay of young and old BMSC can be performed. Following the 7 day incubation period, media from young undifferentiated BMSC can be collected and β-glycerolphosphate, dexamthasone and L-asoribic acid can be added to produce osteoblast differentiation medium. The osteoblast differentiation media can be added to old BMSC during an osteoblast differentiation assay. A similar experiment can be performed between old medium added to young BMSC during osteoblast differentiation. A western blot will detect changes in β-catenin protein levels. Osteoblast differentiation and bone mineral formation will be detected with ALP and Alizarin Red staining.

### 4.3.5 TGF-β and Fracture Repair in Old mice

Systemic levels of TGF-β have been demonstrated to increase with age. Multiple experiments will be performed to determine if increased levels of TGF-β in old mice have an effect on fracture repair. In old and young mice, a TGF-β neutralizing antibody could be administered to disrupt systemic TGF-β protein levels. Utilizing a *TGF-β receptor 1 (TGF-βR1) fl/fl* mouse, TGF-β signaling could be disrupted when crossed with a Cre expressing mouse line. Crossing the *TGF-βR1* *fl/fl* mouse line with the *Prx1-Cre* mouse will disrupt TGF-β signaling in early
mesenchyme cells or *Coll-Cre* mouse will disrupt TGF-β in immature osteoblasts. Fracture repair quality will be assessed with X-rays, histology, μ-CT and mechanical testing. Tissue fibrosis in the fracture callus will also examined. Changes in β-catenin protein levels will be detected with a β-catenin western blot.

To replicate the increased TGF-β in old mice, recombinant TGF-β can be administered to young mice before a fracture is induced and during the fracture repair process. As a control, LiCl can be administered. Fracture repair quality will be assessed and histology will detect tissue fibrosis. Changes in β-catenin protein levels will be detected with a β-catenin western blot.

Recombinant TGF-β can be added to BMSC’s during the 7 day incubation period before osteoblast differentiation and during the osteoblast differentiation process in both young and old BMSC. Osteoblast differentiation and bone mineral formation will be detected with ALP and Alizarin Red staining.

### 4.3.6 LiCl and Fracture Repair

Previous work by Chen and colleagues demonstrated that β-catenin must be precisely regulated for proper fracture repair. Our work has demonstrated that dysregulated β-catenin may be responsible for tissue fibrosis and delayed fracture union in old mice. Combining these observations would result in a combination of treatments to optimize fracture repair in old mice. To inhibit the increase in β-catenin protein levels during fracture repair in old mice, nefopam will be administered from days 0-4. Following administration of nefopam, fracture repair can be enhanced with the administration of LiCl from days 7-21. Fracture repair quality will then be assessed as previously described.

### 4.3.7 Future Fracture Repair Experiments

To further elucidate the clinical effectiveness of nefopam on old fractures, large animal experiments will be performed. Mid-shaft tibia and gap fractures will be performed initially in old rats, upwards to old sheep. The combination of nefopam and LiCl will also be administered.
Chapter 5

5 Appendix

5.1 Introduction to Fracture Quality

Bone is composed of multiple materials, including crystalline mineral (hydroxyapatite), type I collagen hydrated matrix and numerous non-collagenous proteins and small molecules. The physical properties of bone include strength, stiffness and toughness. Strength and stiffness are derived from the non-organic components and collagen contributes mainly towards toughness and supporting bone strength (Burr, 2002). Bone requires stiffness to prevent deformation during mechanical loading and flexibility to absorb energy without cracking (Seeman and Delmas, 2006). Bone mineral density (BMD) is a measurement of mineral (calcium) density per square centimeters and an increase in BMD increases stiffness and sacrifices flexibility. However, bone strength is a combination of the amount of bone and bone quality, which is maintained by the dynamic interplay by osteoblasts and osteoclasts, resulting in bone modeling and remodeling respectively.

Fracture risk is related to changes to both the structural and material characteristics of bone, which are responsible for absorbing fracture energy (Seeman and Delmas, 2006). In a laboratory environment, the structural and material characteristics of bone are measured using bone histomorphometry and mechanical testing to determine fracture risk and the quality of fracture repair. Clinically, BMD is determined using dual energy x-ray absorptiometry (DXA) deduce fracture risk in patients. However, BMD tests can only account for a 16% reduction in fracture risk (Cummings et al., 2002). An alternative approach to determine fracture risk is the mechanical response tissue analyzer (MRTA), which is a non-invasive test that measures the cross-sectional bending of bones under low frequency vibration (Roberts et al., 1996).
5.2 Methods

To quantify the quality of fracture repair in old and young, bone histomorphometry was performed on a 3 month old mouse, 16 month old mouse, 16 month old mouse treated with saline, 16 month old mouse treated with LiCl and 16 month old mouse treated with Nefopam. Static histomorphometry was be used to assess fracture repair at the tissue level, in particular bone volume (BV/TV, %), cortical volume (CV/TV, %) and trabecular volume (TB/TV, %). Parameters were detected utilizing the Leitz Bioquant morphometry system, courtesy of Dr. Marc Grynpas. H&E stained figures were scanned and included Figure 3.6, 3.11, 3.12 and 3.13. There is one sample a group (n=1).
5.3 Results

To quantify bone volume, cortical bone volume and tissue volume, bone histomorphometry was performed on 3 month old mouse, 16 month old mouse, 16 month old mouse treated with saline, 16 month old mouse treated with LiCl and 16 month old mouse treated with nefopam (Table 4.1).

<table>
<thead>
<tr>
<th>Group</th>
<th>BV/TV</th>
<th>CV/TV</th>
<th>TB/TV</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 Month</td>
<td>0.265</td>
<td>0.184</td>
<td>0.081</td>
</tr>
<tr>
<td>16 Month</td>
<td>0.203</td>
<td>0.078</td>
<td>0.125</td>
</tr>
<tr>
<td>16 Month Saline</td>
<td>0.174</td>
<td>0.057</td>
<td>0.117</td>
</tr>
<tr>
<td>16 Month LiCl</td>
<td>0.450</td>
<td>0.118</td>
<td>0.333</td>
</tr>
<tr>
<td>16 Month Nefopam</td>
<td>0.297</td>
<td>0.174</td>
<td>0.123</td>
</tr>
</tbody>
</table>

Tabe 4.1
Static bone histomorphometry data of fracture calluses 21 days post-fracture.

16 month LiCl treated mouse had the largest BV/TV % of 0.450 and the largest TB/TV% of 0.333. The 3 month old mouse had the largest CV/TV% of 0.184 and the lowest TB/TV% of 0.081. The nefopam treated mouse had the second highest BV/TV% of 0.297, second highest CV/TV% of 0.174. Similar to the 3 month old mouse, the nefopam treated mouse has a larger percentage of CV/TV then TB/TV.
Figure 5.1
Nefopam rescued bone histomorphometry properties in the 16 month old mouse. The nefopam treated mouse had a larger percentage of cortical bone volume compared to trabecular bone volume. This pattern is similar in the 3 month old mouse.
5.4 Discussion

In this study, we have identified that increased levels of β-catenin and β-catenin signaling may be responsible for delayed fracture union and tissue fibrosis in 16 month old mice. Inhibition of β-catenin in 16 month old mice with the β-catenin blocking agent nefopam decreased tissue fibrosis and improved fracture repair outcome. Bone histomorphometry confirmed that nefopam increased bone volume, cortical volume and decreased trabecular bone volume compared to the controls. Collectively, these results demonstrate that nefopam improves fracture repair in 16 month old mice by reducing tissue fibrosis and improving bone formation and fracture repair.

The increased cortical bone volume and decreased trabecular bone volume observed in the 16 month old nefopam treated mice could be a result of increased bone remodeling during the fracture repair. Increasing Wnt/β-catenin signaling in the bone marrow microenvironment decreases osteoclastogenesis (Qiang et al., 2009). Furthermore, stabilization of β-catenin (Catnb<sup>lox(ex3)</sup>) in osteoblasts resulted in higher bone mass. Deletion of β-catenin (Catnb<sup>tm2kem</sup>) in undifferentiated osteoblasts resulted in lower bone mass, osteopenia and increased bone remodeling. This suggests that β-catenin promotes osteoblast differentiation and inhibits osteoclastogenesis and bone remodeling (Glass et al., 2005). During the early stages of fracture repair when nefopam is administered, decreased β-catenin may allow myloid progenitor cells to differentiate to osteoclasts. Following the repair phase, the newly differentiated osteoclasts are able to remodel the 16 month old fracture callus.

Future experiments include performing the bone histomorphometry with a minimum of 10 samples (n=10) and three sections per sample. Furthermore, μ-CT will provide 3-D quantitative measurements of BV, CV and TB. To detect if there is increased bone remodeling in the nefopam treated group, calcein green can be administered at various time points throughout the fracture repair process. Calcein Green is a bone mineralization marker and will determine how nefopam affects dynamic histomorphometry parameters such bone formation rate and bone remodeling rate will be determined. TRAP staining of histology sections will detect osteoclast remodeling.
References:


Day, T.F., Guo, X., Garrett-Beal, L., and Yang, Y. (2005). Wnt/beta-catenin signaling in mesenchymal progenitors controls osteoblast and chondrocyte differentiation during vertebrate skeletogenesis. Dev Cell 8, 739-750.


Tufan, A.C., and Tuan, R.S. (2001). Wnt regulation of limb mesenchymal chondrogenesis is accompanied by altered N-cadherin-related functions. FASEB J 15, 1436-1438.


