The Role of Discoidin Domain Receptor 2 in Bone Regeneration

by

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DEDICATION

To my father and mother, brothers and sisters, my wife and my daughter who have shown their support, love, and encouragement throughout my life and education. Without your inspiration, support and motivation this dream would not have been possible to achieve.

{ هذا من فضل رَبِّي }

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فيشرفني أن اهدي هذا العملَ لوالديّ العزيزين ولأسرتي الكريمة اخوة واخوات ولزوجتي الحبيبة ولكل غالي افتقدنا طيلة أيام هذه الغربة وافتقدناه وأسألُ الله تعالى أنْ يوفقنا لما يحُبهُ ويرضاه وأن يجعلَ هذا العمل خالصاً لوجههِ الكريم.

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ABSTRACT

Bone is a dynamic tissue with self-healing capabilities that allow repair of most fractures with restoration of original architecture. However, large bone defects, such as those caused by tumor resections or severe trauma, do not regenerate spontaneously and represent a major clinical challenge for craniomaxillofacial and orthopedic surgeons. Bone grafts are usually used to manage such conditions. Bone autografts consist mainly of bone extracellular matrix (ECM) and associated cells. Recent research has unveiled many unique characteristics of ECM that play a key role in tissue regeneration. ECM enhances cell recruitment through cell surface receptors, which determine cell-ECM interactions and trigger specific cellular functions such as adhesion, proliferation, and differentiation. Discoidin domain receptor 2 (DDR2) is a collagen-activated receptor tyrosine kinase shown to be essential for skeletal development in humans and mice. *Ddr2*-deficient mice exhibit dwarfism and defective bone formation in the axial, appendicular and cranial skeletons. However, the role of DDR2 in bone regeneration has not yet been investigated. Here we evaluated the requirement for DDR2 in bone regeneration by using two well-established regeneration models; a calvarial subcritical-defect and tibial fracture.

In a calvarial subcritical defect model, we showed that DDR2 is essential for regeneration of a subcritical-size defect. Smallie mice (*Ddr2*$^{slie/slie}$), which contain a nonfunctional *Ddr2* allele, are unable to heal a subcritical-size (0.5 mm) calvarial defect that, in WT mice, can spontaneously heal within 4 weeks. Also, *Ddr2* expression during calvarial bone regeneration was defined using *Ddr2*-LacZ knock-in mice and β-galactosidase staining. *Ddr2* expression, which was restricted to

periosteal surfaces of uninjured calvarial bone, greatly expanded with injury. Similar results were seen when the lineage of *Ddr2*-expressing cells was examined using *Ddr2cre^{ERT}, Ai14 TdTomato* mice. Ddr2+ cells and their progeny expanded within the defect three days and two weeks post-surgery. Furthermore, three days post-surgery, *Ddr2$^{slie/slie}$* mice showed a significant decrease in cell proliferation in the calvarial defect when compared with WT littermates. Lastly, levels of the preosteoblast markers, Osterix and phosphorylated RUNX2 (S319-P) decreased in *Ddr2$^{slie/slie}$* mice, which suggests that osteoblast differentiation was arrested.

In the tibial fracture model, we first defined the expression pattern of DDR2 during fracture healing using *Ddr2-LacZ* knock-in mice and *Ddr2-Cre^{ERT}; Tdtomato* mice. LacZ expression was first detected in select regions of the fracture site 2- and 5-days post fracture and expanded throughout the fracture callus after 1.5 and 3 weeks. Similar results were observed *in Ddr2-Cre^{ERT};Tdtomato* mice. Ddr2+ cells and their progeny began to expand in the developing fracture callus 1.5-weeks post-fracture and continued to expand after 3 weeks. *Ddr2$^{slie/slie}$* mice exhibited significantly less fracture union than WT mice, and this defect was related to a decrease in cartilage formation as measured by safranin O staining. In addition, mutant mice developed significantly less callus tissue at 6-weeks post-fracture. To examine the role of DDR2 in skeletal progenitor cells (SPCs), we purified PDGFRα+ CD51+ SPCs from bone marrow of *Ddr2$^{fl/fl}$* mice using FACS followed by treatment with AdCre. *Ddr2* deletion resulted in defective osteoblast differentiation and accelerated adipogenesis. On the other hand, DDR2 overexpression in a mesenchymal cell line (ST2 cells) increased osteoblast differentiation

Together, our study demonstrates that DDR2 is necessary for normal calvarial bone regeneration as well as for optimal fracture healing. This requirement may be explained in part by effects of DDR2 on proliferation, SPC function and osteoblast differentiation.

CHAPTER 1

Introduction

The Extracellular Matrix

Tissues are typically comprised of extracellular matrix (ECM), cells, blood-filled vascular space, in addition to a collection of other proteins used for signaling between cells, but the proportions differ depending on anatomical location. The ECM is "the non-cellular component present within all tissues and organs and provides not only essential physical scaffolding for the cellular constituents but also initiates crucial biochemical and biomechanical cues that are required for tissue morphogenesis, differentiation and homeostasis" [1]. Each tissue has a unique ECM structure and topography created during embryonic development via dynamic and mutual biochemical and biophysical interactions between the different cellular components. In general, ECM consists of water, proteins and polysaccharides[1].

Polysaccharides, which are usually linked to protein in the form of proteoglycans, form chains called glycosaminoglycans (GAGs). The fibrous proteins, which have both structural and adhesive functions, include collagen, elastin, fibronectin, and laminin[2]. The proteoglycans, in which fibrous proteins are embedded, fill most of the extracellular interstitial space of the organ or tissue and have a variety of functions such as hydration, binding force resistance, and buffering[3]. Indeed, the ECM topological, physical, and biochemical composition is not only tissue-specific, but is also markedly heterogeneous.

Collagens are the most abundant class of proteins in the body making up to 30% of the total protein mass[2]. Collagens provide tensile strength, regulate cell adhesion, support chemotaxis and migration, and direct tissue development[2]. Collagens contain three polypeptide chains (α-chains) that interact to form a triple-helix structure. To date, more than 28 types of collagen have been identified in vertebrates[2]. Some of them (types I, II, III, V, and XI) are arranged in fibrils and are found in tissues that must be able to resist tensile, shear, or compression forces, including tendon, bone, cartilage, and skin[4]. These collagen fibrils are strengthened by covalent crosslinking between lysine residues of the constituent collagen molecules mediated by lysyl oxidases (LOX)[1].

Type I collagen is by far the most abundant protein in all vertebrates[2]. It provides the structure and mechanical matrix for skin, tendons, bone, cornea, blood vessel walls and other connective tissues[4]. Approximately 90% of the entire collagen content of bone is type I collagen (Table 1)[5,6]. Type I collagen molecules are essential for the mechanical strength of bone, especially for bone toughness (capacity to absorb energy) [5]. Any abnormalities in the collagen structure, which can be induced by genetic mutations (osteogenesis imperfecta) or pharmacologic agents (lathyric agents), affect bone quality[5].

TABLE 1.1 Characteristics of Collagen-Related Genes and Proteins Found in Bone Matrix
Adopted from Gehron P. et al. (2008) [6]

Collagens	Gene	Protein	Function
Type I	COL1A1 17q21.3-22 18kb, 51 exons, 7.2 and 5.9 kb mRNA COL1A2 7q21.3-22 35kb, 52 exons, 6.5 and 5.5kb mRNA	$[\alpha1(I)_2\alpha2(I)]$ $[\alpha1(I)_3]$	Most abundant protein in bone matrix (90% of organic matrix), serves as scaffolding, binds and orients other proteins that nucleate hydroxyapatite deposition
Type X	COL10A1	$[\alpha1(x)_3]$	Present in hypertrophic cartilage but does not appear to regulate matrix mineralization
Others Type III Type V FACITS ?	COL3A1 2q24.3-q31 COL5A1 9q34.2 COL5A2 2q14 COL5A319p13.2	$[\alpha1(III)]_3$ $[\alpha1(V)_2\alpha2(V)]$ $[\alpha1(V)\alpha2(V)\alpha3(V)]$	Present in bone in trace amounts, may regulate collagen fibril diameter; their paucity in bone may explain the large diameter size of bone collagen fibrils

Role of the Extracellular Matrix in Bone Remodeling

The skeleton provides structural support, a reservoir of calcium and phosphate, and a niche for the hematopoietic system. Given the importance of these functions, skeletal homeostasis is crucial to maintaining systemic homeostasis [7]. To maintain a healthy skeleton with optimal mechanical integrity, bone is constantly remodeled throughout life through the coupled activities of bone resorbing osteoclasts and bone forming osteoblasts [7, 8]. Therefore, any imbalance (pathological) between these cellular process leads to disease conditions of excessive bone loss or formation[7]. Osteoclasts, the bone resorbing cells, arise from hemopoietic progenitors and function to dissolve bone mineral and enzymatically degrade ECM protein. In contrast, osteoblasts arise from mesenchymal stem cells (MSC) and deposit a collagen-rich ECM that mineralizes to become bone [8]. A fraction of total osteoblasts become entombed in the bone matrix as osteocytes. These post-mitotic cells function as mechanosensors and endocrine cells, producing factors such as

FGF23, RANKL and Sclerostin, which modulate bone activity and mineral homeostasis [9]. The fourth cell type is the bone lining cell, whose functions are not well-understood, although it is thought to have some role in coupling bone formation to bone resorption [10].

Bone cells aren't the main components of the bone by weight. Rather, the ECM, which is produced by osteoblasts, accounts for most of the dry weight of bone[7]. The bone ECM consists of a mineralized portion containing calcium and phosphate in the form of hydroxyapatite that provides rigidity and hardness and organic components, mainly type I collagen that imparts flexibility. Therefore, it is the bone ECM that determines the mechanical properties of the skeleton[7].

In general, the bone remodeling process is controlled by three factors; the need to couple bone formation to bone resorption, systemic factors controlling calcium and phosphate homeostasis, and mechanical force [8]. Coupling bone formation to bone resorption requires signaling between osteoblast and osteoclast, which occurs within a basic multicellular unit (BMU). This signaling is mediated by cell-cell contact, diffusible paracrine/endocrine factors and cell-ECM interactions [8]. An example of regulation by cell-cell contact is the receptor tyrosine kinase, ephrin B (EphB) and its ligand, EphB [11]. When the EphB ligand binds the EphB receptor, it activates bidirectional signaling which induces signaling in both the ligand-expressing and the receptor-expressing cells [8,11]. For instance, in osteoblast, when EphB 4 receptor interacts with ligand on an adjacent cell, it produces a forward signaling that activates a RhoA-dependent pathway to promote osteoblastogenesis. At the same time, in osteoclast, EphB 2 receptor interacts with its ligand resulting in reverse signaling that downregulates c-Fos and NFATc1 to inhibit osteoclast function.

The systemic/endocrine regulation of bone remodeling is controlled by four principal hormones: PTH, vitamin D_3 (1,25-dihydroxyvitamin D_3), calcitonin and oestrogen [8]. The first three hormones are involved in the regulation of serum calcium levels with bone acting as a mineral reservoir [8]. PTH plays an important role in regulating calcium-phosphate metabolism and low serum calcium levels stimulates PTH production [8]. PTH has skeletal and non-skeletal roles in controlling serum calcium levels. In bone, PTH binds to its receptor (expressed on bone stromal cells, osteoblasts and osteocytes) and activates cAMP responsive element binding protein (CREB) signaling. This results in increased secretion of MCSF and RANKL, thereby indirectly stimulating osteoclastic bone resorption [8]. In addition, PTH enhances the number and activation of osteoblasts through different pathways [12]. PTH also controls serum calcium by stimulating renal reabsorption of calcium, which, together with the increased calcium released by bone resorption, restores physiological serum calcium levels [8].

The last factor having a substantial role in bone remodeling is mechanical stimulation. Mechanical force is a key regulator of bone remodeling and bone architecture. Numerous animal studies have established the importance of mechanical stimulation on bone metabolism and bone formation or resorption. This will be discussed in greater detail later in this chapter.

Role of the Extracellular Matrix in Skeletal Stem Cell Differentiation

Skeletal stem cells (SSC), also known as stromal stem cells or mesenchymal stem cells, reside in the postnatal bone. SSCs are multipotent and have the ability to differentiate to all the major non-hematopoietic cell types associated with bone (i.e. osteoblasts, , chondrocytes, and marrow adipocytes) as well as the ability for self-renewal [13]. The first evidence for the existence of SSCs in in bone marrow stroma was provided by Friedenstein and colleagues in the late 1960s[16].

SSCs, which are fibroblast-like cells, have the ability to form colonies from single cells, referred to as colony forming units-fibroblasts (CFU-Fs) [15, 18]. Individual CFU-Fs had the capacity to differentiate ex-vivo into the principal skeletal lineages (osteogenic, adipogenic, and chondrogenic) [15, 17].

In addition to the in vitro characterization of SSCs described above, more recent work has focused on identifying SSCs in vivo. A number of SSC surface markers were defined including nestin (Nes), the leptin receptor (Lepr), osterix (Osx), myxovirus resistance-1 (Mx1), platelet-derived growth factor receptor α (Pdgfra), paired-related homeobox gene-1 (Prx1) homeobox gene a11 (Hoxa11), Gremlin1 (Grem1), Gli1 and Axin2[14]. Nestin was identified as an SSCs marker using a Nestin-GFP mouse [14, 19]. Fluorescence-activated cell sorting (FACS) purification of GFP+ cells identified a relatively rare stromal cell population enriched in CFU-F activity and having the ability for multipotency and self-renewal [14, 19]. Lineage tracing experiments suggest that this population of Nes-GFP+ cells is derived from type II collagen-expressing chondrocytes via a RUNX2 and Indian hedgehog (Ihh)-dependent mechanism [14, 20].

Another SSCs surface marker that could be used to isolate stromal cells enriched in SSCs activity is PDGFRα. It is commonly used alone or in combination with standard stem cell markers [14, 21]. PDGFRα+ CD51+ bone marrow stromal cells were shown to recapitulate the SSCs activity of Nes-GFP+ cells [14, 22]. Also, the leptin receptor (LepR) was shown to be an important SSC marker specifically in adult mice [14, 23]. Lineage tracing of LepR+ marrow cells demonstrated that these cells mainly arise postnatally and serve as precursors for osteoblasts and adipocytes [14, 23]. After fracture or ablation/irradiation injury, LepR+ cells are necessary for bone marrow homeostasis and tissue repair [14, 23, 24].

Cranial sutures are active growth sites for intramembranous bone formation in the cranial vault and the location of a SCC population that participates in craniofacial growth and repair [14, 25, 26]. Two types of mesenchymal cells are present in the cranial suture; mesenchymal cells adjacent to the osteogenic front of the calvaria bones that differentiate into osteoblasts and become incorporated into the growing bones and mid-suture mesenchymal cells that remain undifferentiated [14, 27]. Lineage tracing studies showed that mid-suture mesenchymal cells are calvaria mesenchymal stem cells [14, 26]. These mesenchymal stem cells express Gli1+ (glioma-associated oncogene 1), an intermediate in hedgehog signaling. Fate mapping of Gli1+ cells shows that they contribute to craniofacial bones during growth and injury repair, and depletion of this cell population leads to craniofacial malformations [14, 28]. Another marker for calvaria mesenchymal stem cells is Axin2, a Wnt pathway intermediate [14, 28]. Axin2+ cells are capable of long-term self-renewal and differentiation to osteoblasts during development, remodeling and regeneration [14, 28].

SSCs interact with components of the microenvironment. Physical factors, including cell shape, external mechanical forces, ECM, among others, have been implicated in SSC fate decisions [18]. Within the SSC niche, environmental signals regulate stem cell behavior. One of the most important components of the SSC niche is the ECM. Many studies reported on how SSCs sense and respond to signals from the ECM at the molecular level, and how these signals regulate SSC fate [29, 30]. SSC interactions with the niche are reciprocal, since SSCs can remodel the niche in response to the signals they receive from it [29, 31].

Native bone ECM secreted by SSCs provides an optimal biological framework that is able to maintain SSC stemness, prevent premature cell differentiation, and preserve the ability of SSCs to differentiate into osteoblasts or adipocytes [32]. On the other hand, SSCs lose their unique properties when they lose their ECM and are cultured on tissue culture plastic [32]. For example,

mice lacking Biglycan (encoded by the gene *Bgn*), which is an ECM proteoglycan that is enriched in bone, exhibit reduced growth rate and decreased bone mass resulting in age-dependent osteopenia [32, 33]. Furthermore, when the number of osteogenic precursors measured in the bone marrow of normal and $Bgn^{-/0}$ mutant mice was compared, the number of colonies (CFU-F) gradually decreased with age in mutant mice [34]. In addition, *Bgn*-deficient SSC showed a combination of decreased proliferation and increased apoptosis [34].

The chemical and physical composition and structure of the ECM, which is unique to each tissue, is important for cell-ECM interactions and cellular function. Soft matrices mimicking the matrix environment of the brain promote neurogenic differentiation. Somewhat stiffer matrices promote myogenic differentiation, while rigid matrices support osteogenic differentiation [35]. In the case of SSCs, stiffer ECM promotes osteogenesis while softer ECM promotes adipogenesis. In the stiffer ECM, cells are exposed to cytoskeletal tension resulting from mechanical interactions between SSC and ECM. This mechanical interaction is mediated by integrins, which are a family of heterodimeric cell surface molecules that regulate intracellular and extracellular signaling pathways to affect survival, migration and differentiation. Integrins activate several downstream pathways, including focal adhesion kinase (FAK), mitogen-activated protein kinase (MAPK) and Rho/Rho-associated protein kinase (ROCK) [14, 36]. When SSCs are in a stiff loading environment, such as when integrin receptors interact with the type I collagen-rich ECM of bone, FAK is stimulated resulting in activation of the MAPK pathway, which subsequently phosphorylates both Runt-related transcription factor 2 (RUNX2) and Peroxisome proliferator-activated receptor gamma (PPARγ). The net effect if these phosphorylations is the activation of osteogenesis and inhibition of adipogenesis [14, 37]. On the other hand, when there is a softer matrix or in the absence of mechanical load, RUNX2 and PPARγ are both dephosphorylated, which results in increased

adipogenesis and inhibition of osteogenesis. At least part of this switch and reciprocal control of osteogenic and adipogenic differentiation is explained by MAPK phosphorylation of RUNX2 and PPARγ transcription factors [37].

RUNX2 belongs to the Runx family of transcription factors, which consists of RUNX1, RUNX2, and RUNX3. RUNX2, which is also known as core-binding factor α-1 (Cbfα1), polyoma virus enhancer-binding protein 2 α-A (PEBP2αA), and acute myeloid leukemia 3 protein (AML3), is the master gene and most important factor involved in the osteogenic differentiation, and essential for chondrocyte maturation. The chromosomal location of human *RUNX2* indicates an association of the gene to cleidocranial dysplasia (CCD), an autosomal dominant bone disease [38]. Heterozygous (*Runx2*$^{+/-}$) mice showed hypoplastic clavicles and nasal bones along with retarded ossification of parietal, interparietal, and supraoccipital bones, which resembles the phenotype of patients with CCD [39, 40]. Mice with a homozygous mutation in *Runx2* died just after birth due to respiratory failure, and their skeleton exhibited a complete lack of ossification and chondrocyte maturation[39].

Runx2 is weakly expressed in uncommitted SSCs, and its expression is upregulated in preosteoblasts, reaches maximal levels in immature osteoblasts, is then down-regulated in mature osteoblasts and is undetectable during the differentiation of osteoblast into osteocytes [41, 42]. RUNX2 enhances the proliferation of SSCs and promotes their commitment into osteoblast lineage cells [41]. Runx2 induces the expression of major bone ECM protein genes including type I collagen (*Col1a1*), Osteopontin (*Spp1*), Bone sialoprotein (*Ibsp*), Osteocalcin (*Bglap2*), and Fibronectin (*Fn1*) [41, 42, 43, 44]. In addition, overexpression of RUNX2 accelerates osteoblast differentiation and inhibits chondrocyte differentiation [41, 45]. Furthermore, RUNX2 is also involved in the regulation of cell proliferation and cell cycle progression [46]. *Runx2*$^{-/-}$ calvarial cells

proliferated faster than WT calvarial cells in vitro [41, 47]. Galindo et al. found RUNX2 expression increased significantly during the slow cell proliferation phase (G_0), but reduced markedly during the rapid cell proliferation (G1 phase)[46, 48]. This suggests that RUNX2 may serve as an inhibitor of cell proliferation [46].

The nuclear receptor PPARγ is a crucial cellular and metabolic switch that regulates many physiologic processes in the bone environment. PPARγ regulates a diverse array of physiologic processes including SSC adipogenesis, lipid metabolism, insulin sensitivity and inflammation, and is also involved in diseases such as diabetes, obesity and atherosclerosis [49, 50]. In addition, PPARγ is a metabolic switch for stem cell fate in both SSCs and hematopoietic lineages [49]. Studies showed that activation of PPARγ shifts the balance of SSCs fate by favoring adipocyte differentiation and inhibiting osteoblast differentiation[49, 51]. PPARγ-deficient embryonic stem cells fail to differentiate into adipocytes, but spontaneously differentiate into osteoblasts; moreover, $PPAR\gamma^{+/-}$ mice display a high bone mass due to increased osteoblast number and bone formation [49, 52]. Furthermore, PPARγ activity and adipogenesis are inhibited by activation of RUNX2 and osteoblastogenesis [52].

MAPKs Mediate Bone Cell Responses to ECM

MAP kinase pathways function as important regulators of cell growth, differentiation and morphogenesis in most tissues including bone. The mitogen-activated protein kinases (MAPKs) include Ras, RAF, the MAP kinase kinases, MEK1 and MEK2, and the terminal MAP kinases, ERK1 and ERK2, p38 kinases, which consist of MAP kinase kinase 3 and 6 (MKK3, MKK6) and p38 α, β, γ and δ and c-June N-terminal kinases (JNK), which consist of MKK 4 and 7 and JNK1 and 2 [52]. MAPKs have the potential to mediate the skeletal response to a wide variety of factors including growth factors, morphogens, ECM components and biomechanical signals. In skeletal

development, transgenic overexpression of a constitutively-active form of the ERK/MAPK intermediate, Mek1, in osteoblasts accelerates formation of the both cranial and appendicular skeletons while a dominant-negative Mek1 slows development [52, 53]. In addition, the hypoplastic clavicles and hypomineralized calvaria characteristic of Runx2 haploinsufficiency in mice was partially rescued when crossing mice expressing constitutively-active MEK1 with $Runx2^{+/-}$ mice [52, 53, 54]. On the other hand, crossing dominant-negative Mek1 mice with $Runx2^{+/-}$ animals led to exacerbated clavicular hypertrophy and calvarial hypomineralization resulting in embryonic lethality [52, 53]. Furthermore, calvarial cells isolated from dominant-negative Mek1 mice exhibited decreased osteoblast differentiation compare with wild type cells while cells from constitutively-active Mek1 mice exhibited enhanced differentiation [52, 53].

In FGF-mediated cranial suture fusion, an important role for ERK/MAPK signaling was also identified. FGF ligands are necessary for normal growth and development of craniofacial structures [52, 55]. Premature suture fusion (craniosynostosis), as is found in Apert syndrome and Crouzon syndromes, is caused by gain of function mutations in FGFR2 [52, 55]. In the Apert syndrome mouse model, harboring the $Fgfr2^{S252W}$ mutation, inhibition of ERK phosphorylation with an shRNA specific to mutant Fgfr2, or with the specific inhibitor, U0126, can block pathological suture fusion [52, 56]. Furthermore, activated FGFRs signal through the ERK/MAPK pathway leading to elevated levels of P-ERK1/2 [52, 57].

Other members of the MAPK family are p38 kinases and JNK pathway intermediates. P38 kinases are critical for skeleton development, maintenance of bone homeostasis, and osteoblast differentiation [52, 58]. They function as down-stream signals activated by the TGF-β and BMP responsive kinase, TAK1 [58, 59]. *Tak1* deletion in mouse preosteoblast results in reduced cortical and trabecular bone, clavicular hypoplasia and delayed fontanelle fusion due to reduced p38

signaling and the p38 intermediates, Mkk3, Mkk6, p38α or p38β [52, 58]. Interestingly, p38β-deficient mice show defects in long bone formation without major effects on calvarial development. Lastly, several studies suggest a role for the JNK pathway in osteoblast differentiation; however, the interpretation of these studies is complicated by the known role of JNK signaling in cell survival and apoptosis [52, 60]. One example of the involvement of JNK in osteoblast differentiation is the demonstration that overexpression of a constitutively-active JNK1 increases in vitro osteoblast differentiation [52, 61].

When SSCs become committed to osteogenic differentiation, MAPK phosphorylates RUNX2 and PPARγ leading to activation of RUNX2 and inactivation of PPARγ [52]. RUNX2 phosphorylation, catalyzed by ERK1/2, occurs on multiple serine residues including S43, S301, S319 and S510 [52]. However, the most important serines for MAPK phosphorylation during osteogenic differentiation are S301, S319 [37, 62]. In rescue experiments using Runx2$^{-/-}$ mouse embryo fibroblasts, transduction with a retroviral vector expressing phosphomimetic mutant Runx2 (S301E, S319E) stimulated osteoblastogenesis while suppressing adipogenesis [37]. In contrast, when RUNX2 phosphorylation decreased or was blocked by transducing cells with a retroviral vector expressing a phosphorylation-deficient RUNX2 mutant (S301A, S319A), osteoblastogenesis was inhibited and adipogenesis was increased [37].

On the other hand, MAPK inhibition of PPARγ activity is mediated by phosphorylation at serine 112 (S112) and this leads to decreased adipocyte gene expression [62, 63]. When PPARγ $^{-/-}$ mouse embryo fibroblasts, which fail to differentiate into adipocytes, but spontaneously form osteoblasts, are transduced with phosphorylation-resistant PPARγ mutant (S112A), this results in stimulation of adipogenesis and inhibition of osteoblastogenesis [37]. Furthermore, when ST2 mesenchymal cells are transduced with dominant-negative MEK1, PPARγ phosphorylation

decreased resulting in increased lipid droplet accumulation and adipogenesis marker expression [37]. These results suggest a crucial role of MAPK signaling in RUNX2/PPARγ phosphorylation and control of osteoblast and adipocyte differentiation by SSCs [37].

Another role of MAPK in SSC differentiation is related to its role in mechanical stimulation.

Mechanical Stimulation of SSCs

Bone is a dynamic tissue that has the remarkable ability to alter its structure in response to changes in mechanical loading. Similar to muscles, bones add mass when we exercise and atrophy if we do not. A tennis player will develop denser bones in his or her playing arm compared to the nonplaying arm [64]. In addition, weight-bearing exercise increases bone mass; however, when mechanical demands are decreased, bone is lost as seen after prolonged bed rest, exposure to microgravity during space flight or spinal cord injury [52, 65, 66, 67, 68]. The skeleton adapts to its mechanical environment throughout life, and this adaptation decreases with age [69, 70].

During mechanical stimulation, the bone mineral apposition rate increases. In addition, it is thought that mechanical load promotes stem cell proliferation followed by differentiation to osteoblast [69]. For example, implant osseointegration is enhanced by physical stimulation, suggesting that strain promotes osteogenic differentiation of stem cells [69, 70]. Also, during distraction osteogenesis when a fracture is created and bone segments are gradually moved apart, the zone between these bone segments where stem cells are located is associated with new bone formation. Furthermore, bone areas calculated to experience low to moderate tension are associated with sites of bone formation [69, 72, 73].

Due to the complexity of the mechanical environment and related responses, it is difficult to determine the specific mechanism by which SSCs respond to physical stimulation in vivo. Therefore, in vitro studies that apply controlled physical stimuli to SSCs are useful for studying potential molecular mechanisms for sensing mechanical stimulation and possible mechanotransduction pathways [74]. In vitro, there are many ways researchers have stimulated cells with mechanical forces [75, 76]. These include stretching (tensile stress) [76, 77], hydrostatic pressure or platen abutment (compressive stress), fluid flow (shear stress) [76, 78, 79], ultrasound [76, 80, 81], high frequency, low magnitude displacement (vibration) [76, 82, 83], and direct cell membrane magnetic stimuli [76, 83]. In addition, there were some in vivo studies that have been done to evaluate the consequences of loss of mechanical loading, such as the mouse tail suspension model, which induces bone loss in skeletally mature mice [83].

Many different bone cells including osteoblasts, SSCs and osteocytes are capable of responding to mechanical forces. However, osteocytes, which are connected through processes extending through the lacuno-canalicular network, are thought to be the primary mechanosensing cell type in bone [69]. Through this network of canalicular processes, osteocytes can communicate through paracrine signaling with osteoblasts, bone lining cells, stem cells and osteoclasts to induce bone formation or resorption [69]. However, some recent in vitro studies showed that stem cells could be directly stimulated by mechanical loading to promote their osteogenic differentiation [69].

For example, exposure of SSCs to 8% cyclic stretch for 1 hour each day for 3 consecutive days resulted in increased osteoblast marker gene expression, including up-regulation of Runx2, alkaline phosphatase (ALP), collagen type 1 (Col1) and bone morphogenetic protein (BMP)2 as well as reduced proliferation [69, 84, 85]. Furthermore, compression induces chondrogenic differentiation of SSCs. One study showed that when SSCs seeded in a fibrin gel are subjected to

dynamic mechanical compression, increases in chondrogenic gene expression and deposition of sulfated glycosaminoglycans are observed [69, 86].

Fluid flow is another method commonly used to apply shear stress in vitro, producing a gradient of stresses along the cell body [69]. This shear stress is affected by the direction of the fluid flow, with cells exposed to unidirectional flow exhibiting different characteristics from cells experiencing oscillatory fluid flow [69]. A study showed that twenty-four hours after oscillatory fluid flow, SSCs proliferation rate was increase and gene expression of *Spp1* and *Bglap* were also increased [87]. Similarly, hydrostatic pressure, both static and dynamic, can also encourage osteogenic differentiation [88]. On the contrary, the absence of mechanical stimulation also affects SSCs differentiation. A study demonstrated that unloaded bone SSCs exhibited a decrease in their osteogenic potential, as indicated by decreased ALP activity and reduced bone nodule formation, compared with loaded bones SSCs [69, 89].

SSCs may sense the mechanical stimuli through different mechanosensors. These include the cytoskeleton, focal adhesions, primary cilia, membrane channels, gap junctions and mechanosomes [69, 90, 91]. The first three will be described. The cytoskeleton provides a structural framework for the cell and is composed primarily of actin, intermediate filaments, and microtubules. Cytoskeletal tension is generated when myosin interacts with actin, which is involved in mechanically induced osteogenesis of SSCs [69, 91]. The cytoskeletal dynamic is regulated by intact cytoskeleton, RhoA, a GTPase, and its effector, ROCK and they are required for fluid flow-induced osteogenic differentiation of SSCs [69, 92]. Any disruption to the cytoskeleton by inhibition of nonmuscle myosin II, actin polymerization, and actin depolymerization prevents fluid flow-induced osteogenic differentiation and promotes differentiation towards the adipogenic and chondrogenic lineages [69, 92]. Furthermore, cytoskeletal contractility, expression of E-cadherin

and distribution of focal adhesions may together influence mechanically induced differentiation of SSCs [89, 93].

Focal adhesions, which contain adapter proteins such as talin and vinculin, link the cytoskeleton to integrins, which attach the cell to the substrate[69]. There are two main components of integrin, alpha and beta, with various isotypes of each [69]. Integrins α1 β1, α2β1 and α11β1 are the main integrins responsible for the binding of bone-forming cells to type I collagen [52]. although other integrins such as α5β1 may also be involved in mechanotransduction [52]. Focal adhesions associate with different signaling pathways, including those mediated by focal adhesion kinase (FAK), an important mediator of signaling at these centers [69, 94]. During mechanical stimulation, integrin activates FAK and Src kinases and stimulates RhoA and Rho-associated coiled-coil containing protein kinase (ROCK) [52, 95]. FAK also activates ERK, p38 and JNK MAPK as well as phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) pathways [55]. Liu et al showed that upon application of fluid shear stress, an increase in ALP activity and expression of osteogenic markers were observed, along with activation of FAK and ERK1/2 [69, 95]. However, FAK and ERK1/2 activation were inhibited when β1 integrins were blocked [69, 95]. Furthermore, Ward et al demonstrated that FAK phosphorylation is important for osteogenic differentiation of human SSCs in response to tension [69, 96].

The third mechanosensor structure is primary cilium, which is a single, immotile, antenna-like structure, generated through the process of intraflagellar transport that extends from the cell into the extracellular space [69, 97]. Primary cilia are very important signaling centers in cells and have shown to be important for mechanosensation in multiple cell types. The primary cilium acts as a microdomain that facilitates biochemical signaling as a result of localization and concentration of various proteins, including ion channels and enzymes [69, 98, 99]. Primary cilia are important for

normal bone development and for sensing a variety of extracellular biochemical and biophysical signals [69, 97, 100]. Recently, it has been demonstrated that primary cilia are involved in mechanical stimulation-induced osteogenic differentiation of SSCs [69]. For example, SSCs exposure to oscillatory fluid flow resulted in an increase in cyclooxygenase 2 and BMP2 gene expression, indicating an early osteogenic response to mechanical stimulation [69, 101]. In addition, a significant increase in proliferation rate was observed. In contrast, SSCs treated with siRNA to inhibit intraflagellar transport 88, an important component of the cilium, did not respond with the flow-induced increases in gene expression [69, 101]. These results suggest mechanosensors are important for mechanically induced osteogenic differentiation.

Multiple pathways mediate SSCs osteogenic lineage commitment during mechanical stimuli, including the MAPK/ERK, Wnt, Hippo, calcium signaling and RhoA/ROCK pathways [69]. The MAPK/ERK pathway is activated during mechanical stimulation. When strain was applied to SSCs, phosphorylation levels of ERK1/2 were elevated and this ERK activity was required for osteogenic differentiation [69, 102]. Also, mechanical strain-mediated increases in mineralization required activation of ERK1/2. However, inhibition of MEK blocked these increases [69, 103]. Lastly, mechanical stimulation of osteoblasts by exposure to fluid flow shear stress rapidly increases P-ERK-dependent phosphorylation of Runx2 at S301 and S319 [52, 104].

Wnt signaling plays an important role in bone development. β-Catenin is a pivotal component of the Wnt signaling pathway and it is tightly regulated at three hierarchical levels: protein stability, subcellular localization and transcriptional activity [105]. β-catenin has been shown to promote osteogenic differentiation in early osteoblast progenitors [69, 106]. Exposure of SSCs to oscillatory fluid flow resulted in translocation of β-catenin and upregulation of Wnt5a, which is

capable of inducing both canonical and non-canonical pathways and necessary to induce activation of RhoA and increase Runx2 gene expression [107].

The Hippo pathway also plays an important part in regulating osteoblast differentiation. It negatively regulates the activity of transcriptional co-activators, Yes-associated protein 1 (YAP) and Transcriptional coactivator with PDZ-binding motif (TAZ). YAP and TAZ exist in an inactive phosphorylated form in the cytoplasm. During Hippo pathway activation, YAP and TAZ are dephosphorylated leading to nuclear translocation [52]. Both YAP and TAZ are additional mediators of cellular responses to static and dynamic loads that function downstream of Rho/ROCK activation [52, 108]. The YAP/TAZ pathway is important for sensing substrate stiffness and cell shape through Rho/ROCK-mediated cytoskeletal stiffening that prevents YAP/TAZ phosphorylation and promotes nuclear translocation [52, 69]. When YAP/TAZ are translocated to the nucleus, they interact with several transcription factors including TEA domain (TEAD) factors, T-box 5 (TBX5) and, interestingly, RUNX2 and PPARγ [52, 109]. In stiff matrix, YAP/TAZ are activated and dephosphorylated resulting in increased RUNX2 gene expression and osteogenic differentiation and suppression PPARγ activity/adipogenesis. [52. 69].

ECM Receptors

Since ECM binding to the cell surface is clearly involved in the response to static and dynamic stimuli, a search for ways to block these interactions was begun in a number of laboratories. One way to interrupt this interaction is by identify blocking antibodies that would inhibit cell adhesion to specific substrates. In order to find out the appropriate antibody, ECM receptors were identified and characterized. The main and most studied ECM receptors are the

integrins, which were discovered in the mid 1980s [110]. While hunting for the integrin receptors, other ECM binding proteins were also discovered. Non-integrin transmembrane receptors include discoidin domain receptors (DDRs), leukocyte-associated immunoglobulin-like receptor-1, glycoprotein VI (GPVI), laminin receptor (LamR), the proteoglycan receptors, and syndecans I-IV. Other cell adhesion molecules such as Emmprin (CD147) were also investigated [110,111].

Integrins

Integrins are crucially important because they are the main receptor proteins that cells use to both bind and respond to the ECM. Integrins are composed of two noncovalently associated transmembrane glycoprotein subunits (α and β) that are both transmembrane type I proteins with large extracellular domains and mostly short cytoplasmic domains capable of interact with multiple intracellular proteins [112]. In vertebrates, the integrin family is composed of 18 α subunits and 8 β subunits that can assemble into 24 different αβ heterodimers[110]. Generally, β1-containing integrins are broadly expressed in most cells, but their α-pairing subunits are more often restricted to specific cell types [113]. Integrins control focal adhesion and cell adherence to the ECM through the assembly of intracellular proteins linked to the cytoskeleton [113]. Upon exposure to ECM stimuli, integrins undergo conformational changes that allow ECM ligand interactions together with tight connections to the inner cytoskeletal network [113].

Osteoclasts and osteoblasts use a diverse range of integrins to interact with bone ECM. The osteoclast, which is a myeloid-derived polykaryon uniquely endowed with the capacity to degrade the organic and inorganic matrices of bone, organizes its actin-containing cytoskeleton to form podosomes which serve as the point of contact with the bone surface [113]. These podosomes contain

a dot-like core of actin filaments surrounded by a loose network of F-actin, as well as integrin receptors and adaptor proteins [114]. In order to form a sealing zone with the bone surface to be resorbed, individual podosomes arrange collectively into superstructures forming an actin ring [113]. This actin-containing sealing zone delineates the active sites of bone resorption and forms a pocket into which protons and bone-resorbing proteases are secreted [113, 115]. Although osteoclast adhesion to the bone surface is essential to form the bone resorption site, the complete repertoire of integrins expressed by osteoclasts has not yet been compiled. The following integrin subunits have been associated with osteoclasts: α5, α2, β1, and β3 [116]. Of these osteoclast integrins, the α5β3 integrin is the most abundantly observed and highly studied [116]. Also, loss of αvβ3-mediated signaling, which regulates cell polarity and cytoskeletal reorganization, results in reduced resorptive activity [117]. Furthermore, another study showed that deletion of kindlin-3, a ubiquitous activator of integrins, leads to loss of α5, β1, and β3 subunits, leading to a severe osteopetrotic phenotype [118].

In osteoblast, early studies indicated that integrin interactions with the major ECM components, fibronectin and collagen type I, control osteoblast differentiation and fate in vitro [119]. The main integrins identified in osteoblast are α1 β1, α2β1, α4β1, α5β1, α5β3, α5β5, α8β1 and α11β1 [52, 113]. Overall, both β1- and α5-paired integrins and α8β1 are the main integrin receptors in osteoblasts (Table 1.2) and may have prominent roles since antibody perturbation experiments blocked osteoblast differentiation and mineralization [113, 119, 120]. Initial investigations showed that an interaction between α5β1 and fibronectin is required for the ability of preosteoblasts to adhere to the ECM and differentiate into mature osteoblasts [119, 121]. Furthermore, α5β1 integrin is involved in osteoblast survival [119, 122]. Also, α5β1 integrin is required for the anabolic effect of increased mechanical load on bone formation as mentioned before. However, other integrins have some role on osteogenesis as well. For example, αvβ3 integrin is involved in the induction of osteoblast

differentiation by dexamethasone and BMP-2 in vitro [119, 123, 124]. Additionally, an α2β1 integrin that induces ROCK, FAK and ERK signaling is involved in both MSC survival and osteogenic differentiation [119, 125, 126, 127].

Binding of integrins to the ECM initiates clustering, cell adhesion and intracellular signaling leading to complex conformational changes that promote high-affinity extracellular ligand binding, as well as activation of signaling proteins including FAK, Rho, Rac and integrin-linked protein kinase (ILK) [119, 128]. FAK activation leads to phosphorylation of a variety of molecules, including Src, PI3K, RACα serine/threonine-protein kinase (Akt), and MAPK (ERK-2 and ERK-1, respectively), among other effectors as mentioned in the section on the mechanical stimulation of SSCs. [119, 128].

Mice with targeted integrin subunit deletion in bone (i.e., β1 and αv) have an embryonic lethal phenotype, making it difficult to analyze their function during osteogenesis [113, 129, 130, 131]. Because of that, integrins have also been ablated during osteoblast differentiation using the Cre/LoxP conditional deletion system. However, the affected mice do not suffer from massive developmental defects [113]. That suggests that other ECM receptors may be required for bone formation. Another potential class of ECM receptor are the Discoidin domain receptors, which we will discuss next.

Table 1.2 Integrin deficiency in mouse and the functional consequence in development or bone formation.
Adopted from Brunner M et al. (2013) [113]

Integrin subunit	Expression in bone cells	Phenotype		References
		General	Bone	
α1	Yes	L/F collagen synthesis/tumor angiogenesis/callus defect	Polymorphism in the locus linked to osteoporosis	Ekholm et al., 2002, Gardner et al., 1996, Lee et al., 2007b, Pozzi et al., 1998, Zemmyo et al., 2003
α2	Yes	L/F platelets adhesion defect on collagen/mammary ductal branching defect/mild kidney defect	No bone defect reported	Chen et al., 2002, Girgert et al., 2010, Grenache et al., 2007
α3	Yes	Perinatal lethal, kidney and lung defects	No bone defect reported	DiPersio et al., 1997, Kreidberg et al., 1996
α4	Yes	Embryonic lethal E11.5–E14.5 placenta defect, cardiac development defect/hematopoietic maintenance and homing defect	No bone defect reported	Arroyo et al., 1996, Yang et al., 1993, Yang et al., 1995, Yang et al., 1996
α5	Yes	Embryonic lethal E9.5: neural crest cell survival, mesoderm defect	No bone defect reported	Goh et al., 1997, Yang et al., 1993
α6	ND	Perinatal lethalskin blistering, brain cortex organization defect	No bone defect reported	Georges-Labouesse et al., 1996, Georges-Labouesse et al., 1998
α7	Yes	Partial lethality/vascular defect/dystrophy/placental defect	No bone defect reported	Mayer et al. (1997)
α8	Yes	Partial perinatal lethality/kidney defect/deafness	No bone defect reported	Littlewood Evans and Muller, 2000, Muller et al., 1997
α9	Yes	Perinatal lethal P6–12, lymphogenesis defect, congenital chylothorax	No bone defect reported	Huang et al. (2000b)
α10	Yes	L/F, dwarfism, mild chondrodysplasia	Growth plate defect	Bengtsson et al. (2005)
α11	Yes	L/F, dwarfism and increase mortality, defective incisor	No bone defect reported	Popova et al. (2007)
αv	Yes	L, embryonic lethal at E12 and birth; defects in placenta and in CNS and GI blood cells, cleft palate	No bone defect reported	Bader et al., 1998, van der Flier et al., 2010
αx	Yes (MSC)	L/F, T-cell defect, increased susceptibility to bacterial infection	No bone defect reported	Ren et al., 2004, Wu et al., 2004

Integrin subunit	Expression in bone cells	Phenotype		References
		General	Bone	
αE	ND	Reduced lymphocyte	No bone defect reported	Schon et al. (1999)
αL	ND	L/F, impaired leucocyte recruitment and tumor rejection, osteoclast development defect	Osteoclast defect	Schmits et al., 1996, Shier et al., 1996
αD	Yes (MSC)	L/F, reduced staphylococcal enterotoxin-induced T-cell response	No bone defect reported	Wu et al. (2004)
αM	ND	L/F, impaired phagocytosis and PMN apoptosis, obesity, mast cell development	No bone defect reported	Coxon et al., 1996, Lu et al., 1997
αIIb	No	L/F, thrombastenic	No bone defect reported	Tronik-Le Roux et al. (2000)
β1	Yes	Embryonic lethal E5.5, inner cell mass deterioration	Reduced bone formation	Fassler and Meyer, 1995, Phillips et al., 2008, Stephens et al., 1995
β2	Yes (MSC)	L/F, impaired leucocyte recruitment, skin infection, osteoporosis	Defect of osteoblast differentiation	Miura et al., 2005, Wilson et al., 1993
β3	Yes weak in preosteoblast	L/F, thrombastenic, osteopetrotic	Defect of osteoclast resorption	Hodivala-Dilke et al., 1999, McHugh et al., 2000
β4	No	Perinatal lethalskin blistering	NA	Dowling et al., 1996, van der Neut et al., 1996
β5	Yes	L/F, no apparent phenotype	No bone defect reported	Huang et al. (2000a)
β6	No	L/F, TGF activation defect, juvenile baldness, asthma	NA	Huang et al., 1996, Munger et al., 1999
β7	No	L/F, Peyer's patches defect, reduced number of intraepithelial lymphocytes	NA	Wagner et al. (1996)
β8	No	L, embryonic lethal at E12 and birth, defects in placenta and in CNS and GI blood cells, cleft palate	NA	Zhu et al. (2002)

Discoidin Domain Receptors

Discoidin domain receptors (DDRs) were discovered during the search for tyrosine kinase proteins expressed in human malignancies [132]. Receptor tyrosine kinases (RTKs) are a large family of single-pass transmembrane receptors, characterized by structurally diverse extracellular ligand-binding regions and conserved cytosolic kinase domains [132]. The discoidin domain receptors are RTKs that contain a discoidin homology domain in their extracellular regions [133]. Ddrs are collagen receptors that differ from collagen-binding integrins in that they have intrinsic tyrosine kinase activity and selective affinity for triple-helical, native fibrillar and non-fibrillar collagens [133].

There are two types of DDRs, DDR1 and DDR2. Ddr1 has broad ligand specificity, which includes all known collagens, and is selectively expressed in epithelia [133]. It is abundant in the brain and is found in the epithelial layer of the colonic mucosa, in keratinocytes, in the thyroid follicles, and in the lung epithelium [132]. In addition, DDR1 can be used as a marker for the formation of neuroectodermal cells during mouse development [133]. In contrast, DDR2 is expressed by mesenchymal cells and principally binds collagens I, II, III and X [133, 134]. Activation of DDR2 by collagen is surprisingly slow, and to reach maximal tyrosine kinase activity, it requires collagen treatment for up to 18 hours [132]. This activation is sustained, and no sign of down-regulation or degradation is observed after up to 4 days [132].

Both DDR1 and DDR2 are involved in multiple physiological processes, such as development, ECM turnover, growth regulation and cancer [133, 134]. In bone, several lines of evidence suggest an important role for DDR2. In humans, *DDR2* mutations cause spondylo-meta-epiphyseal dysplasia (SMED), a skeletal disorder associated with dwarfism, bowing of long bones, craniofacial abnormalities, short fingers, and abnormal calcifications [133, 135]. Also, in a Han

Chinese population, polymorphisms in *DDR2* are associated with low bone mineral density (BMD) and fracture risk [136]. Consistent with the human phenotype, mice with a spontaneous *Ddr2* mutation, called smallie mice (*Ddr2*$^{slie/slie}$), have a SMED-like phenotype characterized by dwarfism and reduction in total bone mineral density [133,137]. Furthermore, detailed analysis of the bone phenotype in *Ddr2*$^{slie/slie}$ mice revealed a dramatic reduction in mineral density of the cranial, axial, and appendicular skeleton due to reduction in osteoblast activity and bone formation in the absence of changes in resorption [133]. Bone changes were accompanied by a large increase in marrow fat.

When Ddr2 is activated, several pathways are stimulated including ERK, JNK and p38 MAPKs, the PI-3 kinase/AKT and NFKβ pathways [52,132]. In osteogenesis, Ddr2 stimulates MAPK-dependent Runx2 phosphorylation [138]. Specifically, overexpression of a constitutively active DDR2 stimulated ERK/MAPK signaling and phosphorylation of RUNX2 and PPARγ leading to increased RUNX2-dependent transcriptional activity and inhibition of PPARγ [133, 138]. Consistent with these results, MSCs from *Ddr2*$^{slie/slie}$ mice showed reduced ability to differentiate into osteoblasts and increased adipogenesis [133]. This defective osteoblast differentiation in *Ddr2*$^{slie/slie}$ cells was due to reduction in ERK/MAPK signaling and RUNX2 S301 S319 phosphorylation [133].

In summary, Ddr2 is a key regulator of bone growth that controls several aspects of the process. Furthermore, DDR2 and integrins may interact during ECM stimulation with DDR2 stimulating integrin-mediated cell adhesion [139]. Also, DDR2 may participate in endochondrial ossification by regulating chondrocyte maturation as well as by stimulating intramembranous ossification by controlling osteoblast differentiation via phosphorylation of RUNX2. However, it is not known if DDR2 is required for bone regeneration.

Bone Regeneration

Bone possesses an intrinsic capacity for regeneration as part of the repair process in response to injury as well as during skeletal development and during the continuous remodeling that occurs throughout adult life [140]. The bone regeneration process consists of a well-orchestrated series of biological events, with a definable temporal and spatial sequence, involving a number of cell types and intracellular and extracellular molecular-signaling pathways [140]. In order to explore these events, two different mouse models for bone regeneration will be discussed, calvarial subcritical-size defect and tibial fracture mouse models

Calvarial Subcritical Size Defect

In order to understand how the calvarial bones regenerate, first we need to understand how they develop. Calvarial bones form the dome-like superior portion of the cranium, comprising the superior portions of the frontal, parietal, and occipital bones. Calvarial bone is formed by intramembranous ossification, where a compact and spongy bone develops directly from sheets of mesenchymal (undifferentiated) connective tissue [141]. The process begins when a group of mesenchymal cells within a highly vascularized area of the embryonic connective tissue proliferate (mesenchymal condensations) and differentiate directly into preosteoblasts and then into osteoblasts [142]. Osteoid, which is secreted by the osteoblasts, is uncalcified matrix that calcifies as mineral salts are deposited on it, thereby entrapping the osteoblasts within. Once entrapped, the

osteoblasts become osteocytes [141]. The sutures, where two opposing bone fronts oppose, are the predominant sites of bone growth.

There are two calvarial defect mouse models depending on the size of the defect, critical-size calvarial defect (CSD) and subcritical-size calvarial defect (SSD). A CSD is defined as a defect that will never heal within the lifetime of the animal, which in the case of a mouse is a defect larger than 2 mm [143]. Since a CSD doesn't heal spontaneously, it allows the researcher to test different kinds of grafting material, cells, or growth factors for ability to promote bone regeneration. On the other hand, SSD is defined as a calvarial defect that will heal spontaneously without any intervention, which is a mouse calvarial defect equal or less that 2mm [144]. The SSD is used by the researchers to investigate the self-healing ability of mice after various genetic manipulations, to trace cells involved in healing, and to further understand the biological events involved in normal bone healing.

Intramembranous bone healing is a well-organized process that includes multiple biological events. After formation of SSD, clot formation is initiated followed by an acute inflammatory response [145,146]. This inflammatory response includes cell proliferation, angiogenesis and inflammatory cell infiltration associated with peaks of growth factor secretion (BMP-2-4-7, TGFβ1, VEGFa), cytokines and chemokines (TNFα, IL-10) [145, 146]. Subsequently, granulation tissue is formed followed by MSCs migration to the injury site [145, 146]. MSCs then differentiate to osteoblasts and start matrix formation followed by mineral deposition until bridging of the defect is completed [145]. During healing, no evidence of cartilage cells or tissue were observed [134]. At this stage, bone formation/maturation markers (RUNX2, ALP, DMP1, PHEX, SOST) were highly expressed [145]. Lastly, bone remodeling takes place to reinforce and strengthen the newly formed bone.

Tibial Fracture Model

Fracture healing using the tibial model has been extensively studied and reviewed. The intense interest in understanding fracture healing is consistent with the fact that fractures are the most common large-organ, traumatic injuries in humans and approximately 10% do not heal properly [147]. The tibia is the second largest bone in the human skeleton, and it forms by endochondral ossification[148]. The initial stage of endochondral ossification is formation of a cartilage model through condensation of mesenchymal cells, followed by chondrogenic differentiation of mesenchymal cells [148]. Then, this cartilage model is invaded by vasculature to form a primary ossification center at the diaphysis, and later a secondary ossification center at the epiphysis [148]. After that, the perichondrium of the cartilage model becomes the periosteum and the cartilage in the ossification center is replaced gradually with osteoblasts, which secret osteoid that is subsequently calcified [148].

Fracture healing consists of a well-orchestrated series of biological events that mirror the primary morphogenetic pathways during embryonic skeletal development [147]. There are two types of fracture healing, direct intramembranous healing (discussed above related to SSD healing) and indirect (secondary) fracture healing, which consists of both intramembranous and endochondral bone formation [146]. Both fracture healing and endochondral bone formation are directly regulated by different growth factors and proteins, including FGF-2, BMPs, PTH and PTH-related protein, hedgehog proteins, TGF-β and Wnts [147, 149-159]. The indirect form of fracture healing is most commonly seen in long bone fractures [146]. Immediately following bone trauma, a hematoma is

generated, and an acute inflammatory immune response is initiated [146, 147]. This immune response leads to secretion of multiple cytokines and chemokines including TNF-α, IL-1, IL-6, IL-11 and IL-18, and infiltration of immune cells, including T cells. Then, a fibrin-rich granulation tissue forms, which allows endochondral bone formation to occur between the fracture ends external to periosteal sites. This results in soft callus formation that stabilizes the injury site. [146, 147]. Soft callus formation depends on the recruitment of MSCs. Current data suggest that there are two key regulators for recruiting and homing specific MSCs to the site of trauma, stromal cell-derived factor-1(SDF-1) and CXCR-4 [146]. Once MSCs are recruited, several peptide signaling cascades are initiated including TGF-β2, -β3 and GDF-5 pathways that participate in chondrogenesis and endochondral ossification, as well as BMP-2, -5, and-6 pathways which promote both chondrogenesis and osteogenesis [146, 147]. This soft callus is replaced by hard callus that provides biomechanical stability and replacement of cartilage by bone. This hard callus does not fully restore the biomechanical properties of normal bone. This requires an extensive bone remodeling process. This remodeling process involves a combination of hard callus resorption by osteoclasts, and lamellar bone deposition by osteoblasts and may take years to achieve a fully regenerated bone structure [146, 147].

Role of ECM in Bone Regeneration

Bone extracellular matrix plays important roles not only in bone development and maintenance, but also in response to bone injury and fracture. Studies of bone healing indicate that resident stem or progenitor cells interact with the ECM to orchestrate a complex signaling cascade leading to tissue regeneration and remodeling [160]. Dysregulation of proteins mediating ECM interactions is implicated in several deformities affecting bone structure and regeneration [161]. Type

I collagen is the core structural protein in bone where collagen-mediated signaling is critical for tissue differentiation and mineralization [161, 162, 163]. Diseases caused by mutations in type I collagen, such as osteogenesis imperfecta, are characterized by uncoupling between bone formation and resorption, impaired osteoblast differentiation and increased osteoclasts activity, as well as defects in fracture repair [160, 164, 165, 166].

ECM enhances cell recruitment during bone regeneration through cell surface receptors. Collagen binding integrins are the main ECM receptor known to be important in the crosstalk between cells and the ECM. Integrin α1β1 is a collagen receptor expressed on many mesenchymal cells, but mice deficient in α1 integrin have no gross structural defects. However, bone fractures in α1-knock-out mice had significantly less callus tissue than the WT mice [167]. Furthermore, safranin O staining revealed a defect in cartilage formation in α1-knockout mice as well as reduced expression of ECM related genes (*Col2a1, Col9a2, Col10a1, Col10a1, Mmp13* and *Acan*) and reduced proliferation of MSCs [167].

Targeting of ECM-cell interactions represents a potential strategy to promote bone regeneration. For example, PepGen P-15, which is a combination natural inorganic bovine-derived hydroxyapatite matrix (ABM) coupled with a synthetic cell-binding peptide (P-15) comprising 15 amino acids of a cell-binding region of collagen type I, enhanced new bone formation in a critical-size tibial cortical bone defect, whereas control defects showed very little newly formed bone [168, 169]. Furthermore, when P-15 was added in scaffold material, it stimulated expression of ALP, BMP-2 and BMP-7 [169, 170]. This suggest that P-15 promotes osteogenic differentiation. Moreover, treating non-union fracture patients with a P-15 containing bone graft substitute result in a 90 % success rate [169, 171].

In addition to P-15, GFOGER (glycine-phenylalanine-hydroxyproline-glycine-glutamate-arginine) is another collagen-mimetic peptide that selectively promotes α2β1 integrin binding, which is a critical event for osteoblastic differentiation [169,172]. Scaffolds coated with GFOGER showed significantly accelerated and increased bone formation in non-healing femoral defects compared to uncoated scaffolds and empty defects [169, 173]. Furthermore, implants coated with GFOGER were found to improve peri-implant bone regeneration and osseointegration [169, 174,175].

Indeed, peptides containing integrin binding domains of type I collagen coupled to tissue engineered scaffolds have been successfully used to stimulate bone regeneration [173, 174, 175, 176]. However, the integrin-activating tissue engineered scaffolds examined to date do not approach the activity of bone autografts, the "gold standard" for bone regeneration [177]. Also, bone-specific knockout of individual integrins in vivo only leads to modest phenotypes [178, 179, 180, 181]. These studies suggest that additional ECM factors/signals critical for bone regeneration remain to be discovered. As discussed in this chapter, the non-integrin collagen receptor, Ddr2, is critical for skeletal development [133, 137]. However, its role in bone regeneration has not yet been investigated.

Summary and Statement of Experimental Goals

The ECM plays a fundamental role in bone development, remodeling, regeneration and response to mechanical loading by binding to cells using specific ECM receptors. The most studied ECM receptors are the integrins, which play a crucial role in cell-matrix and intercellular interactions mediated by ECM adhesion molecules including collagen. DDR2 is a second class of

collagen receptor that differs from integrins in having a selective affinity for triple helical, fibrillar collagen and intrinsic tyrosine kinase activity. Interestingly, DDR2 and integrins may interact during ECM stimulation, and possibly DDR2 positively modulates integrin-mediated cell adhesion. Recently, our lab showed that *Ddr2* has a major role in bone development and deficiency of *Ddr2* causes craniofacial and long bone defects. In addition, *Ddr2* is important for maintenance of osteoblast activity.

Based on the known role of DDR2 in bone development and its possible interaction with integrins, which are known to be required for bone regeneration, we asked the question-Is *Ddr2* required for bone regeneration? This thesis will evaluate this requirement using 2 well-established regeneration models; a calvarial subcritical defect and tibial fracture. Studies will first determine the temporospatial expression of DDR2 during bone regeneration, examine the participation of Ddr2-expressing cells and their progeny in the regeneration process and use a gene knockout approach to determine the requirement for DDR2 in regeneration.

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Chapter 2

The Role of Discoidin Domain Receptor 2 in Calvarial Bone Regeneration

Abstract

Bone is an archetypical example of an organ that can completely heal itself by activating an intrinsic regeneration program. However, large bone defects caused by trauma, tumor resection or pathological bone resorption cannot spontaneously heal. Their treatment is a major clinical orthopedic challenge and global health problem. Cranial bone regeneration requires differentiation of mesenchymal stem cells to osteoblasts and remodeling of the bone extracellular matrix (ECM). Discoidin domain receptor 2 (DDR2) is a collagen-activated receptor tyrosine kinase shown to be essential for skeletal development in humans and mice. *Ddr2*-deficient mice exhibit dwarfism and defective bone formation in the axial, appendicular and cranial skeletons. However, the role of DDR2 in bone regeneration has not yet been investigated. Here we show that Smallie mice (*Ddr2*$^{slie/slie}$), which contain a nonfunctional *Ddr2* allele, are unable to heal a subcritical-size (0.5 mm) calvarial defect that, in wild type mice, can spontaneously heal within 4 weeks. Also, *Ddr2* expression during calvarial bone regeneration was defined using *Ddr2*-LacZ knock-in mice and β-galactosidase staining. *Ddr2* expression, which was restricted to periosteal surfaces of uninjured calvarial bone, greatly expanded with injury. Similar results were seen when the lineage of *Ddr2*-expressing cells was examined using *Ddr2cre*^{ERT}*, Ai14 TdTomato* mice. Ddr2 positive cells and their progeny expanded within the defect three days and two weeks post-surgery. Furthermore,

three days post-surgery, *Ddr2$^{slie/slie}$* mice showed a significant decrease in cell proliferation in the calvarial defect when compared with WT littermates. Lastly, levels of the preosteoblast markers, Osterix and phosphorylated RUNX2 (S319-P) decreased in *Ddr2$^{slie/slie}$* mice consistent with there being a defect in osteoblast differentiation. Together, our study demonstrates that DDR2 is necessary for normal calvarial bone regeneration and this requirement may be explained in part by effects of DDR2 on proliferation and osteoblast differentiation.

Introduction

Bone is a dynamic tissue with self-healing capabilities that allow repair of most fractures with restoration of original architecture. However, large bone defects, such as those caused by tumor resections or severe trauma, do not regenerate spontaneously and represent a major clinical challenge for craniomaxillofacial and orthopedic surgeons.[1] Bone grafts are usually used to manage such conditions. Bone autografts consist mainly of bone ECM and associated cells. [2] Recent research has unveiled many unique characteristics of ECM that play a key role in tissue regeneration. ECM enhances cell recruitment through cell surface receptors, which determine cell-ECM interactions and trigger specific cellular functions such as adhesion, migration, proliferation, and differentiation [3-5]. Therefore, a clear understanding of these ECM receptors will facilitate the development of new bone regeneration strategies.

Specific cell surface receptors mediate interactions between the collagenous ECM and cells. Collagen binding integrins are the main ECM receptors known to be important in the crosstalk between bone cells and the ECM. Integrin α2β1 is a collagen receptor expressed on many mesenchymal cells. Studies showed that stimulation of this integrin, using scaffolds that selectively promote α2β1 integrin binding, significantly accelerated and increased bone formation in non-healing femoral defects [7, 8]. Integrins have clear roles in bone formation and regeneration,

but they don't explain the entire response of bone cells to ECM since bone-specific knockout of individual integrins only partially prevents bone formation [8-11]. The discoidin domain receptors 1 and 2 (DDR1 and DDR2) are another important class of ECM receptor [12]. Unlike integrins, DDRs have intrinsic tyrosine kinase activity and are selectively activated by triple-helical collagens [13, 14].

Both DDR1 and DDR2 are involved in multiple physiological processes, such as development, ECM turnover, growth regulation and cancer [15-17]. In bone, several lines of evidence suggest an important role for DDR2. In humans, *Ddr2* mutations cause spondylo-meta-epiphyseal dysplasia (SMED), a skeletal disorder associated with dwarfism, bowing of long bones, craniofacial abnormalities, short fingers, and abnormal calcifications [16, 18]. Also, in a Han Chinese population, polymorphisms in *Ddr2* are associated with low bone mineral density (BMD) and fracture risk [19]. Consistent with the human phenotype, mice with a spontaneous *Ddr2* mutation, called smallie mice ($Ddr2^{slie/slie}$), have a SMED-like phenotype characterized by dwarfism and reduction in total bone mineral density [16,17]. Furthermore, detailed analysis of the bone phenotype in $Ddr2^{slie/slie}$ mice revealed a dramatic reduction in bone volume of the cranial, axial, and appendicular skeleton due to reduction in osteoblast activity and bone formation in the absence of changes in resorption [16]. Bone changes were accompanied by a large increase in marrow fat [16].

DDR2 is a key regulator of bone formation that controls several aspects of the process. However, it is not known if DDR2 is required for bone regeneration. In this study, we investigated the functional importance of *Ddr2* in the calvarial bone subcritical size defect regeneration using a *Ddr2*-deficient mouse model and defined the expression of *Ddr2* during calvarial bone regeneration. These findings provide new insights into the role of cell-ECM interactions in bone regeneration and may have important implications for understanding and enhancing bone regeneration.

Materials and Methods

Mice

Generation and genotyping of *Ddr2*$^{slie/slie}$ mice, which contain a spontaneous 150 kb deletion in the *Ddr2* locus to produce an effective null, and *Ddr2-LacZ* (*Ddr2*$^{+/LacZ}$) knock-in mice, where the bacterial LacZ gene is knocked into the *Ddr2* locus, were previously described [16, 17, 21]. Studies also used *Ddr2Cre*^{ERT} mice recently developed by Dr. Barry Greenberg (UC San Diego). These mice contain *Cre*^{ERT} knocked in-frame into exon 2 of *Ddr2*. *Ddr2Cre*^{ERT} mice were crossed with *Ai14 TdTomato* mice provided by Dr. Noriaki Ono (University of Michigan, Ann Arbor) [22]. *TdTomato* mice harbor a targeted mutation of the Gt (ROSA) 26Sor locus with a loxP-flanked STOP cassette preventing transcription of a CAG promoter-driven red fluorescent protein variant (tdTomato); TdTomato is expressed following Cre-mediated recombination.

Calvarial Subcritical-Size Surgery

All mouse experimental procedures conformed to standards for the use of laboratory animals and were approved by the institutional Animal Care and Use Committee (IACUC) of the University of Michigan. Eight-week-old mice were maintained with general anesthesia (2% isoflurane/100% O_2) during the procedure and received Buprenorphine (0.02mg/kg ip) for analgesia. Under sterile conditions, a sagittal incision was made, parietal bones were exposed, and a 0.5 mm subcritical size defect (SSD) was created with a carbide bur at low speed in wild-type (WT), *Ddr2*$^{slie/slie}$, *Ddr2*$^{+/LacZ}$ and *Ddr2cre*^{ERT}*; Ai14 TdTomato* mice. Incisions were closed using 4-0 black silk interrupted sutures. The day after surgery, another buprenorphine injection was given to the mice. Bone regeneration of the SSD was measured 2, 4, 6, and 12 weeks post-surgery. For *Ddr2cre*^{ERT}*; Ai14 TdTomato* mice, three daily intraperitoneal injections with tamoxifen

(75mg/kg) were given. One day after the last injection, SSD surgery was performed, and mice were sacrificed 3 days and 2 weeks post-surgery.

Micro–computed tomography analysis of bone regeneration

After fixation with 10% neutral buffered formalin for 24 hours, SSD bone regeneration were measured by micro-computed tomography using a Scanco Model 100 (Scanco Medical, Bassersdorf, Switzerland). Scan settings were: voxel size 12 μm, 70 kVp, 114 μA, 0.5 mm AL filter, and integration time 500 ms. All scans were analyzed using fixed thresholds (180 for trabecular bone and 280 for cortical bone). For bone volume quantification of the SSD, a circular region of interest (ROI) of 0.5mm diameter was defined for the assessment of newly formed bone at the defect area. To avoid examiner bias, the genotype of mice was not specifically highlighted during quantification analysis.

Detection of β-gal (Lacz) expression

Samples dissected from heterozygous *Ddr2-LacZ* ($Ddr2^{+/LacZ}$) mice were processed for X-gal staining using standard procedures. Briefly, 10 μm frozen sections were stained with freshly prepared X-gal solution and counterstained with Vector® Nuclear Fast Red.

Cell Proliferation

Three days post-surgery, mice were intraperitoneally injected with 5-ethynyl-2'-deoxyuridine (EdU, 100 mg/kg) and sacrificed after 4h [25]. Calvarial samples were fixed with 4% PFA (paraformaldehyde) overnight and decalcified in 10% EDTA for 1–2 weeks. Decalcified bone tissues were embedded in paraffin and sectioned at 5 μm thickness. EdU+ signals were detected with a Click-iT® EdU Alexa Fluor® 488 Imaging Kit (C10337; Invitrogen) following the recommended protocol. Cell nuclei were stained with DAPI. For quantification, we counted the number of EdU^+/total nuclei in each section.

Tissue Preparation and Histological Analysis

One week post-surgery SSD calvarial bones were fixed in 4% paraformaldehyde and processed for paraffin sections. For immunofluorescence, histological sections were incubated with primary antibodies to Osterix (Osx) (Abcam, ab 22552), P-RUNX2 (specifically detects RUNX2 phosphorylated at S319 [(26)]) or Gli1 (Novus Biologicals, NBP1-78259) overnight at 4°C (1:100 dilution), and then with Alexa Fluor 555-conjugated donkey anti-rabbit IgG (Invitrogen A-31572). Fluorescence detection was accomplished using an Olympus BX51-P microscope and imaging system.

Statistical Analysis

All data was analyzed using the GraphPad Prism software version 7.0e, La Jolla California USA. Values were reported as mean ± SD. Student's t test was used for statistical comparison between the two experimental groups. Differences were considered significant at $P < 0.05$. A sample size of five to eight mice (including both sexes) was used for experiments unless indicated otherwise.

Results

Ddr2 Signaling is Necessary for Optimal SSD Bone Regeneration

To investigate the functional role of Ddr2 in bone regeneration, we created a subcritical-size calvarial defect in both WT and *Ddr2*$^{slie/slie}$ mice. To show that we were able to create similar defects in both WT and *Ddr2*$^{slie/slie}$ mice, samples were harvested three days and one-week post-surgery and evaluated with Hematoxylin and Eosin (H&E) staining. Our results showed that the calvarial defects were initially identical in both groups (Figure 2.1 A). Reduced bone regeneration

in *Ddr2*$^{slie/slie}$ mice relative to WT animals was seen as early as two weeks post-surgery (Figure 1B). This delay in bone regeneration continued to be seen four- and six-weeks post-surgery. In contrast, defects in WT mice were completely healed after four weeks (Figure 2.1 C and D), which is in agreement with previous reports [23, 24]. Surprisingly, even after twelve weeks, the calvarial defect in *Ddr2*$^{slie/slie}$ mice still had not completely healed (Figure 2.1 E). Results were quantified by micro-CT, which showed a significant reduction in bone volume/total volume in *Ddr2*$^{slie/slie}$ defects two, four, six, and twelve weeks post-surgery (Figure 2.1 F). Interestingly, no further healing of defects was seen in *Ddr2*$^{slie/slie}$ mice after the six week time point. This suggests that the healing process had already arrested by this time, making it unlikely the defect would heal even after extended times.

Ddr2 is Expressed During Calvarial Bone Regeneration

We next examined *Ddr2* expression during calvarial bone regeneration using a *Ddr2-lacZ* reporter mouse line (*Ddr2*$^{+/lacZ}$ mice). Prior to injury, LacZ was expressed in periosteum and select marrow cells (Figure 2.2A). At 3 days post-surgery, LacZ positive cells were observed at the defect margin (Figure 2.2 B) and, by 1 week, were present throughout the defect area (Figure 2.2 C). As the defect continued to heal and new bone formation was initiated (2 weeks post-surgery), strong *Ddr2* expression continued to be observed throughout the defect area , and some of the LacZ+ cells were lining the newly formed bone in the defect margin as well as center (Figure 2.2 D arrowhead). This expression continued in areas still actively forming new bone at 4 and 6 weeks (Fig 2.2 D-F). Note that, unlike wildtype mice that had totally healed the defect after 4-6 weeks, healing was still incomplete in *Ddr2*$^{+/lacZ}$ mice at these later times probably due to *Ddr2* haploinsufficiency. Note that no X-gal staining was seen in calvarial defects from WT littermates,

confirming the specificity of the X-gal staining (Figure 2.2 G). From these results, we conclude that Ddr2 is expressed throughout the period of calvarial defect healing where it is necessary to support injury repair, and its expression varies depending on the healing stage.

Fate Mapping of Ddr2+ Cells

To determine the fate of DDR2^{+} cells and their progeny during calvarial defect healing, an SSD was generated in *Ddr2-Cre^{ERT}; Tdtomato* mice treated with tamoxifen for 3 consecutive days prior to surgery. TdTomato+ cells were then followed for up to 2 weeks (Figure 2.3). Similar to the localization of LacZ-positive cells in Figure 2.2, in unoperated control calvaria, TdTomato^{+} cells were located mainly in the periosteum and marrow of calvaria (Figure 2.3 B). As early as 3 days post-surgery, TdTomato^{+} cells had migrated into the defect area presumably from the adjacent periosteum (Figure 2.3 C). These cells or their progeny persisted in the defect site at two weeks post-surgery where they were associated with the surface of newly formed bone and in the middle of the defect (Figure 2.3 D). Thus, our results indicate that Ddr2 positive cells can rapidly respond to injury by migrating into the defect site where they support injury repair.

Deletion of Ddr2 Decreases Cell Proliferation

To investigate the cellular response to injury, we analyzed cell proliferation in the calvarial SSD region. We performed EdU incorporation analysis, which utilizes EdU to label newly synthesized DNA and applies a “click” chemistry reaction to detect the thymidine analog in native tissues using fluorescence [25]. Three days post-surgery, *Ddr2$^{slie/slie}$* and WT mice were injected with EdU and sacrificed 4 hours after injection. As shown in Figure 2.4, DNA synthesis and, by inference, cell proliferation was reduced in defects from *Ddr2$^{slie/slie}$* mice by approximately 65

percent. These results suggest that Ddr2 is required for proliferation of cells at the injury site involved in regeneration of the calvarial defect.

Preosteoblast Markers are Reduced During Bone Regeneration.

Bone regeneration requires differentiation of mesenchymal stem cells to osteoblasts. To determine if Ddr2 status affected osteoblast differentiation during SSD healing, we measured osteoblast differentiation markers by immunofluorescence in 1-week post-surgery SSDs. Osterix/Sp7 (Osx), which is critical for osteoblast differentiation and bone formation [27], was significantly decreased in Ddr2- deficient mice (Figure 2.5 A and B). In addition, phosphorylation of RUNX2, which is associated with osteoblast activity) and bone formation [26, 27], trended toward a decrease in Ddr2- deficient mice (p = 0.074, Figure 2.5 C and D). Gli1, which is a skeletal stem cell marker, did not significantly decrease in this study. Together, these results suggest that DDR2 is required for osteogenic differentiation during bone regeneration, but may not control levels of skeletal stem cells in the defect site.

Discussion

Bone ECM plays important roles not only in bone development and maintenance, but also in the healing of bone injuries. Type I collagen is the core structural protein in the bone where collagen-mediated signaling is critical for tissue differentiation and mineralization [28-30]. Previous studies have been largely focused on collagen-binding integrins as mediators of the response to this ECM component. Knockout of individual collagen-binding integrins in mice resulted in relatively mild phenotypes[31-33] suggesting the involvement of other receptors in mediating collagen signaling

DDR2, a non-integrin collagen activated receptor tyrosine kinase, is critical for skeletal bone growth and development. However, its role in calvarial bone regeneration has not been previously explored. Our analysis of *Ddr2*-deficient mice indicates that proper DDR2 signaling is required for optimal calvarial bone regeneration. Whether measured by μCT or histology, bone regeneration was clearly reduced in *Ddr2*$^{slie/slie}$ mice. Complete bridging occurred in WT controls after as little as 4 weeks while in *Ddr2*$^{slie/slie}$ mice some bone formation was seen on the margins of the defect, but complete bridging was never observed, even after 12 weeks (Figure 2.1 B-E). Quantitation of defect bone area by μCT revealed that, as early as 2 weeks post-surgery, there is a significant decrease of bone formation in the *Ddr2*$^{slie/slie}$ mice when compared with WT (Figure 2.1 F). Four weeks post-surgery, there was a 50% reduction in new bone volume in *Ddr2*$^{slie/slie}$ mice versus controls and bone formation appeared to plateau after 6 weeks with no additional bone being present in 12 week samples.

Using LacZ staining, we localized *Ddr2* expression during calvarial bone regeneration. LacZ positive cells, which were present mainly in the periosteum of calvaria before surgery, were

expanded during the calvarial bone regeneration at the defect site (Figure 2.2). Also, LacZ positive cells were lining the new formed bone in the defect margin as well as center (Figure 2.2 D). To determine the fate of DDR2+ cells and their progeny during bone regeneration, we generated calvarial defects in *Ddr2-Cre^{ERT}; Tdtomato* mice and followed TdTomato+ cells for up to 2 weeks. Consistent with the *Ddr2-lacZ* studies, in calvaria without defects, TdTomato+ cells were mainly found in the periosteum with some additional positive cells in the bone marrow (Figure 2.3 B). However, 3 days post-surgery, TdTomato+ cells were detected in the defect area (Figure 2.3 C). The TdTomato+ area further expanded at the 2-week time point (Figure 2.3 D). Our result indicate that Ddr2 positive cells can respond to injury rapidly and expand to support injury repair. It has been reported that skeletal progenitor cells involved in cranial repair mainly resided in the periosteum, dura and the sutural mesenchyme [34], the same areas where we see DDR2 expression. In addition to skeletal progenitor cells, it is possible that DDR2 is expressed in other skeletal cells, such as in preosteoblast and osteoblast. These cells were present in the defect area 2 weeks post-surgery when active bone formation started and positive cells were detected lining newly formed bone in the middle of the defect as well as defect margins.

We demonstrated that Ddr2 is required for normal cell proliferation during calvarial defect regeneration. We showed that *Ddr2$^{slie/slie}$* mice have less EdU positive cells when compared to WT (Figure 2.4). This result is consistent with other studies that showed the *Ddr2*-deficient mice exhibited shortening of long bones and defective wound healing caused by a reduced proliferative response of chondrocytes and skin fibroblasts, respectively [35-38]. This is could be one of the explanations for the defective bone regeneration seen in *Ddr2*-deficient mice.

Similar to bone injury healing, *Ddr2*-deficient mice exhibit delayed dermal wound healing, indicating that this receptor is also necessary for regeneration of skin[37]. A possible cause of

impaired calvarial bone regeneration is a defect in osteoblast differentiation and/or function [16]. Our group and others showed that DDR2 plays an essential role in osteoblast differentiation [16, 39]. Bone changes in *Ddr2$^{slie/slie}$* mice were attributed to decreased bone formation due to defective osteoblast function [16]. Also, calvarial cells from *Ddr2$^{slie/slie}$* mice showed defective osteoblast differentiation in cell culture [16]. Consistent with our previous finding, we showed that preosteoblastic markers, Osx and p-RUNX2, were decrease during calvarial defect regeneration in *Ddr2$^{slie/slie}$* mice. These findings support the hypothesis that DDR2 is required for osteoblast differentiation and function. Bone healing is a well-organized process that includes multiple biological events and factors. More investigations are required to further understand DDR2 functions in bone regeneration.

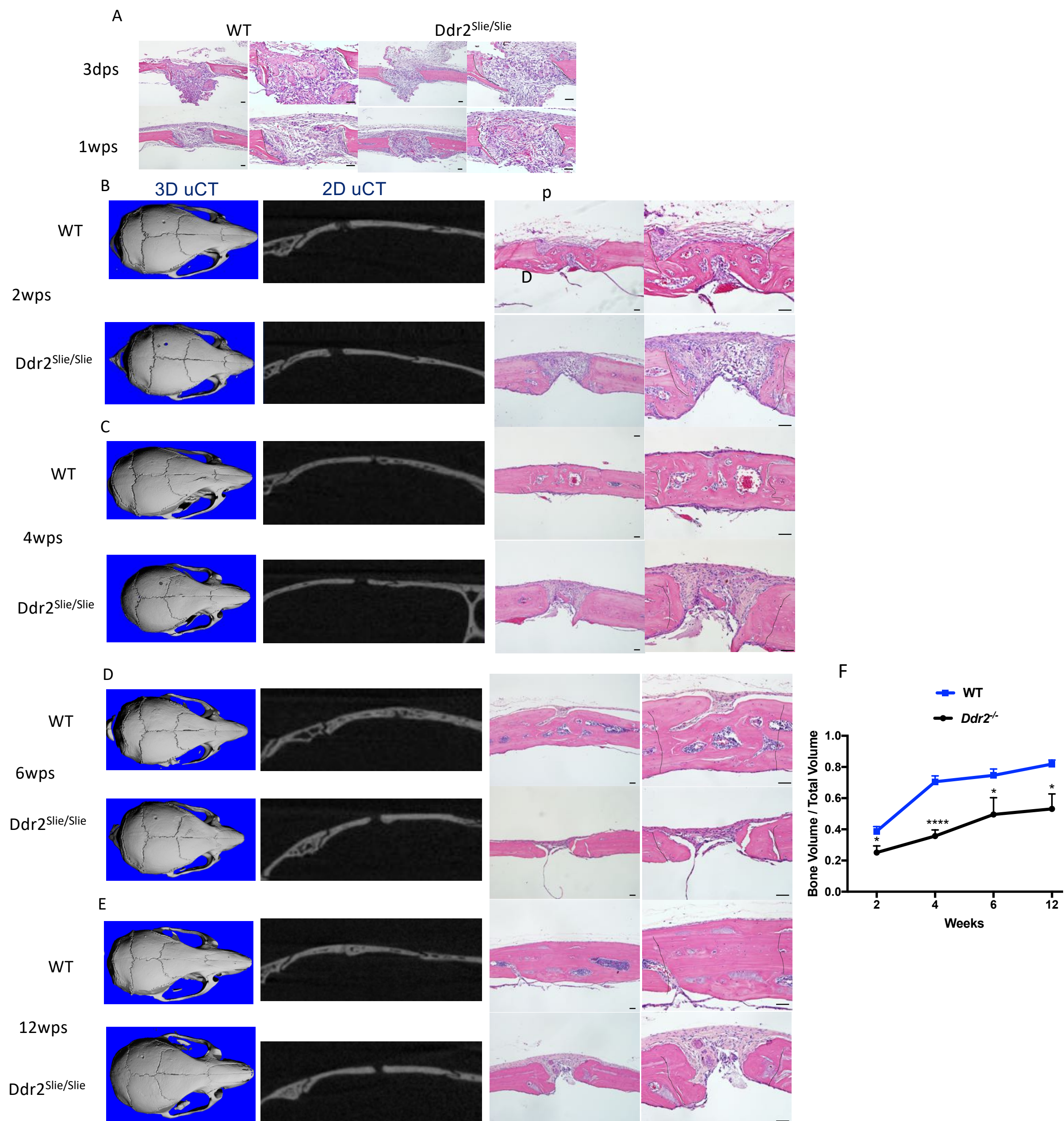

Figure 2.1 ***Ddr2$^{slie/slie}$*** **mice fail to heal a calvarial SSD**. **(A)** H&E staining 3 days and 1 week post-surgery of SSD for WT and Ddr2$^{slie/slie}$ mice. **(B-E)** Representative micro-CT and histological images of the defect area showing amount of bone regeneration. **(F)** Measurements are shown for regenerated bone volume/total volume. n = 6-8. dps= day post-surgery, wps = weeks post-surgery *P<0.05, ****P<0.0001. Scale bar 50um. Dash lines are estimated defect margin. P: periosteum. D: Dura

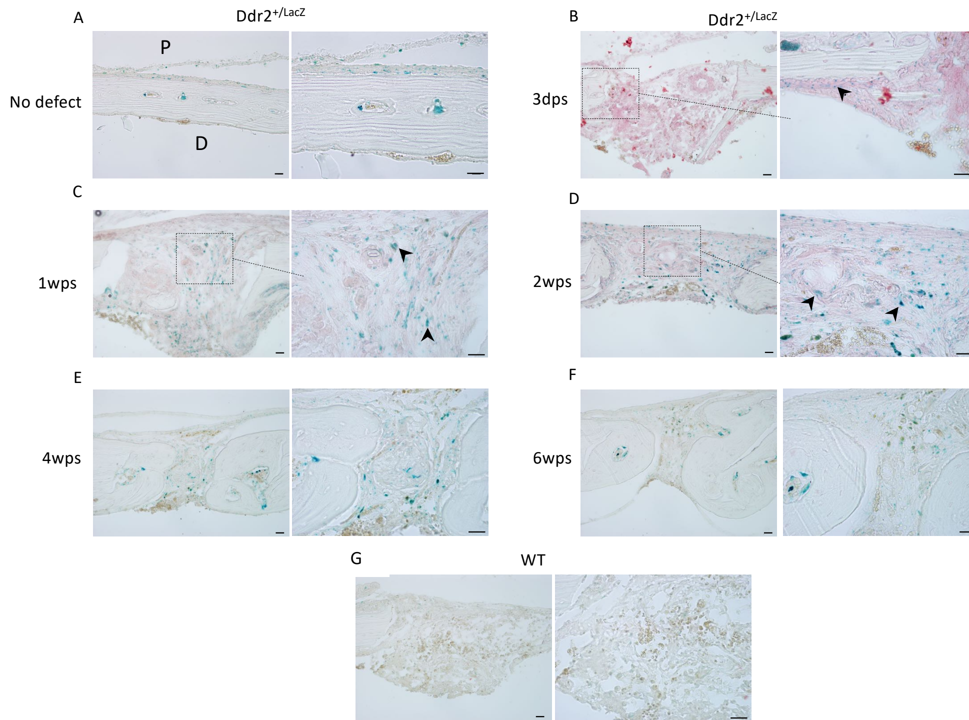

Figure 2.2 Localization and expression of DDR2 during calvarial bone regeneration. X-Gal staining of *Ddr2+/LacZ* mouse calvaria. **(A)** No defect. **(B)** At 3dps, Ddr2 expression is observed in the dura and the margin of the defect (arrowhead). **(C-F)** X-gal staining found in the defect at 1wps, markedly increase at 2wps, and continue to exist at 4wps and 6wps. Notice the arrowhead in **(D)** pointed to a LacZ+ cell lining the newly formed bone. **(G)** No X-Gal staining in the Ddr2+/+ control. Scale bar 25um. P: periosteum. D: Dura

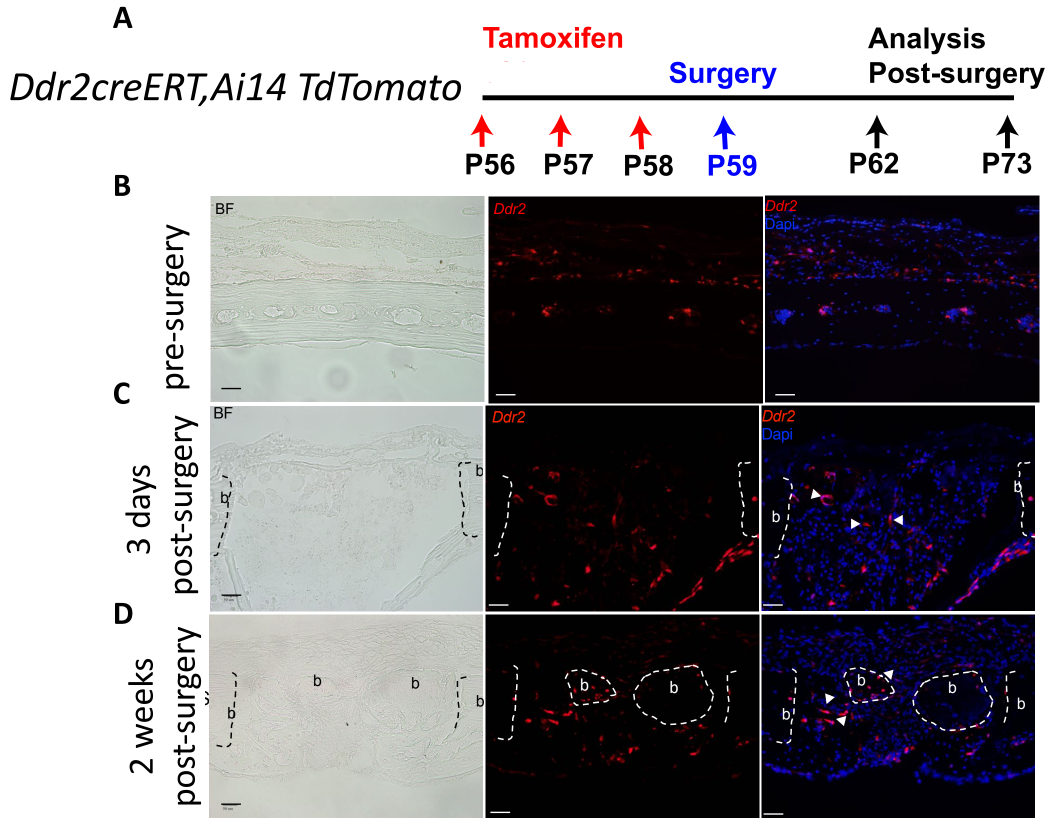

Figure 2.3 Ddr2 positive cells migrate to the calvarial defect. (A) Time scheme for Ddr*2cre*^{ERT}, *Ai14 TdTomato* mice tamoxifen injections and calvarial SSD surgery. **(B)** A 10um thickness frozen section of mouse calvaria shows the distribution of Ddr2 positive cells without defect. **(C)** Three days post-surgery calvarial SSD shows that Ddr2 positive cells have migrated into the defect site. **(D)** Two weeks post-surgery of calvarial defect shows expansion of DDr2 positive cells or their progeny at the defect site, with some of them lining the newly formed bone in the defect. White arrowhead points to Ddr2 positive

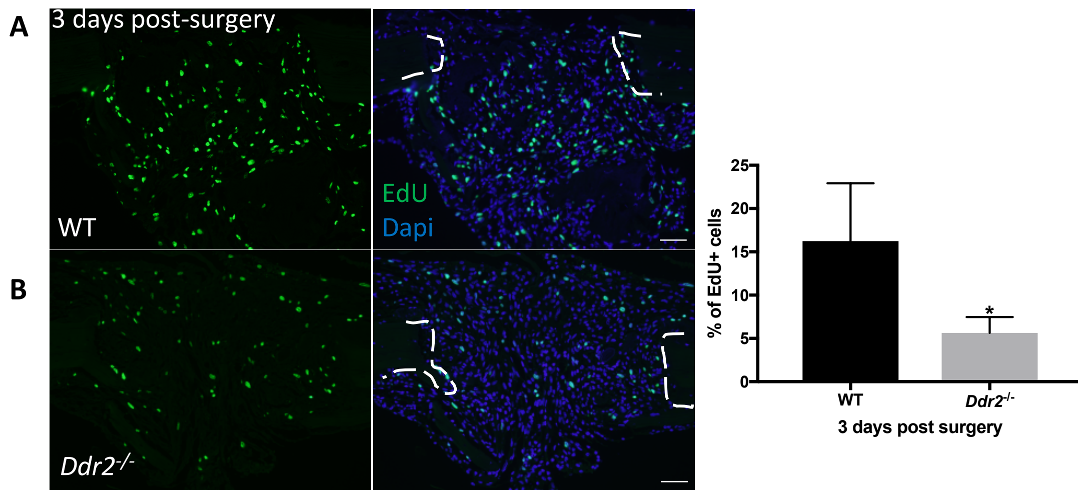

Figure 2.4 DDR2 is required for cell proliferation during SSD healing. (A and B) Paraffin sections of calvarial defects from *Ddr2$^{-/-}$* and WT mice calvarial defect showed EdU+ cells in green and cell nuclei in blue (DAPI). **(C)** Quantification of EdU+ cells in both and WT mouse calvarial defect sections. * $P<0.05$. n = 4. Scale bar 50um

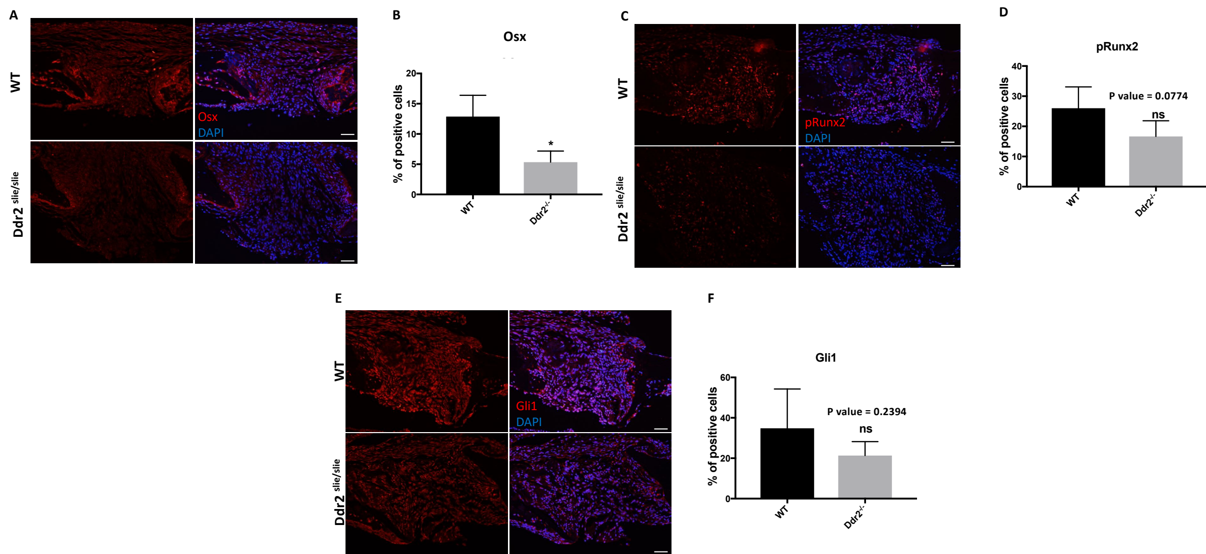

Figure 2.5 *Ddr2* deficiency results in decrease osteogenic differentiation markers during bone regeneration. Immunofluorescence staining (IF) 1-week post-surgery. **(A, C, and E)** IF for Osterix, p-RUNX2 and Gli1. **(B, D and F)** Percentage of positive cells over total cells. *P<0.05. Scale bar 50um. ns= not statistically significant, n = 4

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Chapter 3

Role of Discoidin Domain Receptor 2 in Tibial Fracture Healing

Abstract

Skeletal fractures are the most common large-organ, traumatic injuries in humans. Defective fracture healing is a major public health problem with approximately 10% of all fractures failing to heal properly. The fracture healing process requires extracellular matrix (ECM)-cell interactions mediated by cell surface receptors. Discoidin domain receptor 2 (DDR2), a non-integrin collagen-activated receptor tyrosine kinase, is an important ECM receptor in bone that is essential for normal skeletal growth and development. Here, we examine whether DDR2 is also required for fracture healing using $Ddr2^{slie/slie}$ mice, which contain a spontaneous 150 kb deletion in the *Ddr2* locus to produce an effective null. We first defined the expression pattern of Ddr2 during fracture healing using Ddr2-LacZ knock-in mice and $Ddr2\text{-}Cre^{ERT}$*; Tdtomato* mice. LacZ expression was first detected in select regions of the fracture site 2- and 5-days post fracture and expanded throughout the fracture callus after 1.5 and 3 weeks. Similar results were observed in $Ddr2\text{-}Cre^{ERT}$*; Tdtomato* mice. Ddr2+ cells and their progeny began to expand in the developing fracture callus 1.5-weeks post-fracture and continued to expand after 3 weeks. $Ddr2^{slie/slie}$ mice exhibited significantly less fracture union than wild-type (WT) mice, and this defect was related to a decrease in cartilage formation as measured by safranin O staining. In addition, mutant mice developed significantly less callus tissue at 6 weeks post-fracture. To examine the role of DDR2 in skeletal progenitor cells (SPCs), we purified PDGFRα^{+} CD51^{+} SPCs from bone marrow of

Ddr2$^{fl/fl}$ mice using FACS followed by treatment with AdCre. *Ddr2* deletion resulted in defective osteoblast differentiation and accelerated adipogenesis. On the other hand, overexpression of DDR2 in a mesenchymal cell line (ST2 cells) increased osteoblast differentiation. Together, these results indicate that DDR2 is necessary for optimal fracture healing and this requirement may be explained in part by effects of DDR2 on SPC function.

Introduction

Bone loss due to trauma, neoplasia, congenital defects or periodontal disease is a major cause of disability and human suffering. For example, delayed healing or non-union, which occurs in 5-10% of all fractures and 20% of high impact fractures, prolongs patient morbidity, causes substantial pain and is associated with a significantly higher rate of healthcare resource use and per patient cost [1, 2]. The repair of a fractured long bone in adults recapitulates many of the steps of endochondral ossification, which occurs during embryonic bone development [3]. This fracture healing process, where sequential cellular and molecular events take place to generate new bone, has been thoroughly studied in humans as well as rodent models [4, 5, 6, 7]. There is increasing evidence that during bone development and regeneration the extracellular matrix (ECM) profoundly influences major cellular programs, such as adhesion, migration, proliferation, and differentiation, via activation of cell surface ECM receptors [8, 9]. A clear understanding of these ECM receptors will facilitate the development of new bone regeneration strategies.

Bone and cartilage ECM are organized around type I and type II collagen fibers, respectively[3]. ECM enhances cell recruitment during bone regeneration through cell surface receptors. Collagen binding integrins are the main ECM receptor known to be important in the crosstalk between cells and the ECM. Integrin α1β1 is a collagen receptor expressed on many mesenchymal cells [3, 4]. Integrins have clear roles in bone formation and regeneration, but they

don't explain the entire response of bone cells to ECM since bone-specific knockout of individual integrins only partially prevents bone formation and fracture healing [3, 10, 11, 12]. Mice deficient in α1 integrin have no gross structural defects as well as show similar fracture union to WT mice [3]. The discoidin domain receptors 1 and 2 (DDR1 and DDR2) are another important class of ECM receptors [13]. Unlike integrins, DDRs have intrinsic tyrosine kinase activity and are selectively activated by triple-helical collagens [14, 15].

Both DDR1 and DDR2 are involved in multiple physiological processes, such as development, ECM turnover, growth regulation and cancer [16, 17]. In bone, several lines of evidence suggest an important role for DDR2. In humans, *DDR2* mutations cause spondylo-meta-epiphyseal dysplasia (SMED), a skeletal disorder associated with dwarfism, bowing of long bones, craniofacial abnormalities, short fingers, and abnormal calcifications [16, 18]. Also, in a Han Chinese population, polymorphisms in *DDR2* are associated with low bone mineral density (BMD) and fracture risk [19]. Consistent with the human phenotype, mice with a spontaneous *Ddr2* mutation, called smallie mice ($Ddr2^{slie/slie}$), have a SMED-like phenotype characterized by dwarfism and reduction in total bone mineral density [16,20]. Furthermore, detailed analysis of the bone phenotype in $Ddr2^{slie/slie}$ mice revealed a dramatic reduction in mineral density of the cranial, axial, and appendicular skeleton due to reduction in osteoblast activity and bone formation in the absence of changes in resorption [16]. Bone changes were accompanied by a large increase in marrow fat [16].

DDR2 is a key regulator of bone growth that controls several aspects of the process. However, it is not known if DDR2 is required for bone regeneration. Here, we investigate the functional importance of DDR2 in tibial fracture healing using a *Ddr2*-deficient mouse model and also define the expression and distribution of DDR2 during the fracture healing process. These

findings provide new insights into the role of cell-ECM interactions during fracture healing and may have important implications for understanding and enhancing bone regeneration.

Materials and Methods

Mice

Generation and genotyping of *Ddr2*$^{slie/slie}$ mice and Ddr2-LacZ (*Ddr2*$^{+/LacZ}$) knock-in mice, where the bacterial LacZ gene is knocked into the Ddr2 locus, was previously described [16, 20, 21]. Studies also used *Ddr2Cre*^{ERT} mice recently developed by Dr. Barry Greenberg (UC San Diego). These mice contain Cre^{ERT} knocked in-frame into exon 2 of *Ddr2*. *Ddr2Cre*^{ERT} mice were crossed with Ai14 TdTomato mice provided by Dr. Noriaki Ono (University of Michigan, Ann Arbor) [22]. TdTomato mice harbor a targeted mutation of the Gt (ROSA) 26Sor locus with a loxP-flanked STOP cassette preventing transcription of a CAG promoter-driven red fluorescent protein variant (tdTomato); TdTomato is expressed following Cre-mediated recombination. *Ddr2*$^{fl/fl}$ mice in which exon 8 of the *Ddr2* gene is flanked by two LoxP sequences were generated from a "knockout-first" ES cell clone *Ddr2*$^{tm1a(EUCOMM)Wtsi}$(EPD0607__B01) obtained from European Mutant Mouse Repository. To generate a *Ddr2* knockout first mouse line, ES cell transplantation was performed by the University of Michigan Transgenic Model core. To generate *Ddr2*$^{fl/fl}$ mice, knockout first mice were crossed with FLPO mice to remove LacZ-Neo elements.

Tibia Fracture Model

All mouse experimental procedures conformed to standards for the use of laboratory animals and were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Michigan. The fracture method was previously described in detail [23, 24]. Eight-week-old mice were maintained with general anesthesia (2% isoflurane/100% O_2) during the procedure

and received Buprenorphine (0.02mg/kg ip) and Carprofen (5mg/kg sc) for analgesia. Under sterile conditions, a small incision was made medial to the tibial tuberosity. A 26-gauge needle was used to bore a small hole into the medial-proximal cortex. The needle was withdrawn and replaced with a sterile 0.009-inch diameter stainless steel wire (McMaster-Carr) that was passed through the marrow space and compacted firmly into the distal tibial bone. The wire was trimmed flush with the femoral notch, the patella reduced, and the capsule and muscle fascia were closed using resorbable suture. Skin closure was performed using glue. While still anesthetized, mice were placed on a custom fabricated guillotine device, with the femoral mid-shaft placed on top of a two-point support surface. An anvil striker was placed on the antero-medial surface of the tibia at approximately mid-shaft. A weight of ~290 g was dropped from 8 cm to fracture the bone. Tibial "splints" were placed with surgical tape to prevent initial rotational instability to the fracture site over the first 48 hours [25].

Micro–computed tomography analysis

Following euthanasia, carefully excised fracture repair constructs were scanned using an eXplore Locus SP microCT system (GE Healthcare). All specimens were scanned in water using the following parameters: voltage 80 kVp; current 80 μA; exposure time 1600 ms; voxel size in the reconstructed image 18 μm, isotropic. The data were processed and analyzed using MicroView (v2.1.2 Advanced Bone Application; GE Healthcare Preclinical Imaging). The fracture union was evaluated using a modified Radiographic Union Score for Tibia (mRUST) [26]. First, the image was reoriented in the MicroView so that the anterior-posterior and longitudinal axes were aligned with the principal image axes. Each tibial fracture on each of 4 cortices on anteroposterior (A-P) and lateral (Lat) were evaluated as follows: 1, no callus; 2, callus present; 3, bridging callus; 4,

remodeled and fracture line not visible. The minimum mRUST score is 4 and the maximum is 16. To avoid examiner bias, the genotype of mice was not known during analysis.

Safranin O staining

Fractured tibiae were fixed in 4% paraformaldehyde, decalcified in 10% ethylenediaminetetraacetic acid, embedded in paraffin, and sectioned longitudinally with a microtome. Sections were stained with 0.001% fast green and 0.1% safranin O. Images were acquired with an Eclipse E800 microscope (Nikon) and safranin O positive area were measured in a randomized and blind manner using the Bioquant Osteo software V17.2.6 (Bioquant Image Analysis Corp., Nashville, TN)

Detection of β-gal (LacZ) expression

Samples dissected from heterozygous *Ddr2-LacZ* (*Ddr2*$^{+/LacZ}$) mice were processed for X-gal staining using standard procedures. Briefly, 10 µm frozen sections were stained with freshly prepared X-gal solution and counterstained with Vector® Nuclear Fast Red.

Lentiviral Transduction

Full length human *DDR2* cDNA was subcloned into PLentiloxRSV-Puro using restriction sites XbaI and EcoRI. LentiDDR2 overexpression was transfected into 293T cells. Supernatant were collected 4 days after transfection. ST2 cells were plated at density 10,000 / cm 2 in 35 mm dishes. 1 ml Lentivirus supernatant was used to transduce ST2 overnight. 200 ug/ ml Puromycin was used to select DDR2 stable transduced ST2 cells for 2 weeks. 50 ug/ ml puromycin was used to maintain DDR2 stable expression ST2 cells.

Flow Cytometry

For PDGFRα CD51 sorting, bone marrow stomal cells (BMSCs) were harvested from tibia and femur of 12 weeks *Ddr2*$^{fl/fl}$ mice by flushing the marrow cavity with digestion buffer (2mg/mL

Collagenase IV and 3mg/mL Dispase in 1xPBS) using 21G needle. Three digestion steps were carried out at 37°C with periodic agitate ion to obtain a single cell suspension. After each period of digestion, cells in suspension were collected into ice-cold staining buffer (1X PBS, 0.5% BSA, 2mM EDTA). For staining, cells were resuspended in staining buffer at a concentration of 1×10^6 cells/30ul in a solution containing the following antibodies: PDGFRα/CD140a-PECF594 (BD, clone APA5, 1:100) and CD51-PE (ebioscience, Clone RMV-7, 1:100). After staining, all samples were washed twice with staining buffer and resuspended in staining buffer with DAPI (1:10,000) for analysis and sorting. Analysis was carried out on an LSRII Fortessa flow cytometer (BD), sorting was performed on a FACSAria II (BD). Results were analyzed with FlowJo (v10.0.7) software [27]. The PDGFRα+ CD51+ BM cells were plated at a density of 5×10^4 cells/cm^2 for adenovirus infection.

Cell cultures and *in vitro* differentiation

ST2 cells were obtained from Dr. Kurt Hankenson (University of Michigan, Ann Arbor) [28]. Osteoblast differentiation was induced in ST2 and sorted BMSCs cells from *Ddr2*$^{fl/fl}$ mice by growth in α-MEM/10%FBS containing 50 µg/ml ascorbic acid, 10 mM β-glycerophosphate and 3 µM Chrion 99021 (Cayman Chemicals, Ann Arbor, MI) in ST2 only [16]. For adipogenesis, cells were grown for 2 days in alpha minimal essential medium containing 10% FBS, insulin (5 µg/ml), dexamethasone (1 µM), IBMX (500 µM), and troglitazone (5 µM), followed by growth in medium containing troglitazone (5 µM) only for up to 9 days [16, 29]. For gene expression analysis, total mRNA was isolated using TRIzol (Invitrogen), cDNA was synthesized, and quantitative RT-PCR was performed on the following mRNAs:*, Ddr2, Runx2, Ibsp, Bglap, Paprγ, Cebp* and *Fabp4* mRNA.

Statistical Analysis

All data was analyzed using the GraphPad Prism software version 7.0e, La Jolla California USA. Values were reported as mean ± SD. Student's t test was used for statistical comparison between the two experimental groups. Differences were considered significant at $P < 0.05$. A sample size of 6 mice (including both sexes) was used for experiments.

Results

DDR2 is up-regulated after bone fracture

Fracture healing consists of a well-orchestrated series of events defined by distinct phases of anabolic (callus expansion) and catabolic (callus remodeling) responses to the injury [27, 30, 31]. Resident MSCs in the skeleton (from the periosteum and bone marrow) provide the major source of progenitors for the repair process [27, 32, 33, 34]. To explore a possible role for *Ddr2* genes in the repair process, the expression of DDR2 was examined following fracture injury of the tibia using *Ddr2*$^{+/LacZ}$ knock-in mice (contain bacterial LacZ knocked into exon 8 of the Ddr2 locus)[21] (Figure 3.1). Prior to injury, LacZ was expressed mainly in the periosteum (Figure 3.1 A and B). As early as 2 days post-fracture, we observed LacZ staining in select condensed regions of cells within the fracture callus (Figure 3.1C-E). The LacZ-positive cell area progressively expanded as the fracture healing process progressed (5 days post-fracture, panels G-I; 1.5-weeks post-fracture, panels J-O; 3 weeks post-fracture, panels P-W). Initially, positive cells appeared to be associated with mesenchymal condensations and early chondrocytes (day 2 and 5) with subsequent association with putative osteoblasts on the bone surface after 1.5 and 3 weeks. From these results, we conclude that DDR2 is expressed throughout the fracture healing process, and its expression varied

depending on the healing stage. In addition, DDR2 may be expressed in skeletal progenitor cells, chondrocytes and osteoblast as healing proceeds.

To further trace the fate of Ddr2 positive cells and their progeny during fracture healing, we induced *Ddr2-Cre^{ERT}; Tdtomato* mice at 2 months of age with tamoxifen and after one day performed fracture surgery (Figure 3.2 A). Numbers of animals and time points were limited in this experiment due to post-surgery mortality. However, results clearly showed that 1.5 week post-fracture, Ddr2+ cells had expanded at the fracture callus (Figure 3.2 C-E). Interestingly, 3 weeks post-fracture a dramatic increase in Ddr2+ cells was seen throughout fracture callus area (Figure 3.2 F-H). Thus, our results indicate that Ddr2 positive cells and their progeny can respond to injury rapidly and expand to support regeneration.

Ddr2 loss-of-function mice display defects during fracture healing

To assess the role of *Ddr2* in fracture repair, tibial fractures were made in wild type and *Ddr2$^{slie/slie}$* mice (referred to as *Ddr2$^{-/-}$*). Histology sections, x-rays and micro-computed tomography (µCT) scans performed at several time points following fracture injury revealed abnormal fracture healing in *Ddr2* mutants. During the early response to injury, 1.5 weeks post-fracture (WPF), there were no apparent differences in the fracture between mutants and WT (Figure 3.3 A). However, safranin O staining revealed a defect in cartilage formation in *Ddr2* mutant mice (Figure 3.3 B and C). By mid-stage healing (3 WPF) *Ddr2* mutant animals demonstrated a delay in fracture gap union. This was manifested as a reduction in mRUST score reflecting fewer bridged cortical sites in *Ddr2* mutants, but no difference in the callus volume (Figure 3.3 D-F). During remodeling stages of healing (6 WPF), two phenotypes were observed in *Ddr2* mutant animals. First, a significant number of animals exhibited non-union fractures; second,

the callus volume was significantly smaller in the *Ddr2* mutants (Figure 3.3 G-I). Thus, DDR2 is required for normal fracture healing.

Ddr2 is enriched in purified skeletal progenitor cells and is required for differentiation to osteoblasts.

We purified skeletal progenitor cells (SPCs) from whole marrow of *Ddr2*$^{loxp/loxp}$ mice by FACS using stem cell markers, PDGFRα and CD51 [36] (Figure 3.4 A). This population has properties of SPCs. It contains most of the colony forming activity of marrow, has high self-renewal capacity, gives rise to bone, supports hematopoiesis and exhibits multipotency (forms osteoblasts, adipocytes and chondrocytes)[36, 37]. In addition, SPCs (from the periosteum and bone marrow) provide the major source of progenitors for the repair process [27, 32, 33, 34]. *Ddr2* mRNA in this population was enriched approximately 10- fold versus total marrow nucleated cells (Figure 3.4 B). Cells were treated with control or Cre-expressing adenovirus and grown in osteogenic and adipogenic medium. AdCre treatment (*Ddr2* knockout) almost completely eliminated *Ddr2* mRNA, clearly reduced osteoblast differentiation markers *Runx2, bone sialoprotein, Bglap,* and increased adipogenesis markers *Paprγ, Cebp* and *Fabp4* as measured by mRNA expression (Figure 3.4 C and D).

As further evidence that DDR2 promotes osteoblast differentiation of progenitor cells, we transduced a mesenchymal cell line (ST2 cells) with a lentivirus vector encoding full-length Ddr2 cDNA. This increased DDR2 protein levels approximately 2-fold and increased osteoblast differentiation as measured by alizarin red staining and induction of the osteoblast markers, *bone*

sialoprotein, and *osteocalcin* (Figure 3.5). Together, these results provide strong evidence that DDR2 stimulates osteoblast differentiation of skeletal progenitor cells.

Discussion

Type I collagen, the core structural protein in bone, plays an important role during tissue differentiation and mineralization [37, 38]. Previous studies have been largely focused on collagen-binding integrins as mediators of the response to this ECM component. Knockout of individual collagen-binding integrins in mice resulted in relatively mild phenotypes [39, 40, 41], however, suggesting the involvement of other receptors in mediating collagen signaling. DDR2, a non-integrin collagen activated receptor tyrosine kinase, is critical for skeletal bone growth and development. Our understanding of DDR2 in the skeleton is largely limited to the analysis of bone growth and development [16]. The aim of this study is to determine the function of DDR2 in the healing of tibial fractures.

Using LacZ staining in $Ddr2^{+/LacZ}$ mice, we localized DDR2 expression during fracture healing. In unfractured bones, DDR2 was expressed in the periosteum (Figure 3 A and B). As early as 2 days post-fracture, when a hematoma is present and an acute inflammatory immune response is initiated, we detected a few LacZ+ cells in select regions of the fracture callus (Figure 3.1 C-E). Five days post fracture, at the end of the hematoma stage and at the beginning of the soft callus stage, we see more LacZ+ cells in the fracture callus possibly associated with chondrocytes (Figure 3.1 G-I). In the soft callus stage 1.5-weeks post- fracture, a further expansion of LacZ+ cells were observed throughout the callus in apparent association with chondrocytes (Figure 3.1 J-O). At 3-weeks post fracture, which is a hard callus stage, we see strong Ddr2 expression throughout the

callus in association with newly formed osteoblasts on the woven bone surface (Figure 3.1 P-W). These results indicate that Ddr2-expressing cells expand dramatically throughout fracture healing in progressive association with skeletal progenitor cells, chondrocytes and osteoblasts.

To determine the fate of DDR2+ cells and their progeny during fracture healing, we conducted fractures in *Ddr2-Cre^{ERT}; Tdtomato* mice and followed TdTomato+ cells for up to 3 weeks. Consistent with the Ddr2-lacZ studies, in unfractured bones, TdTomato+ cells were mainly found in the periosteum with some additional positive cells in the bone marrow (Figure 3.2 B). However, 1.5 weeks post-fracture, TdTomato+ cells were detected in the fracture callus (Figure 3.2 C-E). The TdTomato+ area further expanded at the 3 week time point such that the label was found throughout the callus (Figure 3.2 F-H). These results are consistent with the previous one and confirm that Ddr2+ cells are involved in fracture healing. They also indicate that the small number of Ddr2+ cells in the periosteum pre-fracture have greatly expanded during fracture healing such that their progeny are found throughout the callus at 3 weeks. This localization is consistent with Ddr2 being initially expressed in an SPC population in the periosteum that subsequently expands with bone injury to provide the chondrocytes and osteoblasts necessary for bone regeneration.

Our analysis of *Ddr2*-deficient mice indicates that proper DDR2 signaling is required for optimal fracture healing. Here, we show that Ddr2 knockout results in a decrease in fracture union 3- and 6-weeks post fracture. In addition, *Ddr2*-knockout mice have lower callus volume at 6 weeks post fracture when compared to WT littermates. Furthermore, safranin O staining revealed a defect in cartilage formation of *Ddr2*-knockout mice at 1.5 weeks post fracture compared with WT littermates. These results are similar to *α1* integrin-deficient (*α1*-KO) mice, where knockout mice show diminished callus size and cartilage synthesis [3]. Moreover, faulty matrix organization,

induced by using a lathyrogenic diet to prevent collagen cross-linking, has an effect similar to that observed in *α1*-KO mice [44], which suggesting that the organization of the collagenous matrix is important for integrin and DDR2 function. A previous study showed that DDR2 and integrins may interact during collagen stimulation with DDR2 enhancing integrin-mediated cell adhesion [45].

Bone formation during adult fracture repair involves activation of SPCs, which then replicate and differentiate into osteoblasts and chondroblasts of the callus. Our results show that DDR2 is enriched in PDGFRα+ and CD51+ SPCs. This is consistent with the localization of Ddr2 in unfractured tibia where LacZ+ cells were mainly seen in the periosteum, which consist mainly of SPCs [46]. This suggests that DDR2 is a SPCs marker. Furthermore, deletion of *Ddr2* in PDGFRα+ and CD51+ SPCs *in vitro* reduced osteoblast differentiation and increased adipogenesis. Moreover, overexpression of *Ddr2* in the ST2 mesenchymal cell line increased osteoblast differentiation. This result indicates that DDR2 overexpression increases bone formation, and could be applied in tissue engineering and bone regeneration

Together, these results provide strong evidence that DDR2 stimulates osteoblast differentiation of skeletal progenitor cells. In addition, DDR2 is necessary for optimal fracture healing. DDR2 could be a possible target to stimulate bone formation and regeneration. However, further investigation is required to explain the mechanism of action of DDR2 in bone regeneration.

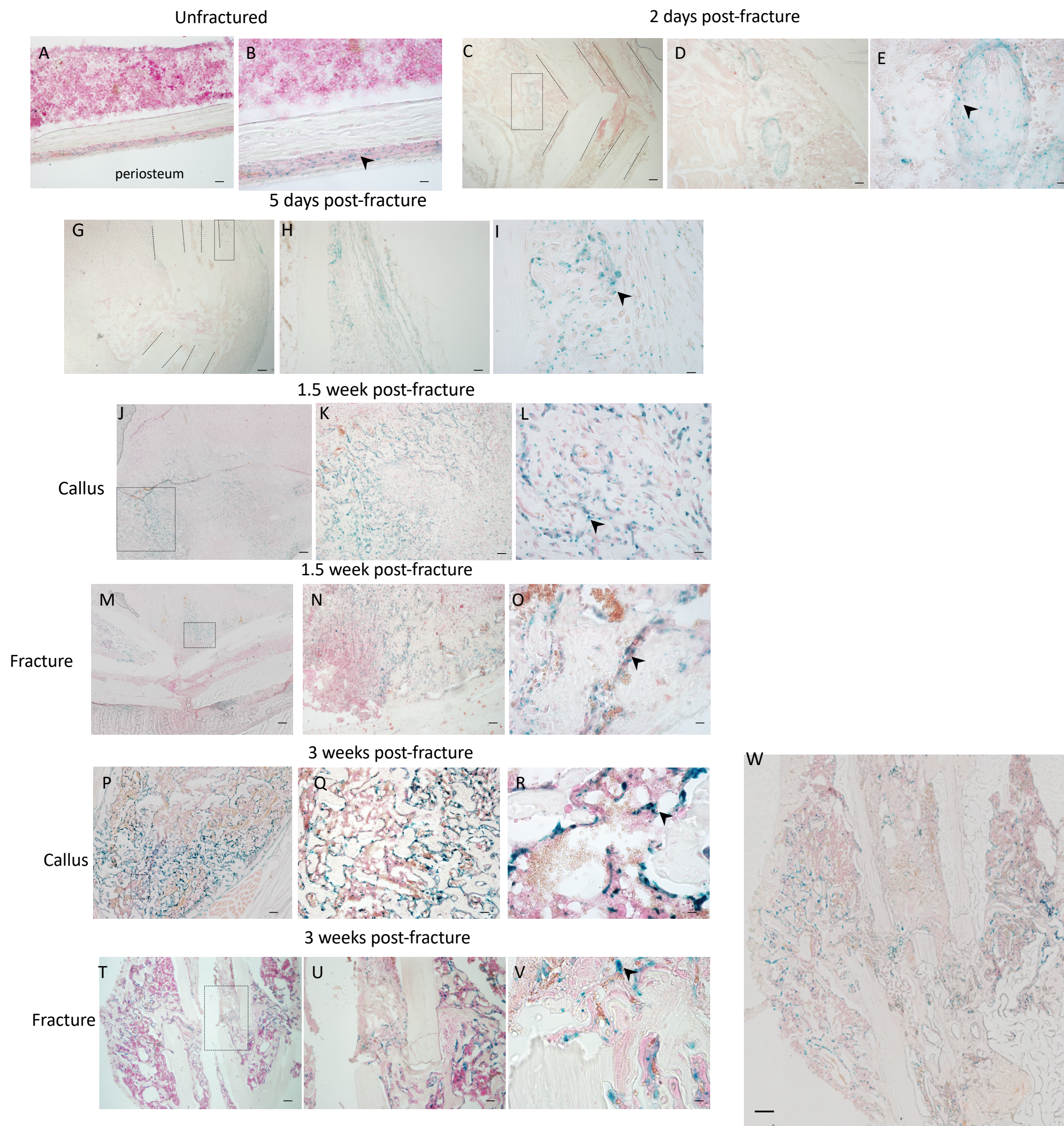

Figure 3.1 Expansion of DDR2 expression after bone fracture. X-Gal staining of *Ddr2*$^{+/LacZ}$ mice **(A and B)** Unfractured control showing periosteal staining (arrowhead). **(C-E)** At 2dpf, Ddr2 expression is first observed next to the fracture area **(G-I)** At 5dps, LacZ+ cells observed next to the bone surface. **(J-L)** Beginning callus area before reaching bone surface and **(M-O)** fracture area of 1.5wpf, notice the expanded DDR2 expression. **(P-R)** Beginning callus area before reaching bone surface and (**T-V)** fracture area of 3wpf, DDR2 strongly expressed throughout fracture callus. **(W)** Scanned image shows the expansion of Ddr2 expression at 3wps. Arrowhead= LacZ+ cells. Scale bar 50um in (B, E, I, L, O, R, and V), 100um in (A, D, H, K, N, Q and U), 200 um (C, G, J, M, P, and T) and 300 um in (W)

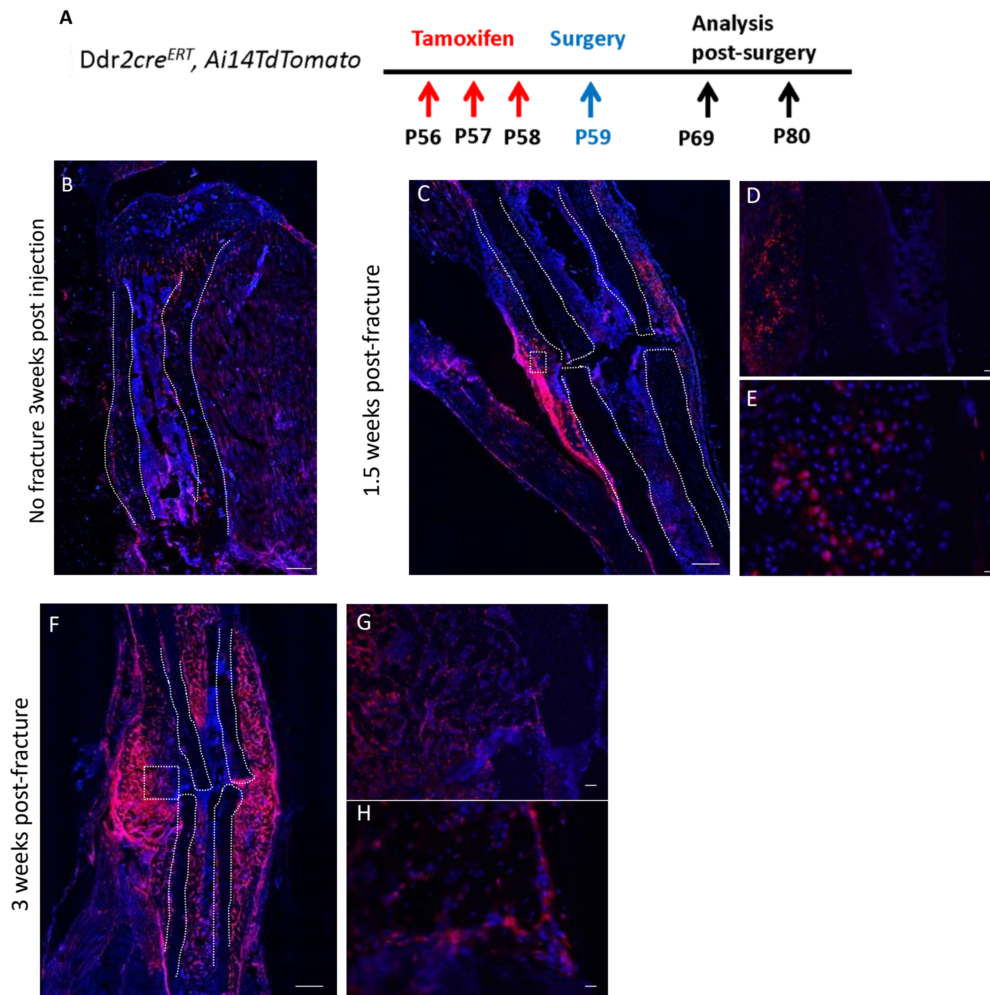

Figure 3.2 Ddr2 positive cells expanded during fracture healing. (A) Time scheme for Ddr*2cre*^{ERT}*, Ai14 TdTomato* mice tamoxifen injections and fracture surgery. **(B)** A 10um thickness frozen section of mouse tibia shows the distribution of Ddr2 positive cells without fracture. **(C-E)** Ten days post-fracture, Ddr2+ cells expanded at fracture callus. **(F-H)** Three weeks post-fracture. Ddr2+ cells highly expanded at the fracture callus. Scale bar 3000um in (B, C, and F), 200um in (D and G), and 50um in (E and H)

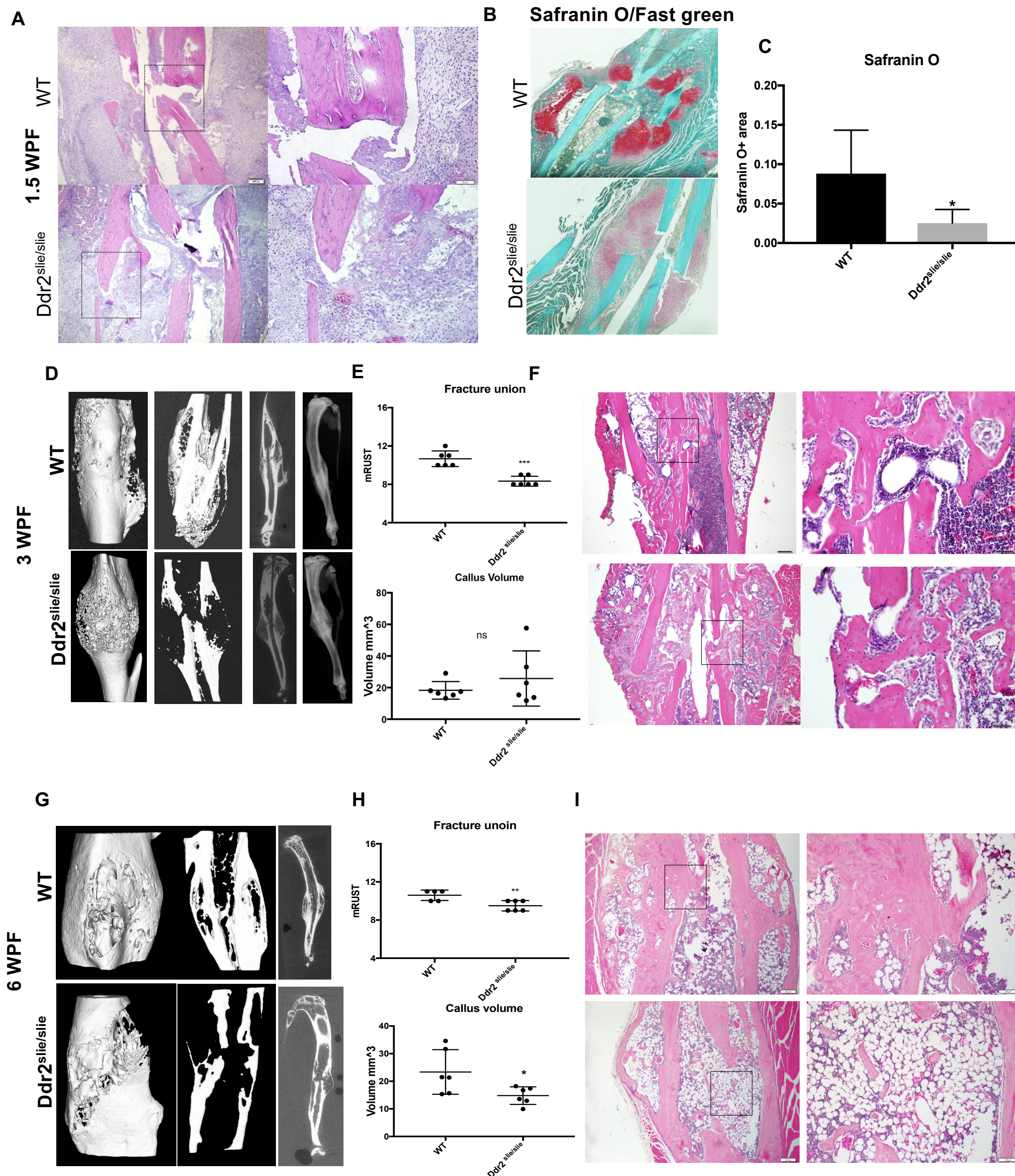

Figure 3.3 DDR2 is necessary for normal fracture healing (A) Representative histological images of the fracture area 1.5-week post-fracture (WPF). **(B and C)** Quantifications of cartilage areas from Safranin O/Fast Green-stained sections at 1.5 WPF. Cartilage was designated by Safranin O. **(D and G)** Representative micro-CT and radiograph images showing amount of bone healing at 3 and 6 WPF. **(E and H)** Measurements are shown for mRUST (radiographic union scoring tibia fracture) and callus volume. **(F and I)** Representative histological images of the fracture area at 3 and 6 WPF. Scale bar 50um in (A, F and I) and 300um in (B), *P<0.05, **P<0.01, ***P<0.0001.

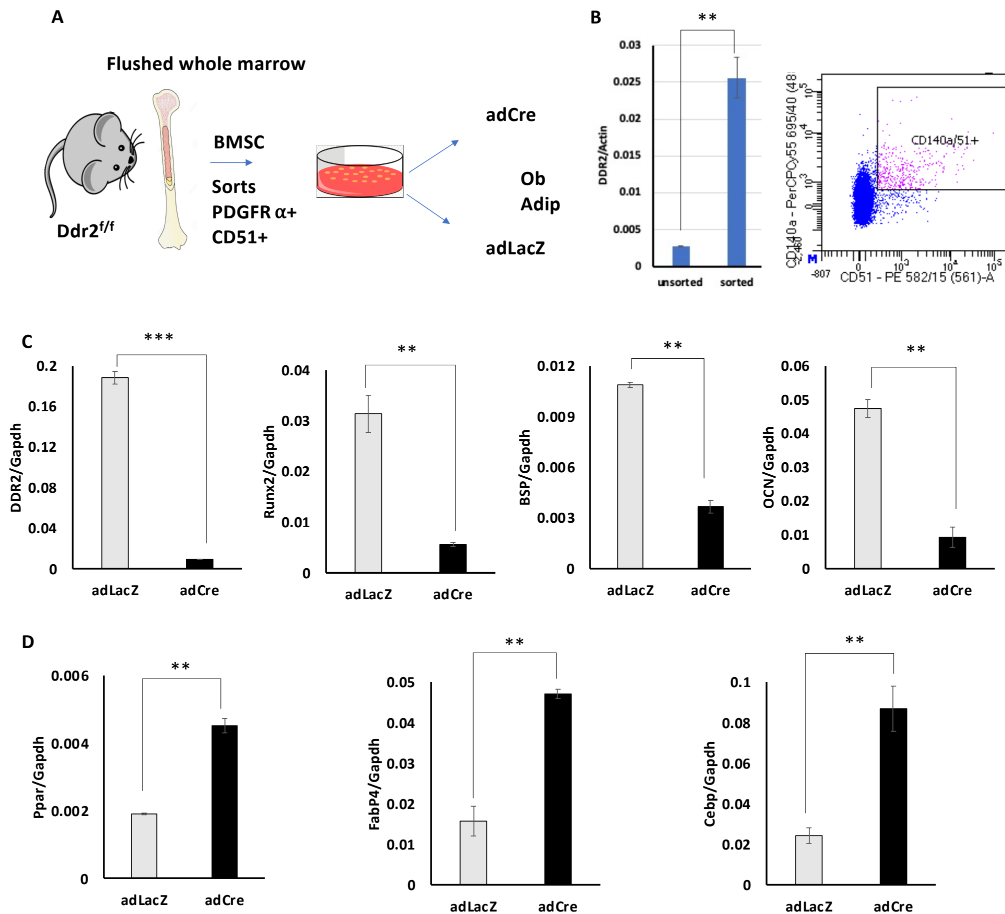

Figure 3.4 Loss of Ddr2 in purified SPCs inhibits osteoblast lineage and stimulate adipogenesis. (A) A Schematic diagram of the experiment design shows nucleated marrow cells from *Ddr2* $^{loxp/loxp}$ mice were enriched for SPCs by FACS (CD140a/PDGFRα+, CD51+), and treated with AdLacZ or AdCre followed by osteogenic or adipogenic differentiation. **(B)** Ddr2 expression in sorted SPCs. **(C)** Ddr2, Runx2, bone sialoprotein and osteocalcin mRNA expression. **(D)** Pparg, Fabp, Cebp mRNA expression. **P<0.01, ***P<0.0001. Provided by Dr. Chunxi Ge

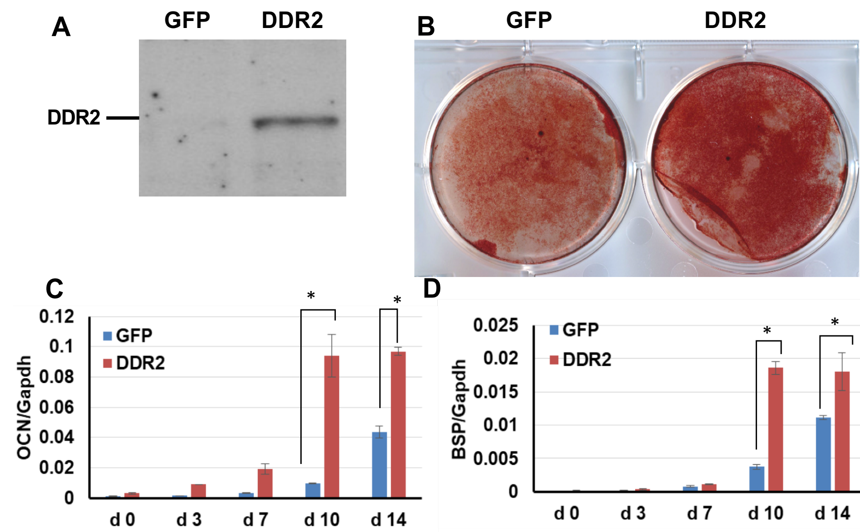

Figure 3.5 DDR2 stimulate osteoblast differentiation. (A) ST2 mesenchymal cells were transduced with a Ddr2 expressing lentivirus (DDR2 Western blot). **(B)** Mineralization at 14 days (Alizarin red stain). **(C and D)** Osteoblast mRNA; OCN, osteocalcin; BSP, bone sialoprotein. *P<0.05. Provided by Dr. Chunxi Ge

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Chapter 4

Summary and Conclusion

Bone extracellular matrix (ECM) plays important roles not only in bone development and maintenance, but also in the healing of bone injuries. Type I collagen is the core structural protein in the bone where collagen-mediated signaling is critical for tissue differentiation and mineralization [1-3]. ECM is formed by deposited a collagen-rich matrix by osteoblasts that mineralizes to become bone [4]. Osteoblasts arise from skeletal stem cell (SSC). The osteogenic differentiation of SSC involves SSC-ECM interactions that promote and support the SSC commitment to a certain cellular lineage. In addition, ECM is one of the most important components of SSC niche. Many studies reported on how SSCs sense and respond to signals from the ECM at the molecular level, and how these signals regulate SSC fate [5, 6]. This SSC-ECM interaction is processed by ECM surface receptors in SSC.

Integrins are the classical receptor proteins that cells use to both bind and respond to the ECM. Integrins α1 β1, α2β1 and α11β1 are the main integrins responsible for the binding of bone-forming cells to type I collagen [7] although other integrins such as α5β1 may also be involved in mechanotransduction[7]. Binding of integrins to the ECM initiates clustering, cell adhesion and intracellular signaling leading to complex conformational changes that promote high-affinity extracellular ligand binding, as well as activation of signaling proteins including FAK, Rho, Rac

and integrin-linked protein kinase (ILK) [8, 9]. Mice with targeted integrin subunit deletion in bone (i.e., β1 and αv) have an embryonic lethal phenotype, making it difficult to analyze their function during osteogenesis [10-13]. Because of that, integrins have also been ablated during osteoblast differentiation using the Cre/LoxP conditional deletion system. However, the affected mice do not suffer from major skeletal defects [10], which suggests that other ECM receptors may be required for bone formation.

Another class of ECM receptors are the discoidin domain receptors (DDRs). DDRs are receptor tyrosine kinases (RTKs) that contain a discoidin homology domain in their extracellular regions [14, 15]. DDRs are collagen receptors that differ from collagen-binding integrins in that they have intrinsic tyrosine kinase activity and selective affinity for triple-helical, native fibrillar and non-fibrillar collagens [14]. There are two types of DDRs, DDR1 and DDR2. Both DDR1 and DDR2 are involved in multiple physiological processes, such as development, ECM turnover, growth regulation and cancer [14, 16]. In bone, several lines of evidence suggest an important role for DDR2. In humans, DDR2 mutations cause spondylo-meta-epiphyseal dysplasia (SMED), a skeletal disorder associated with dwarfism, bowing of long bones, craniofacial abnormalities, short fingers, and abnormal calcifications [14, 17]. Also, in a Han Chinese population, polymorphisms in DDR2 are associated with low bone mineral density (BMD) and fracture risk [18]. Consistent with the human phenotype, mice with a spontaneous *Ddr2* mutation, called smallie mice (*Ddr2$^{slie/slie}$*), have a SMED-like phenotype characterized by dwarfism and reduction in total bone mineral density [14, 19]. Furthermore, detailed analysis of the bone phenotype in *Ddr2$^{slie/slie}$* mice revealed a dramatic reduction in mineral density of the cranial, axial, and appendicular skeleton due to reduction in osteoblast activity and bone formation in the absence of changes in resorption [14]. Bone changes were accompanied by a large increase in marrow fat.

Bone possesses an intrinsic capacity for regeneration as part of the repair process in response to injury as well as during skeletal development and during the continuous remodeling that occurs throughout adult life [20]. Bone extracellular matrix plays important roles not only in bone development and maintenance, but also in response to bone injury and fracture. Studies of bone healing indicate that resident stem or progenitor cells interact with the ECM to orchestrate a complex signaling cascade leading to tissue regeneration and remodeling [21]. Dysregulation of proteins mediating ECM interactions is implicated in several deformities affecting bone structure and regeneration [22]. Type I collagen is the core structural protein in bone where collagen-mediated signaling is critical for tissue differentiation and mineralization [21-23]. Collagen receptor, DDR2, is a key regulator of bone growth that controls several aspects of this process. However, it is not known if DDR2 is required for bone regeneration. In this thesis, we examined the functional importance of the collagen receptor, DDR2, in bone regeneration using 2 regeneration models; a calvarial subcritical defect and tibial fracture.

In calvarial bone regeneration, we show that DDR2 is essential for regeneration of a subcritical-size 0.5mm defect. $Ddr2^{slie/slie}$ mice are unable to heal a defect that, in wild type mice, can spontaneously heal within 4 weeks. Also, *Ddr2* expression during calvarial bone regeneration was defined using *Ddr2*-LacZ knock-in mice and β-galactosidase staining. *Ddr2* expression, which was restricted to periosteal surfaces of uninjured calvarial bone, greatly expanded with injury. Similar results were seen when the lineage of *Ddr2*-expressing cells was examined using $Ddr2cre^{ERT}$*, Ai14 TdTomato* mice. Ddr2 positive cells and their progeny expanded within the defect three days and two weeks post-surgery. Furthermore, three days post-surgery, $Ddr2^{slie/slie}$ mice showed a significant decrease in cell proliferation in the calvarial defect when compared with

WT littermates. Lastly, levels of the preosteoblast markers, Osterix and phosphorylated RUNX2 (S319-P) decreased in *Ddr2$^{slie/slie}$* mice, which suggests that osteoblast differentiation was arrested.

The regeneration of a fractured long bone was also studied in Ddr2$^{slie/slie}$ mice. As was done with the calvarial defect, we first defined the expression pattern of DDR2 during fracture healing using *Ddr2-LacZ* knock-in mice and *Ddr2-Cre^{ERT}; Tdtomato* mice. LacZ expression was first detected in select regions of the fracture site 2- and 5-days post fracture and expanded throughout the fracture callus after 1.5 and 3 weeks. Similar results were observed in *Ddr2-Cre^{ERT}; Tdtomato mice*. Ddr2+ cells and their progeny began to expand in the developing fracture callus 1.5-weeks post-fracture and continued to expand after 3 weeks. *Ddr2$^{slie/slie}$* mice exhibited significantly less fracture union than wild-type (WT) mice, and this defect was related to a decrease in cartilage formation as measured by safranin O staining. In addition, mutant mice developed significantly less callus tissue at 6 weeks post-fracture. To examine the role of DDR2 in skeletal progenitor cells (SPCs), we purified PDGFRα^{+} CD51^{+} SPCs from bone marrow of *Ddr2$^{fl/fl}$* mice using FACS followed by treatment with AdCre. *Ddr2* deletion resulted in defective osteoblast differentiation and accelerated adipogenesis. On the other hand, overexpression of DDR2 in a mesenchymal cell line (ST2 cells) increased osteoblast differentiation. These results suggest that DDR2 can directly act on SPCs to promote osteoblast differentiation and may at least in part explain the regenerative defects we observed.

Conclusion

The information gained from these studies has the potential to positively affect and enhance bone regeneration. Understanding the link between cell-ECM interactions mediated by cell surface receptors and bone regeneration may lead to the development of new regeneration strategies. DDR2, which binds and is activated by a triple-helical fibrillar collagen, is required for calvarial bone regeneration as well as fracture healing. This requirement may be explained in part by effects of DDR2 on proliferation, SPCs function and osteoblast differentiation. We showed that SPCs are enriched in DDR2, and DDR2 was expressed in periosteum of tibia and calvaria, which consist mainly of SPCs. In addition, unpublished data from our lab (data not shown) shows that DDR2+ cells are present in the calvarial suture, which has been reported to be the niche for SPCs for craniofacial bone homeostasis and repair [21]. These results suggest that DDR2 could be a SPCs marker for a specific SPCs subpopulation, since recent study provided evidence that bone contains multiple pools of stem cells, each with distinct physiologic functions [24]. In addition to SPCs, we think that DDR2 is expressed in other skeletal cells, such as preosteoblast and prechondrocyte, and osteoblast according to the LacZ localization.

Cell-ECM interaction is important for multiple cellular functions including cell proliferation, cell adhesion, migration, and ECM remodeling.[25-27]. The decrease in cell proliferation in *Ddr2*-deficent mice we observed could be due to defective cell-ECM interaction. We speculate that one of the reasons for defective cell-ECM interaction is abnormal collagen cross-linking in *Ddr2*-deficient mice. Khosravi and colleagues showed that DDR2 mediates collagen induction of lysyl oxidase enzyme-dependent collagen cross-links [28], and knockdown of DDR2 with shRNA in differentiating primary rat osteoblasts largely suppressed collagen induction of lysyl oxidase [28]. Also, the authors suggest that integrin signaling potentially cooperates with DDR2

in collagen induction of lysyl oxidase [28]. This notion is supported by a study that shows DDR2 interacts with integrins in mediating cell adhesion to collagen [29]. In unpublished studies, we observed abnormal organization of type II collagen in growth plates of Ddr2-deficient mice which could affect activation of both DDR2 and integrins. Defective cell-ECM interactions could affect multiple cellular functions, such as cell proliferation, migration, differentiation, and ECM remodeling. It is possible that in the absence of Ddr2, an abnormal collagen matrix is formed which reciprocally alters the ability of cells to respond to matrix either through integrins or other matrix receptors. How Ddr2 affects matrix structure could be the focus of future studies.

Proper differentiation and functioning of osteoblast is essential for bone homeostasis and regeneration. Our group and others showed that DDR2 plays an essential role in osteoblast differentiation [14, 30]. Bone changes in *Ddr2*$^{slie/slie}$ mice were attributed to decreased bone formation due to defective osteoblast function [14]. Also, calvarial cells from *Ddr2*$^{slie/slie}$ mice showed defective osteoblast differentiation in cell culture [14]. Consistent with our previous finding, we showed that preosteoblastic markers, Osx and p-RUNX2, were decrease during calvarial defect regeneration in *Ddr2*$^{slie/slie}$ mice. In addition, deletion of *Ddr2* in PDGFRα+ and CD51+ SPCs *in vitro* reduced osteoblast differentiation and increased adipogenesis. These findings support the hypothesis that DDR2 is required for osteoblast differentiation and function

There are several experiments that could be done to help us understanding the function of DDR2 in certain cell populations during bone regeneration. Our lab developed *Ddr2*$^{loxp/loxp}$ mice that can be combined with tissue selective Cre mice to disrupt *Ddr2* in specific cell populations during bone regeneration. *Ddr2*$^{loxp/loxp}$ mice could be crossed with the following TAM-inducible Cre mice: Gli1CreERT [31] for knockout in cranial and long bone SPCs [32], *Col2a1CreERT* for

knockout in chondrocytes [33] or 2.3*Col1a2CreERT* for knockout in osteoblasts [34]. By performing these experiments, it will be possible to determine the major cellular sites of Ddr2 action.

Despite the unique capacity of bone tissue for self-repair, there are situations in which its regenerative potential is limited or associated with complications. In my field, dental practice, the bone loss that develops after tooth loss, periodontal disease, or trauma often requires extensive horizontal and/or vertical regeneration procedures. Tissue engineered scaffolds that improve cell-ECM interactions have been used and studied in order to enhance bone regeneration. For example, peptides containing integrin binding domains of type I collagen (GFOGER) coupled to tissue engineered scaffolds have been successfully used to stimulate bone regeneration [35-38]. However, the integrin-activating tissue engineered scaffolds examined to date do not approach the activity of bone autografts, the "gold standard" for bone regeneration [39]. We showed that overexpression of DDR2 in the ST2 mesenchymal cell line increases osteoblast differentiation. This suggests that manipulation of DDR2 levels or activity represent potential new routes for stimulating SPC osteogenic differentiation and bone regeneration. DDR2 binds and is activated by a unique triple-helical sequence present in fibrillar collagens (core sequence GVMGF-HO-Pro) [40]. Peptides containing this sequence do not bind integrins or directly stimulate integrin activity, but increase integrin-mediated collagen binding and downstream signaling [29, 40]. A possible future direction for our research would be to couple the DDR2-binding peptide sequence (GVMGF) alone or combined with integrin peptide sequence (GFOGER) to tissue engineered scaffolds to stimulate and enhance bone regeneration. In addition, dental implants could be coated with DDR2 and integrin peptides to improve peri-implant bone regeneration and osseointegration [36, 37, 41].

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