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University of Turku

Faculty of Medicine
Institute of Biomedicine
Medical Biochemistry and Genetics
Turku Doctoral Programme of Molecular Medicine

Supervised by

Associate Professor Riku Kiviranta, M.D, Ph.D
Institute of Biomedicine
University of Turku, Finland
Department of Endocrinology
Turku University Hospital, Finland

Adjunct Professor Terhi Heino, Ph.D
Institute of Biomedicine
University of Turku
Turku, Finland

Reviewed by

Professor Juha Tuukkanen, D.D.S, Ph.D
Department of Anatomy and Cell Biology
Institute of Biomedicine
Faculty of Medicine
University of Oulu, Oulu, Finland

Dr. Hanna Taipaleenmaki, Ph.D
Molecular Skeletal Biology Laboratory
Department of Trauma and Orthopedic Surgery
University Medical Center Hamburg-Eppendorf
Hamburg, Germany

Opponent

Associate Professor Sara Windahl, Ph.D
Department of Laboratory Medicine
Karolinska Institute, Stockholm, Sweden

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To my family

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ABSTRACT

Bone is a dynamic organ that is remodeled in a continuous cycle of bone formation by osteoblasts and bone resorption by osteoclasts. Extensive studies have revealed that bone remodeling can be modulated by multiple signaling pathways. Wnt ligands are a family of 19 lipid-modified glycoproteins that play essential roles during development, homeostasis and in cancer. The current evidence indicates that Wnt signaling pathway is also a central pathway for regulating skeletal development.

Our group and others recently reported that heterozygous or homozygous mutations in Wnt1 gene in human cause early-onset osteoporosis or osteogenesis imperfecta, respectively. This thesis investigates the role of Wnt1 and its molecular mechanisms in regulating bone and cartilage development. *In vivo*, the mesenchymal cell derived Wnt1 is a key regulator of bone metabolism affecting both trabecular and cortical bone compartments. Moreover, Wnt1 has a dual function to induce osteoblast differentiation and activity as well as to suppress osteoclast differentiation. In adult mice, osteoblastic Wnt1 regulates diaphyseal cortical bone mass and cortical thickness by regulating periosteal bone formation. Furthermore, Wnt1 deficiency in mesenchymal progenitors in mice does not lead to defective phenotype of growth plate or articular cartilage, while results in low subchondral bone mass. Mechanistically, Wnt1 functions in osteoblast and osteoclast in a juxtacrine manner, requiring physical contact between the source and its target cells.

These novel data significantly contribute to our understanding of the role of Wnt1 in regulating bone and cartilage metabolism. Furthermore, our data identifies Wnt1 as a potential target for the treatment of musculoskeletal diseases.

KEYWORDS: Wnt1, bone, cartilage, osteoblast, osteoclast, chondrocyte.

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TIIVISTELMÄ

Luu on dynaaminen kudos, jota muokataan jatkuvasti luuta muodostavien osteoblastien ja luuta hajottavien osteoklastien toimesta. Tutkimukset ovat osoittaneet, että useat erilaiset molekyylitason signalointireitit säätelevät luun aineenvaihduntaa. Wnt-ligandit, joita on kaikkiaan 19, kuuluvat lipidimuokattujen glykoproteiinien perheeseen. Niillä on oleellinen merkitys kudosten kehityksen ja toiminnan säätelyssä sekä syövän synnyssä. Nykytietämyksen mukaan Wnt-signalointireitti säätelee keskeisesti myös luuston kehitystä.

Tutkimusryhmämme julkaisi vastikään yhdessä muiden tutkijoiden kanssa uutta tietoa siitä, miten Wnt1-geenin mutaatiot voivat aiheuttaa varhain kehittyvän osteoporoosin tai osteogenesis imperfecta -luustosairauden. Tämän väitös-tutkimuksen tavoitteena oli tutkia Wnt1:n ja siihen liittyvien solu- ja molekyylitason mekanismien osuutta luun ja ruston kehityksessä. Tutkimuksen tulokset osoittavat, että mesenkymaalisesta linjasta peräisin oleva Wnt1 on luun aineenvaihdunnan keskeinen säätelijä, joka vaikuttaa sekä hohka- että kuoriluuhun. Wnt1 vaikuttaa sekä luuta muodostaviin osteoblasteihin lisäämällä niiden erilaistumista ja toimintaa, että luuta hajottavin osteoklasteihin estämällä niiden erilaistumista. Aikuisilla hiirillä tehdyissä kokeissa havaittiin, että osteoblasteista peräisin oleva Wnt1 säätelee luun varsiosan kuoriluun massaa ja paksuutta vaikuttamalla periosteaaliseen luunmuodostukseen. Vaikka Wnt1:n puute hiirten mesenkymaalisissa esiaste-soluissa ei johtanut muutoksiin rustoisessa kasvulevyssä tai nivelrustossa, se vähensi ruston alaisen luun massaa. Soluviljelykokeita käyttäen havaittiin, että Wnt1 toimii osteoblastien ja osteoklastien välisessä viestinnässä jukstakriinisesti eli vaatii kontaktin Wnt1:ä erittävän solun ja Wnt1:n kohdesolun välillä.

Väitöstutkimuksessa saavutettiin uusia, kiinnostavia tuloksia, joiden avulla ymmärrämme paremmin Wnt1:n merkitystä luun ja ruston aineenvaihdunnassa. Tutkimustulosten perusteella Wnt1 voisi olla myös mahdollinen kohdemolekyyli lääkekehityksessä etsittäessä uusia hoitomuotoja tuki- ja liikuntaelinsairauksiin.

AVAINSANAT: Wnt1, luu, rusto, osteoblasti, osteoklasti, kondrosyytti.

Table of Contents

Abbreviations

ALP	Alkaline phosphatase
BFR	Bone formation rate
BMD	Bone mineral density
BV/TV	Bone volume/tissue volume
COMP	Cartilage oligomeric matrix protein
Cort.Th	Cortical thickness
Ctsk	Cathepsin K
DC-STAMP	Dendritic cell specific transmembrane protein
DKK	Dickkopf
DMP1	Dentin matrix protein 1
ECM	Extracellular matrix
Fzd	Frizzled
Lrp5/6	Low density lipoprotein receptor-related protein 5/6
JNK	C-Jun amino-terminal kinase
MAR	Mineral apposition rate
M-CSF	Macrophage colony-stimulating factor
MMP9	Matrix metalloproteinase 9
MMP13	Matrix metalloproteinase 13
MS/BS	Mineralizing surface/bone surface
MSCs	Mesenchymal stem cells
mTORC1	Mammalian target of rapamycin complex 1
Nfatc1	Nuclear factor of activated T-cells, cytoplasmic 1
N.Ob	Osteoblast number
N.Oc	Osteoclast number
OA	Osteoarthritis
OI	Osteogenesis imperfecta
OCN	Osteocalcin
OPG	Osteoprotegerin
OPN	Osteopontin
OSCAR	Osteoclast-associated immunoglobulin-like receptor
Osx	Osterix

PBS	Phosphate buffered saline
PCP	Planar cell polarity
PORCN	Porcupine
PTH	Parathyroid hormone
PTHrP	Parathyroid hormone-related peptide
RANKL	Receptor activator of nuclear factor-κB ligand
ROI	Regions of interests
Runx2	Runt-related transcriptional factor 2
SOC	Secondary ossification center
SOST	Sclerostin
Tb.N	Trabecular number
Tb.Sp	Trabecular separation
Tb.Th	Trabecular thickness
TCF	T-cell factor
TGF-β	Transforming growth factor β
Tracp	Tartrate-resistant acid phosphatase
Wls	Wntless
Wnt	Wingless-type MMTV integration site family
μCT	Microcomputed tomography

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I Fan Wang, Kati Tarkkonen, Vappu Nieminen-Pihala, Kenichi Nagano, Rana Al Majidi, Tero Puolakkainen, Petri Rummukainen, Jemina Lehto, Anne Roivainen, Fu-Ping Zhang, Outi Mäkitie, Roland Baron and Riku Kiviranta. Mesenchymal Cell-Derived Juxtacrine Wnt1 Signaling Regulates Osteoblast Activity and Osteoclast Differentiation. *Journal of Bone and Mineral Research*, 2019; No.6: 1129–1142.

II Fan Wang, Petri Rummukainen, Terhi J. Heino and Riku Kiviranta; Osteoblastic Wnt1 Regulate Periosteal Bone Formation in Adult Mice. *Bone*, 2021 Feb; 143:115754.

III Fan Wang, Petri Rummukainen, Matias Pehkonen, A.-M. Säämänen, Terhi. J. Heino and Riku Kiviranta. Wnt1 Deficiency in Mesenchymal Progenitors Decreased the Subchondral Bone Mass but has no Impact on Growth Plate or Articular Cartilage in Mice. Manuscript.

1 Introduction

Bone is a dynamic organ that consists of several different cell types, such as osteoblasts, osteoclasts and chondrocytes. The abnormal proliferation, differentiation and apoptosis of bone cells causes skeletal related disease, such as osteoporosis, osteogenesis imperfecta (OI) and osteoarthritis (OA) (Baron, R., & Kneissel, M., 2013). Even though many signal transduction cascades are involved in bone development, such as bone morphogenetic protein (BMP) signaling, transforming growth factor β (TGF-β) signaling and parathyroid hormone (PTH) signaling (Janssens, K. et al., 2005; Rubin, M. R., & Bilezikian, J. P., 2003; Urist, 1965), Wingless-type MMTV integration site family (Wnt) signaling pathway has emerged as a central pathway to induce bone formation.

Wnt ligands are a family of 19 secreted lipid-modified glycoproteins that are involved in embryonic development, tissue homeostasis and cancer development. Clinical studies and genetically modified mouse models have suggested that many Wnt ligands regulate bone mass. Mutations in several Wnt ligands result in skeletal malformations in humans and mice. For example, mutations in Wnt3, Wnt3a or Wnt7a result in a range of limb malformations in humans, while local misexpression of Wnt14 induces morphological and molecular changes characteristic of the first step of joint formation (Hartmann, C., & Tabin, C. J., 2001; Niemann, S., et al., 2004; Woods, C. G., et al, 2006).

Recently, our group and others identified Wnt1 mutations from patients with early-onset osteoporosis and severe OI and demonstrated that Wnt1 is an important ligand in the regulation of bone metabolism (Fahiminiya, S., et al., 2013; Keupp, K., et al., 2013; Laine, C. M., et al., 2013; Pyott, S. M., et al., 2013). However, the cellular source of Wnt1 in bone microenvironment and the molecular mechanisms by which Wnt1 regulates bone and cartilage metabolism remains open. Moreover, the role of Wnt1 in adult skeleton is still unknown.

In this study, by generating global and cell-specific conditional knockout mouse model, we revealed that limb bud mesenchymal progenitor cell-derived Wnt1 is a key regulator of bone metabolism. Importantly, we showed that Wnt1 functions only in short range in inducing osteoblast differentiation and activity as well as suppressing osteoclast differentiation. Furthermore, we found that Wnt1 improves

cortical thickness in adult mice by stimulating periosteal modeling-based bone formation. Finally, we discovered that the role of Wnt1 in articular cartilage and growth plate is very modest, while it plays an essential role in subchondral bone. Taken together, this thesis study provides important novel insight into the role of Wnt1 in the regulation of bone and cartilage metabolism.

2 Review of the Literature

2.1 Bone and cartilage

In higher vertebrates, the skeletal structure consists mainly of bone and cartilage, which are formed by osteoblasts and chondrocytes, respectively, during embryogenesis.

Bone is a dynamic organ remodeled in a continuous cycle of bone formation and resorption. It has essential functions in the body, such as movement, storing calcium and phosphate, providing attachment points for soft tissues, and harboring of bone marrow (Florencio-Silva, R., et al, 2015). There are two types of bone in adult skeleton: cortical and cancellous (or trabecular) bone (**Figure.1**). Cortical bone, which is also called compact bone, forms the external layer of all bones, particularly in diaphysis of long bones. It represents approximatively 80% of total skeletal mass and contains a high matrix mass. These characteristics give cortical bone its strength and therefore supports the mechanical role of bone. Cancellous bone, also known as trabecular bone, is typically located at the epiphyses and metaphysis of long bones and in the vertebral bodies. Trabecular bone has honeycomb-like structure with high porosity, and a large surface exposed to the bone marrow and blood flow, therefore facilitating its higher turnover compared to cortical bone. Due to its porosity, trabecular bone has weaker mechanical strength than cortical bone (Ott, S. M., 2018). However, both cortical and trabecular bone are essential for bone strength, and their relation is complex.

Cartilage is flexible connective tissue found e.g in the external ear, synovial joints, ribs, nose and in the growth plate of children and adolescents. During the embryogenesis, cartilage either remains as cartilage or functions as a template for endochondral ossification (**Figure 1**). Three types of cartilage are identified in human body: hyaline, fibrous and elastic. All these cartilage types are made by chondrocytes, which synthesize and produce the major components of the extracellular matrix (ECM), such as cartilage oligomeric matrix protein (COMP), type II collagen (Col2a) and aggrecan (Krishnan, Y., & Grodzinsky, A. J., 2018). The surface zone of articular cartilage is made by flat chondrocytes producing lubricating molecules, and the middle and bottom zone of articular cartilage are made by large round chondrocytes surrounded by a typical cartilage matrix. The

epiphyseal growth plate is comprised of three histologically distinct cell layers: resting, proliferative and hypertrophic zone. The chondrocytes in resting zone are round and small, acting as a renewing population of progenitors that give rise to proliferating chondrocytes. Proliferating chondrocytes line up in clonal columns that differentiate into hypertrophic chondrocytes. Hypertrophic chondrocytes increase in size, mineralize ECM, and subsequently undergo apoptosis. The terminal chondrocytes express matrix to induce vascular invasion and osteoblast recruitment, which then leads the formation of primary spongiosa (Hallett, S. A., et al, 2019). The growth plate chondrocytes contribute to bone extension by proliferation, secretion and mineralization of ECM, and hypertrophy. Unlike in mouse, the epiphyseal growth plate is transient and eventually disappears during puberty in human.

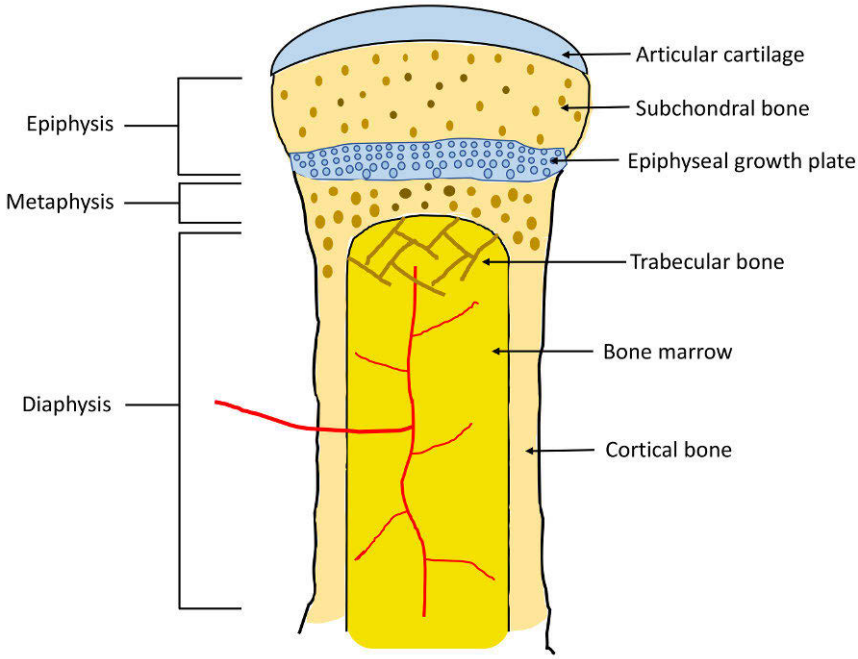

Figure 1. Structure of long bone.

2.1.1 The composition of bone and cartilage

Bone is mainly composed of mineral, collagen, water and small amounts of other substances, such as other proteins and inorganic salts. Mineral components account for approximately 60-70% of total bone mass and the main mineral component is hydroxyapatite. The function of mineral is to give bone its compressive strength. It also serves as a source of calcium, phosphate, magnesium, sodium and carbonate for

the body (Shekaran, A., & García, A. J., 2011; Zhu, W., et al., 2008). Type I collagen (Col1) is the main fibrous protein of the bone ECM (Bou-Gharios, G.,et al, 2019), accounting for 90-95% of the organic matrix. It gives bone tensile strength and its elasticity improves fracture resistance. The remaining part of organic matrix consists of proteoglycans such as decorin, biglycan and glypican, as well as noncollagenous proteins such as osteocalcin (OCN), bone sialoprotein (BSP) and osteopontin (OPN), which regulate the mineralization process. In addition, bone ECM contains small amounts of different growth factors, enzymes and cytokines. Each of the components of the bone ECM can influence bone quality by directly regulating matrix mineralization and/or bone cellular activity.

Cartilage is composed of a large amount of collagenous ECM and abundant ground substance that is rich in proteoglycans and collagen fibers. The main extracellular protein of cartilage in mammals is Col2. In hyaline cartilage, Col2 is up to 40% of its dry weight. Elastic cartilage includes Col2 fibers with elastic fibers, while fibrocartilage contains also Col1. In cartilage, aggrecan is the most abundant proteoglycan with high molecular weight. It binds to hyaluronan (HA) through link proteins to form large proteoglycan aggregates. Aggrecan supports cartilage with its permeability to resist compressive loads (Sophia Fox, et al., 2009).

2.2 Bone formation

Bone formation occurs via two major mechanisms: intramembranous and endochondral ossification. In intramembranous ossification, mesenchymal cells differentiate directly into osteoblasts to form mature bone. This process takes place in membranous neuro- and viscerocranium and in part of the clavicle. In endochondral ossification, osteochondral progenitors initially differentiate into chondrocytes to form cartilage, providing a framework for the future bone. Endochondral bone formation occurs in axial and appendicular skeleton, the skull base and the posterior part of the skull (Berendsen, A. D., & Olsen, B. R., 2015).

2.2.1 Cellular composition of bone

2.2.1.1 Osteoblast

Osteoblasts that are differentiated from multipotent mesenchymal stem cells (MSCs) are generally round or cuboidal in shape and line the bone surface (**Figure 2**). The main function of osteoblast is bone formation, thus playing a crucial role in establishment and maintenance of bone mass. Osteoblast differentiation can be divided into three stages: mesenchymal progenitors, pre-osteoblast and mature osteoblasts. To distinguish the stages of osteoblast differentiation, the expression

levels of osteoblast markers are used, including *Col1*, alkaline phosphatase (*ALP*), *OPN* and *OCN* (Harada, H., et al., 1999; Kern, B., et al., 2001). ALP is an enzyme, which is involved in bone mineralization and is considered to be a marker of pre-osteoblasts. The expression of *ALP* increases with the progression of osteoblast differentiation. *OCN* is strongly expressed during the mineralization phase of ECM and it is generally used as a marker of mature osteoblasts.

Both the proliferation and differentiation of osteoblast is controlled by specific transcription factors, such as Runt-related transcription factor 2 (*Runx2*) and Osterix (*Osx*). Runx2 induces the differentiation of mesenchymal progenitors into immature osteoblasts. Studies in genetic mouse models have shown that Runx2-deficient mice lack osteoblasts and are unable to form hypertrophic chondrocytes (Otto, F., et al., 1997). Moreover, *Runx2* regulates the expression of *Osx,* which is the important transcriptional factor promoting differentiation of immature osteoblasts to mature osteoblasts. *Runx2* regulates *Osx* by directly binding to its promoter, demonstrated by normal *Runx2* expression in *Osx*-deficient mice while *Runx2*-deficient mice have no *Osx* expression (Nakashima, K., et al., 2002; Nishio, Y., et al., 2006). Some osteoblasts further differentiate into osteocytes, then embedded into the bone matrix and remain viable there. In addition, some osteoblasts remain as flattened bone-lining cells that cover the surface of new bone, while most osteoblasts undergo apoptosis (De Gorter, et al., 2018).

2.2.1.2 Osteoclast

The osteoclasts are multinucleated bone-resorbing cells. Osteoclasts are differentiated from mononuclear progenitors of the hematopoietic stem cell lineage (Suda T., et al, 1999) (**Figure 2**). The differentiation and activity of osteoclasts are regulated by multiple growth factors and cytokines. Macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor-κB ligand (RANKL) are necessary for osteoclast differentiation (Boyle W.J., et al., 2003). Both factors, which are produced by marrow stromal and osteoblast lineage cells, promote gene expression and the activation of transcription factors in osteoclast progenitors to induce their differentiation (Lacey, D. L., et al., 1998). M-CSF stimulates proliferation of osteoclast progenitors and inhibits apoptosis of osteoclast by binding to its receptor (cFMS), while RANKL induces osteoclast formation by interacting with its receptor RANK expressed in osteoclast precursors. Osteoblasts secrete RANKL that induces osteoclast precursors to merge with one another and form multinucleated mature osteoclasts. The fusion requires the expression of ATP6v0d2, a part of proton pumps in the plasma membranes of osteoclasts (Lee, S. H., et al., 2006) and of dendritic cell specific transmembrane protein (DC-STAMP) by osteoclast precursors (Yagi, M., et al., 2005).

Many kinds of cell types, such as osteoblasts, stromal cells and periodontal ligament fibroblasts (Sokos D, et al., 2015), produce osteoprotegerin (OPG), which is a decoy receptor of RANKL. In mice, OPG overexpression inhibits osteoclast differentiation and results in osteopetrosis, whereas knockout of OPG results in enhanced osteoclastogenesis and osteoporosis (Simonet, W. S., et al., 1997). Therefore, the RANKL/RANK/OPG signaling is an essential regulator of bone resorption.

Bone resorption takes place when osteoclasts bind to mineralized bone surface. When the mature osteoclast contacts bone, it has a capacity to polarize and then forms a "ruffled membrane" to increase the cell surface area in contact with bone (Teitelbaum, 2000). Osteoclasts form a "sealing zone" against bone surface around the ruffled membrane to form an isolated microenvironment to facilitate bone resorption. The compartment between osteoclast and the underlying bone matrix is acidified by an electrogenic H^+-ATPase and a charge-coupled Cl^- channel. The acidic milieu first mobilizes mineral component of bone and releases acidic proteases, such as cathepsin K (*Ctsk*), matrix metalloproteinase 9 (MMP9) and matrix metalloproteinase 13 (MMP13) to degrade demineralized organic component of bone (Teitelbaum, Steven L., & Ross, F. P., 2003). Bone degradation products are then released into bone environment, degraded by lysosomes, or secreted into extracellular area via transcytosis (Salo, J., et al., 1997).

2.2.1.3 Osteocyte

Osteocytes, the most abundant cell type in mature bone, account for approximately 90-95% of all bone cells in the adult human skeleton (Aarden, E. M.,et al., 1994). Osteocytes are derived from osteoblasts and represent their terminally differentiated stage. They are stellate, dendritic shaped cells embedded in mineralized bone matrix (Dallas, S. L.,et al., 2013) (**Figure 2**). The tiny canaliculi form the osteocytes connect osteocytes to each other, as well as to the cells on the bone surface. Bone fluid travels through the lacuno-canalicular system to provide nutrients and oxygen to maintain the viability of osteocytes in the enclosed space (Bonewald, 2011). The characteristic morphology of lacuno-canalicular system allows the biochemical signals to pass from one osteocyte to another (Knothe Tate et al., 2004). In addition, osteocytes are very sensitive to the pressure applied to bone and they are the key players in the process of bone mechanotransduction (Qin, L., et al., 2020). The process of mechanotransduction in osteocytes is complicated and many cell parts of osteocytes, including cytoskeleton, dendritic processes, primary cilium, ion channels and ECM are essential for the function of osteocytes as mechanosensors (Santos, A., et al., 2009).

It is known that osteocytes can directly control the differentiation and activity of both osteoclasts and osteoblasts. Osteocytes regulate bone formation and

mineralization by expressing mineralization promoters, including phosphate regulating endopeptidase homolog X-linked (Phex) and dentin matrix protein 1 (Dmp1), and by expressing inhibitors of mineralization and bone formation, including matrix extracellular phosphoglycoprotein (MEPE) and sclerostin (SOST). Osteocyte is also a regulator of bone resorption by both suppressing and promoting osteoclast differentiation. Osteocytes and osteocyte-like cell line MLO-Y4 have been shown to express RANKL and thus are able to induce osteoclastogenesis (Kurata et al., 2006; Silvestrini et al., 2005). Osteocytes also send signals inhibiting osteoclast activation (Tatsumi, S., et al., 2007). In addition to regulating bone homeostasis, osteocyte has another important function: regulating phosphate metabolism. Osteocytes express Phex that regulates phosphate homeostasis and bone mineralization. Knockout of Phex gene in mouse results in X-linked hypophosphatemic rickets (Strom, T. M., et al., 1997). Therefore, the osteocyte network can be considered as an endocrine gland that regulates phosphate metabolism by expressing Phex. Recently, more endocrine functions of osteocytes have been reported, such as regulating immune system and energy metabolism (Sato, M., et al., 2013).

2.2.2 Bone remodeling

Bone is a highly dynamic organ that undergoes continuous regeneration, and its formation can be modeling-based and remodeling-based. Bone modeling is responsible for changing bone size and shape by independent actions of osteoblast and osteoclast. Modeling-based bone formation (MBF), which contributes to the periosteal expansion, primarily occurs during development, growth and in response to mechanical loading, but it continues throughout life (Langdahl. B., et al., 2016). Bone remodeling is a lifelong process that functions to remove old or damaged bone tissue and replace it by new bone tissue. This activity depends on the coupling of bone resorption by osteoclasts and bone formation by osteoblast lineage cells (lining cells, osteoblasts and osteocytes) (**Figure 2**). The remodeling cycles consist of four phases: resting, resorption, reversal and formation (Hadjidakis, D. J., & Androulakis, I. I., 2006). In the resting phase, lining cells stay on the bone surface and bone remodeling is inactivated (Feng, X., & McDonald, J. M., 2011; Kenkre, J. S., & Bassett, J. H. D., 2018; Raggatt, L. J., & Partridge, N. C., 2010). Bone remodeling is initiated by remodeling signal that can take several forms as mechanical strain caused bone deformation or systemic hormones (e.g. PTH or estrogen) (Hadjidakis, D. J., & Androulakis, I. I., 2006). Osteocytes send signals, such as M-CSF and RANKL when sense microdamage or bone deformation, to attract osteoclast precursors attaching to the specific bone site and to support osteoclast formation. The remodeling cycle moves on to the next phase after the formation of osteoclasts.

In the resorption phase, partially differentiated mononucleated osteoclast precursors differentiate into mature multinucleated osteoclasts and bone resorption occurs. In the reversal phase, MSCs derived from bone marrow and/or osteoprogenitors are recruited to the bone surface. Bone formation phase is the final stage of remodeling cycle. During this phase, pre-osteoblasts lay down bone and differentiate to mature osteoblasts, which then produce new bone matrix to replace resorbed bone. When the bone formation phase is completed, osteoblasts terminally differentiate into osteocytes embedded into bone matrix or remain as bone lining cells. The resting phage lasts until a new remodeling cycle is initiated.

The exact mechanism that couples bone resorption to formation is not fully understood, however, several coupling factors have been identified during the past years (Sims, N. A., & Martin, T. J., 2015). For example, TGF-β, BMP-2 and insulin-like growth factors (IGFs) which are released from bone matrix during bone resorption could stimulate recruitment, migration and differentiation of osteoblast progenitors. Osteoclasts also produce coupling factors, such as interleukin 6 and the Ephrin receptor family to induce osteoblast differentiation and thereby promote bone formation.

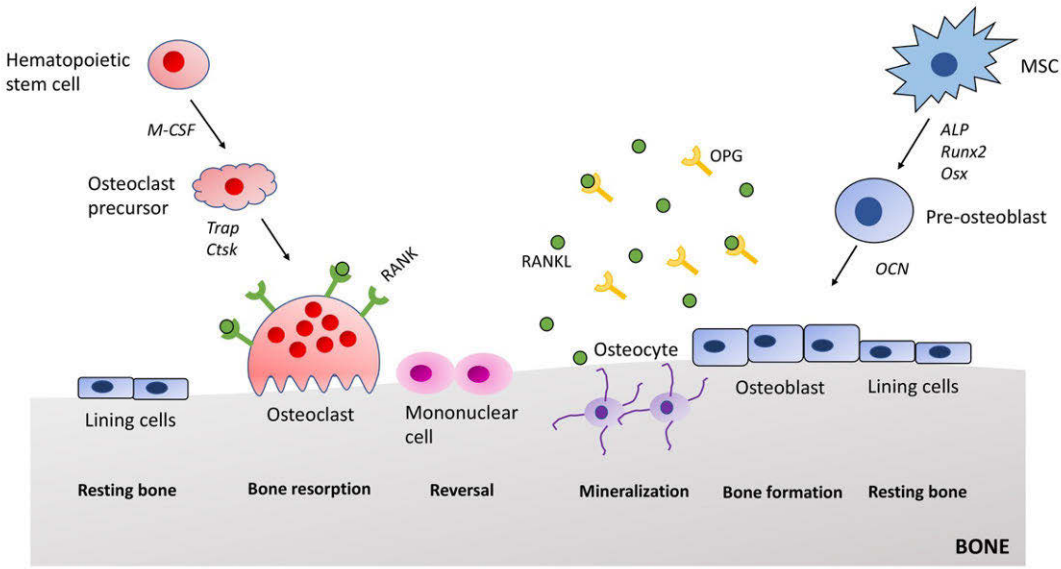

Figure 2. Schematic diagram illustrating bone remodeling. M-CSF, Macrophage colony-stimulating factor; Trap, Tartrate-resistant acid phosphatase; Ctsk, Cathepsin K; RANK, Receptor activator of nuclear factor kappa-B; RANKL, Receptor activator of nuclear factor kappa-B ligand; OPG, Osteoprotegerin; MSC, Mesenchymal stem cell; ALP, Alkaline phosphatase; Runx2, Runt-related transcription factor 2; Osx, Osterix; OCN, osteocalcin.

2.3 Cartilage formation

2.3.1 Cartilage formation in endochondral ossification

Endochondral ossification is a principal process for the formation and growth of long bone in mammals. There are three key stages in endochondral ossification: mesenchymal condensation, chondrocyte differentiation and maturation, and osteoblast differentiation (**Figure 3**). During mesenchymal condensation, mesoderm-originated chondroprogenitors congregate into a cluster, forming an outer boundary that limits the area of chondrogenesis. The increased cell density results in higher number of cell-cell contacts and upregulation of genes related with cell-cell adhesion, including N-cadherin and neural cell adhesion molecular (N-CAM). The condensed mesenchymal cells then differentiate into chondrocytes to form hyaline cartilage. Hyaline chondrocytes express Sox5, 6 and 9 to regulate the expression of Col2 and aggrecan, which are key structural components in cartilage (Lefebvre, 2002; Lefrebvre, V., & de Crombrugghe, B., 1998). Next, chondrocytes in the center proliferate rapidly, and then undergo hypertrophy associated with the expression of type X collagen (Col10) (Poole, C. A., et al., 1991). The chondrocytes in hypertrophic zone increase in size and induce matrix mineralization. Subsequently, hypertrophic chondrocytes undergo apoptosis and express MMP13, which induces the vascular invasion in hypertrophic cartilage. Invasion of blood vessels in hypertrophic cartilage leads to further degradation of calcified cartilage matrix by chondroclasts (Vu, T.H., et al., 1998). The invading vasculature also brings along osteoprogenitors that differentiate into osteoblasts, subsequently form trabecular bone in the primary ossification center. Recently, a novel concept was raised, showing that lineage-specified ($Col10a1^{+}$) hypertrophic chondrocytes can differentiate into preosteoblasts, mature osteoblast and osteocytes, embedded with the cartilage matrix in fetal and postnatal endochondral bone (Yang. L, et al., 2014). These cells can commit to osteogenic fate during normal endochondral bone formation and remodeling.

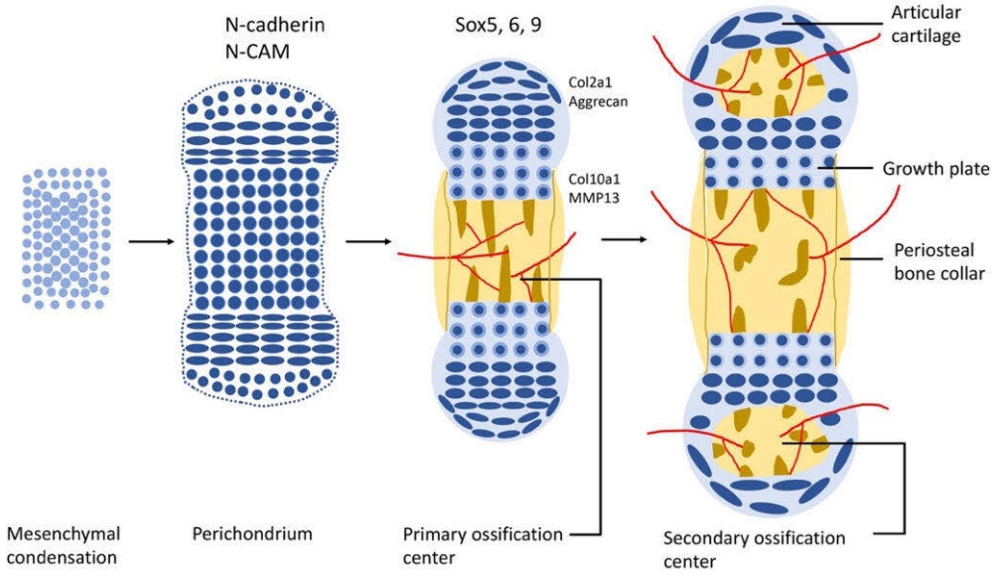

Figure 3. Schematic diagram illustrating cartilage development and endochondral ossification. N-CAM, Neural cell adhesion molecular; Sox 5,6,9, SRY-related HMG box; Col2a1, Collagen type II alpha 1 chain; Col10a1, Collagen type X alpha 1 chain; MMP13, Matrix metalloproteinase 13.

Secondary ossification center (SOC) forms at the epiphyses of long bones via similar processes as primary ossification center: matrix mineralization, apoptosis of chondrocyte, vascular invasion, and bringing osteoprogenitors that differentiate to osteoblast (**Figure 3**). However, the formation of SOC is originated by the formation of cartilage canals, where the vascularization occurs prior to the hypertrophy and matrix mineralization of the cartilage (Alvarez, J., et al., 2005). Moreover, unlike primary ossification center, the bone collar is not formed in SOC (Blumer, M. J. F., et al., 2008).

2.3.2 Articular cartilage formation

Articular cartilage located at both ends of long bones has an important role in maintaining joint function. Unlike growth plate, articular cartilage is a permanent structure without being replaced by bone (Chen, C. G., et al., 2012). There are fewer chondrocytes in articular cartilage compared to growth plate, and the cells are embedded in the ECM that is rich in collagen crosslinks. Mature articular cartilage can be divided into four layers: superficial layer, mid layer, deep layer and calcified layer (Las Heras, F., et al., 2012). Due to its poor regenerative capacity, articular cartilage can degenerate once damaged. OA is the most common disease of articular cartilage, which occurs widely in ageing population (see Chapter 2.4.3). During OA,

the chondrocytes in articular cartilage lose their resting phenotype and differentiate into terminal hypertrophic chondrocytes, expressing Col10 and MMP13 (Martel-Pelletier, J., et al., 2016; Wang, M., et al, 2013).

Different proliferation and differentiation potential of chondrocytes results in different morphology and function of cartilage tissue across the lifespan of an individual. There are many cytokines that participate in regulating the proliferation and differentiation of chondrocytes, including the TGF-β superfamily, Wnts, fibroblast growth factor (FGF), parathyroid hormone-related peptide (PTHrP), Hedgehog family, and Notch (Gao, L., et al., 2013; Long, F., & Ornitz, D. M., 2013).

2.4 Skeletal disorders

2.4.1 Osteoporosis

Osteoporosis is a common human skeletal disease characterized by decreased bone mass, micro-architectural deterioration and increased bone porosity, leading to bone fragility and susceptibility to fractures (Alexeeva, L., et al., 1994). The increasing age and female gender are related with the prevalence of osteoporosis. Data from World Health Organization (WHO) showed that 35% of woman older than 65 years old have osteoporosis (Alexeeva, L., et al., 1994). Dual-energy X-ray absorptiometry (DEXA scan) used for measuring bone mineral density (BMD) is considered the golden standard for the diagnosis of osteoporosis. In 1994, WHO produced diagnostic criteria using SD scores of BMD related to peak bone mass in healthy young women. Osteoporosis is diagnosed when T-score is less than or equal to -2.5 comparing to healthy young woman (30-40 years old). However, a comprehensive evaluation of risk factors for fracture is necessary to define individual risks. The Fracture Risk Assessment tool (FRAX) is a web-based tool that integrates clinical risk factors and BMD at the femoral neck to calculate individual's 10-year absolute risk of hip or major osteoporotic fractures. The advantage of FRAX is incorporating the risk factors other than BMD, including age and previous fracture, and thus the decision for therapy could be made more easily (Kanis, J. A., et al., 2008).

Several types of drugs are currently available for osteoporosis treatment and they are classified as either anabolic (stimulating new bone formation) or anti-resorptive (suppressing bone resorption). PTH is approved for treatment of osteoporosis, its therapy first induces bone formation and subsequently induces both bone formation and resorption but the net result being anabolic (Arlot, M., et al., 2005; Lindsay, R., et al., 2006; McClung, M. R., et al., 2005). Treatment with teriparatide, an active fragment of PTH, has been demonstrated to reduce risk of vertebral and non-vertebral fractures among postmenopausal women with osteoporosis (Black, D. M., & Rosen, C. J., 2016). Concerning about a dose-dependent increase in the risk of

osteosarcoma observed in rodents, the treatment duration with teriparatide is limited by U.S Food and Drug Administration (FDA) to 24 months (Andrews, E. B., et al., 2012).

Wnt/β-catenin signaling pathway is one of the most important regulators of bone mass. Inhibition of sclerostin, an endogenous inhibitor of Wnt signaling, with monoclonal antibodies can enhance Wnt canonical signaling, leading to increased bone mass and enhanced resistance to bone fractures (Li, Xiaodong, et al., 2008). Romosozumab is the most promising anti-sclerostin antibody for the treatment of severe osteoporosis with a high risk of fracture. It promotes bone formation as well as suppresses bone resorption by binding to sclerostin to inhibit its activity. Romosozumab (AMG-785) is approved by FDA and European Medicines Agency (EMA) to treat osteoporosis in postmenopausal women at high risk of fracture.

Anti-resorptive therapies are used to suppress osteoclasts activity, thus reducing the rate of bone remodeling. They include five principal classes of agents: bisphosphonates, estrogens, calcitonin, selective estrogen-receptor modulators (SERMs), as well as biologicals, such as RANKL antibody Denosumab. Bisphosphonates, which are chemically stable analogues of pyrophosphate compounds, are most widely used to treat osteoporosis. Bisphosphonate treatment reduces the risk of vertebral fractures by 40-70% and the risk of hip fractures by 40-50% (Black, D. M.,et al, 1996; Brumsen, C., et al., 2002). There are still side effects, even though the treatment is effective, including osteonecrosis of the jaw and atypical fractures of femur that limit their high-dose and long-term use. Denosumab, a human monoclonal antibody to RANKL, is an anti-resorptive drug for the treatment of osteoporosis and other bone diseases. It inhibits bone resorption by binding to RANKL, leading to decreased differentiation of osteoclasts (Cummings, S. R., et al., 2009).

Estrogen replacement therapy (ERT) both prevents and is a supportive treatment for osteoporosis in postmenopausal women. Estrogen directly regulates osteocytes, osteoclasts as well as osteoblasts, resulting in suppression of bone resorption and maintenance of bone formation. However, because of non-skeletal risks of estrogen use that were revealed in the Women's Health Initiative (WHI), including breast cancer, cardiovascular and thrombotic events, estrogen therapy is not suggested in women who have been menopausal for more than 10 years (Cauley, J. A., et al., 2003). Calcitonin, an endogenous peptide, suppresses bone resorption by binding to its receptor on osteoclasts. It was approved for prevention and treatment of osteoporosis. However, it is now not recommended to use for osteoporosis prevention or treatment because of the risk of cancer with long-term use (Chesnut. C. H., et al., 2004). Raloxifene, a prototypic drug of SERMs, is FDA-approved for prevention and treatment of osteoporosis. It prevents bone loss, increases BMD slightly, and reduces the risk of vertebral fractures, but not non-vertebral or hip

fractures. Although it was shown to reduce the risk of breast cancer, raloxifene increases menopausal symptoms and the risk of venous thromboembolic events. Thus, raloxifene is not suggested to treat osteoporosis for the patients who have pronounced vasomotor menopausal symptoms or have risk factor for venous thrombosis (Compston. J. E., et al., 2019).

2.4.2 Osteogenesis imperfecta (OI)

OI is a genetically heterogeneous disorder of connective tissue characterized by increased bone fragility and deformity. The clinical range of OI phenotype is extremely wide, ranging from nearly asymptomatic individuals to early osteoporosis, severe bone deformities and perinatal lethality. Patients with OI exhibit different disorders, including atypical skeletal development, defective tooth formation, hearing loss, and hypermobile joints. The most classical form of OI (80-90%) is an autosomal dominant condition caused by mutations in *Col1* gene. OI was originally classified into four types (I-IV) based on clinical and radiographic criteria (Sillence, D. O., et al., 1979). More recently, the types of OI have been extended from type V to XVIII according to either phenotype or genetic defect.

A combination physical therapy, surgical invention and medical treatment are currently used for OI management. The treatment aims to improve bone strength, decrease fracture risk, improve mobility and independence of parents.

2.4.3 Osteoarthritis

OA, characterized by progressive damage of articular cartilage, changed structure of subchondral bone as well as surrounding joint tissues, is a painful joint disease. Clinically, the typical symptoms of OA are pain, transient stiffness in the morning and a grating sound when joint is in motion that impair quality of life. OA can be classified into primary and secondary. Primary OA is caused by multiple risk factors, including increasing age, obesity, genetics, female sex, biomechanics and low-grade systemic inflammation (Hunter, D. J., & Bierma-Zeinstra, S., 2019; Martel-Pelletier, J., et al., 2016). Secondary OA is caused by a predisposing condition, such as a previous joint injury, joint deformity or an underlying systemic disease (Sellam, J., & Berenbaum, F., 2013). Gender has some influence on the distribution of OA. Knee or hand OA is more common in women, while hip OA is more prevalent in men.

The pathogenesis of OA is complicated, involving inflammatory, mechanical loading, and metabolic factors that lead to damage of the synovial joint. Its lesions stem from an imbalance between the degeneration and the repair of joint tissues. During the OA process, the composition of cartilage is changed, which leads to its altered material properties and increased susceptibility to disruption (Loeser, R. F.,

et al., 2016). In the early stage of OA, cartilage degeneration is only at the surface; then the cracks progressively deepen, resulting in the expansion of calcified cartilage zone. Hypertrophic chondrocytes generate matrix degradation products as well as inflammatory mediators, such as collagens, proteoglycans, and proteases. In subchondral bone, bone remodeling is increased, leading to the formation of subchondral bone cysts (synovial fluid-filled holes) and osteophytes. In addition, vascular invasion arises, going from subchondral bone, through tidemark into the cartilage. Abnormal subchondral bone remodeling and angiogenesis contribute to the development of OA (Hunter, D. J., & Bierma-Zeinstra, S., 2019).

There are still no optimal preventive methods for primary OA and there are no effective ways to slow its progression. Therefore, the goals of OA treatment are to reduce pain, minimize joint damage and improve the quality of life. Currently, the management of OA includes non-pharmacological interventions, pharmacological interventions and surgical interventions (Martel-Pelletier, J., et al., 2016). The pharmacological treatments of OA are to relieve pain and reduce joint inflammation (Hunter, 2011). Paracetamol and non-steroidal anti-inflammatory drugs (NSAIDs) are the most common medical management for OA. However, they are associated with considerable side effects, especially related to cardiovascular and gastrointestinal events. Therefore, the treatment of oral NSAIDs should be limited to short-term use at the minimum dose (Martel-Pelletier, J., et al., 2016).

Intra-articular injection of corticosteroids is another therapy for OA to reduce inflammation. The local administration of corticosteroid can temporarily relieve pain, but it will become less effective as arthritis worsens.

If pharmacological treatments fail to sufficiently improve the symptoms of OA, surgery should be considered for end-stage OA. Surgical options for knee OA include arthroscopy, osteotomy and arthroplasty (joint replacement). The choice of surgical treatment is based on several factors, such as the level of pain, disease stage, and patient age (Hunter, D. J., & Bierma-Zeinstra, S., 2019).

2.5 Wnt signaling

Wnt signaling pathway is a highly conserved signal transduction cascade, playing a central role in cell proliferation, differentiation, polarization and migration during development. In 1976, scientists Sharma and Chopra reported a mutation in *Wingless* gene affecting the development of wings and halteres in *Drosophila melanogaster* (Sharma, R. P., & Chopra, V. L., 1976). Not long after that, the first member of Wnt family was identified by Nusse and Varmus in 1982 (Nusse, R., & Varmus, H. E., 1982). They showed that the mammary cells infected by mouse mammalian tumor virus (MMTV) slowly transformed to malignant cells. They named this proto-oncogene as *Int1* (first common integration site). After realizing that the sequence of *Int1* was also

present in Drosophila melanogaster, scientists cloned Drosophila *Int1* homologue and discovered that the *Int1* and *Wingless* genes were in fact identical (Rijsewijk, F., et al., 1987). Thereafter the genes belonging to *Int1/Wingless* family were unified as '*Wnt*' (*Wingless-related integration site*) in 1992 (Nusse, R., & Varmus, H. E., 1992).

Currently, 19 different Wnt ligands (Wnts), which belong to a family of lipid-modified glycoprotein of around 40kDa, have been identified. Wnts are expressed in different cell types or tissues; however, the expression of Wnts is cell- and tissue-specific (Marchetti, B., & Pluchino, S., 2013). During synthesis, Wnts, rich in cysteines, can be modified by an acyl group termed palmitoleic acid (Rios-Esteves, J., & Resh, M. D., 2013; Rios-Esteves, J.,et al., 2014; Takada, R., et al., 2006; Willert, K., et al., 2003), allowing Wnts to become hydrophobic and to be tethered to cell membranes. Therefore, the spreading of Wnt and its range of action is restricted. During the synthesis of Wnts, O-acyltransferase porcupine (PORCN) and Wntless (Wls) are necessary for transporting Wnts to the plasma membrane (Bänziger, C., et al., 2006; Kadowaki, T., et al., 1996). Subsequently, Wnts can be secreted through exocytic vesicles or exosomes into extracellular space to function either in an autocrine or a paracrine manner on cells (Bartscherer, K., et al., 2006).

Although Wnt proteins are traditionally assumed as long-range acting morphogens, increasing data suggests that Wnt signaling acts in the cells that are in contact with each other. Recently, Alexandre and colleagues have confirmed that membrane-tethered Wingless (Wg, the main *Drosophila* Wnt) acting in short-range is sufficient to rescue the Wg loss-of-function phenotype in *Drosophila* (Alexandre, C., et al., 2014). This discovery was supported by the study showing that membrane-bound Wnt3 signals in a short-range gradient to induce differentiation of intestinal stem cells (Farin, H. F., et al., 2016).

According to their downstream effects, all the Wnt ligands have been classified into two branches: canonical Wnt ligands that induce a Wnt/β-catenin signaling pathway (Wnt1, Wnt2, Wnt3, Wnt8a, Wnt8b, Wnt10a and Wnt10b) and non-canonical Wnt ligands that induce a Wnt/ β-catenin-independent pathway (Wnt4, Wnt5a, Wnt5b, Wnt6, Wnt7a, Wnt7b and Wnt16).

2.5.1 Canonical Wnt signaling pathway

The canonical Wnt signaling, also named as Wnt/β-catenin-dependent signaling, is initiated by binding of Wnt ligands to a dual receptor complex consisting of one of the seven transmembrane receptors of Frizzled family (Fzd) and low-density lipoprotein receptor-related protein 5/6 (Lrp5/6). In the absence of Wnt ligands, cytoplasmic β-catenin is phosphorylated by the destruction complex, consisting of the scaffold protein Axin, adenomatous polyposis coli (APC), phosphorylating enzyme glycogen synthase kinase-3β (GSK-3β) and casein kinase 1 (CK1). The

inactivated β-catenin is then polyubiquitinated by E3-ubiquitin ligase β-TrCP, following with degradation by the proteasome (Aberle, H., et al, 1997; Kitagawa, M., et al., 1999) (**Figure 4, A**).

In the presence of Wnt ligands, as soon as Wnts bind to co-receptors Fzd and Lrp5/6, Axin is attracted to the phosphorylated site of Lrp5/6 at the membrane by interacting with the scaffold protein dishevelled (Dsh). Consequently, the phosphorylation of β-catenin that is induced by destruction complex is prevented, resulting in the accumulation of activated β-catenin in cytoplasm. The non-phosphorylated β-catenin (activated β-catenin) then transports to the nucleus, where it binds to T-cell factor (TCF) and lymphoid enhancer binding factors (LEF), to induce the transcription of downstream genes (Choi, S. H., et al., 2013) (**Figure 4, B**).

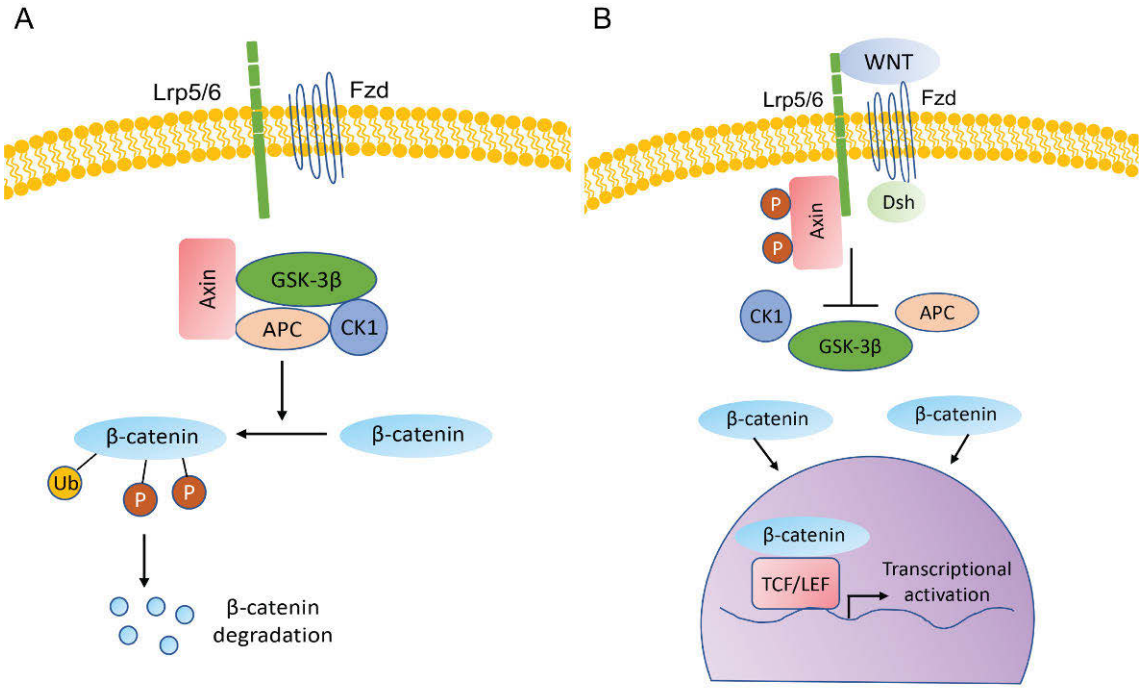

Figure 4. Schematic view of canonical Wnt/β-catenin signaling. **A.** In the absence of Wnt ligands. **B.** In the presence of Wnt ligands. Lrp5/6, Low-density lipoprotein receptor-related 5/6; Fzd, Frizzled; GSK-3β, glycogen synthase kinase-3β; APC, adenomatous polyposis coli; CK1, Casein kinase 1; Ub, Ubiquitin; WNT, Wingless-type MMTV integration site family; Dsh, Dishevelled; TCF/LEF, T-cell factor/lymphoid enhancer binding factor.

Multiple molecules that either promote or inhibit Wnt signaling can modulate canonical Wnt signaling. R-spondins, a family of Wnt agonists, enhance Wnt signaling by binding to three family members of 7-transmembrane receptors,

Leucine Rich Repeat Containing G Protein-Coupled Receptor 4/5/6 (Lgr4/5/6) (Kim, K. A., et al., 2006). Zinc and ring finger 3 (Znrf3) and ring finger 43 (Rnf43) are transmembrane proteins acting as ubiquitin E3 ligases for Fzd. The Lrg4/5/6-R-spondin complex induces degradation of Znrf3/Rnf43, resulting in enhancement of Wnt signaling as Fzd receptor becomes available (de Lau, W., et al., 2014).

In addition to the positive regulators, several well-known inhibitors that bind to either Wnt ligands or their receptors also regulate Wnt signaling. The Fzd-related proteins (sFRPs) and Wnt inhibitory factors (WIFs) are secreted Wnt inhibitors that directly bind to Wnt ligands and block the binding of Wnts and Fzd receptor, and thereby suppress Wnt signaling (Bovolenta, P., et al., 2008; Malinauskas, T., et al., 2011). The Dickkopf (DKK) protein family inactivate Wnt signaling by competitively binding to Lrp5/6. Extracellular part of Lrp5/6 consists of four β-propeller domains (BP1 to BP4 domains). DKK1 binds to the BP1 and BP3 domains of Lrp5/6 and forms a complex with Kremen receptor, accelerating internalization of Lrp5/6. DKK1 inhibits activation of Wnt signaling by blocking the binding of Wnt-Fzd to Lrp5/6 (Mao, B., et al., 2001; Mao, B., et al., 2002). Sclerostin is also an inhibitor of Wnt signaling that was identified as the gene mutated in sclerosteosis (Balemans, W., et al., 2001; Cruciat, C. M., & Niehrs, C., 2013). It is mainly expressed in osteocytes, encoded by *SOST* gene. Similar to DKK1, sclerostin suppresses Wnt signaling pathway by binding to co-receptor Lrp5/6 to disrupt the formation of Lrp5/6-Fzd complex. Recent studies have shown that Lrp4 is another receptor for sclerostin and sclerostin binding to Lrp4 negatively regulates Wnt signaling in osteoblasts, and thus, suppresses bone formation and promotes bone resorption (Chang, M. K., et al., 2014; Xiong, L., et al., 2015).

2.5.1.1 Canonical Wnt signaling pathway in bone development

Previous human genetic studies and animal studies have revealed the central role of Wnt signaling in regulating bone development and homeostasis. In the early 2000s, three independent research groups reported that loss-of-function and gain-of-function mutations in *Lrp5* caused extreme bone loss and high bone mass in patients, respectively (Gong, Y., et al., 1996; Heaney, C., et al., 1998; Johnson, M. L., et al., 1997). Subsequent studies discovered the roles of Wnt receptors and other components of the canonical Wnt pathway in bone development and disease. For example, osteoblast-specific deletion of Lrp6 or *Ctnnb1* (encodes β-catenin) in mice leads to low bone mass (Kramer, I., et al., 2010; Riddle, R. C., et al., 2013), while conditional activation of β-catenin results in significantly increased bone mass (Glass, D. A., et al., 2005; Holmen, S. L.,et al., 2005; Kramer, I., et al., 2010). On the other hand, alterations in the Wnt antagonists also affect bone mass in both humans and mice. For example, mutation of the SOST gene in human leads to

endosteal hyperostosis (van Buchem disease), and increased bone mass (Balemans, W., et al., 2002; Koide, M., & Kobayashi, Y., 2019). Deletion of SOST in mice results in increased bone mass, mimicking the human sclerosteosis phenotype (Li, X., et al., 2008). In addition, mice with deficiency in DKK1, another antagonist of Wnt signaling, also exhibit high bone mass (MacDonald, B. T., et al., 2007).

Recent genetic studies have shown the important roles of specific Wnt ligands that activate canonical Wnt signaling pathway in regulating bone development. Heterozygous mutations in *Wnt1* gene in human causes early-onset osteoporosis, while homozygous mutations of *Wnt1* lead to OI (Fahiminiya, S., et al., 2013; Keupp, K., et al., 2013; Laine, C. M., et al., 2013; Pyott, S. M., et al., 2013). In addition, homozygous mutations of Wnt3a in patients result in absence of all four limbs, while mutations in non-canonical ligand Wnt7a causes limb malformations (Niemann, S., et al., 2004; Woods, C. G., et al, 2006). Wnt10b has also been demonstrated to promote postnatal bone formation by inducing osteoblast differentiation. The Wnt10b deficiency in mice results in progressive bone loss with age, due to impaired differentiation of mesenchymal progenitor cells (Bennett, C. N., et al., 2005; Bennett, C. N., et al., 2007; Stevens, J. R., et al., 2010). Thus, canonical Wnt signaling pathway is crucial for bone development and homeostasis.

2.5.1.2 Canonical Wnt signaling pathway in Cartilage development

Extensive human genetic studies and studies using animal models have revealed that Wnt signaling has essential roles in regulating cartilage and skeletal development. Overexpression of Wnt1, Wnt4 or Wnt8a that generally activate Wnt/β-catenin signaling, in the developing limbs of chick embryo results in inhibition of chondrogenesis, while overexpression of Wnt5a, 5b or Wnt antagonist (Frzb/Frp3) that inhibit Wnt/β-catenin signaling, induces chondrogenesis (Church, V., et al., 2002; Duprez, D.,et al., 1999; Enomoto-Iwamoto, M., et al., 2002; Hartmann, C., & Tabin, C. J., 2000; Rudnicki, J. A., & Brown, A. M. C., 1997). However, unlike the actions of Wnt/β-catenin on chondrogenesis, overexpression of β-catenin promotes chondrocyte hypertrophy and endochondral ossification (Hartmann, C., & Tabin, C. J., 2000; Tamamura, Y., et al., 2005). On the other hand, overexpression of Wnt5a or Frzb/Frp3 results in inhibition of chondrocyte hypertrophy and endochondral ossification. These studies suggest that canonical Wnt/ β -catenin signaling suppresses chondrogenesis but stimulates chondrocyte hypertrophy. Interestingly, both activation and inactivation of β -catenin result in abnormal skeletal development in mice. Constitutive activation of β -catenin in developing cartilage in mice results in progressive loss of articular cartilage and disorganized growth plate (Guo, X., et al., 2004; Tamamura, Y., et al., 2005). However, targeted inactivation of β-catenin in limb bud mesenchymal cells using *Prrx1* or *Dermo* promoter leads

to impaired long bone formation with defected chondrocyte hypertrophy and mineralization (Day, T. F., et al., 2005; Hill, T. P., et al., 2005; Hu, H., et al., 2005). These findings indicate that a certain level of Wnt/β-catenin signaling is necessary for normal cartilage development, while insufficient or excessive activation can cause skeletal disorders.

A number of studies suggest that Wnt/β-catenin signaling is involved in cartilage degradation in OA patients. Nakamura et al. found an upregulation of Wnt7b in OA articular cartilage and in synovium in rheumatoid arthritis (Nakamura, Y., et al., 2005). Moreover, the expression of Wnt-induced signaling protein 1 (WISP-1) is increased in human OA cartilage and synovium and in OA mice model (Blom, A. B., et al., 2009). Francesco and colleagues used microarray analysis to characterize the response of human cartilage to injury and they showed upregulation of Wnt16 and several Wnt target genes, accompanied with downregulation of Wnt inhibitor Frzb and nuclear accumulation of β-catenin (Dell'Accio, F., et al., 2008). These data suggest that acute injury activates Wnt/β-catenin signaling in human articular cartilage. The expression of Wnt inhibitor *DKK1* is decreased in articular cartilage and synovium of OA patients (Honsawek, S., et al., 2010), while expression of *SOST*, an inhibitor of canonical Wnt/β-catenin signaling, is reported in late stage of OA (Chan, B. Y., et al., 2011).

2.5.2 Non-canonical Wnt signaling pathway

The non-canonical Wnt signaling, which is also known as β-catenin-independent Wnt signaling pathway can be subdivided into two well-characterized pathways: the Wnt/planar cell polarity pathway (PCP) and the Wnt/Ca^{2+} pathway. Neither of these pathways involve β-catenin and both of them inhibit canonical Wnt/β-catenin signaling pathway. The Wnt/PCP pathway is initiated by Wnts binding to Fzd and a co-receptor, which can be tyrosine kinase-like orphan receptor 2 (ROR2), related to receptor tyrosine kinase (RYK) or tyrosine protein kinase-like 7 (PTK7) (**Figure 5, A**). Wnt ligand-receptor complex induces the recruitment of Dsh to Fzd, leading to accumulation of dishevelled-associated activator of morphogenesis 1 (DAMM1). Damm1 subsequently activates either small G-protein Rho or Rac1. Rho stimulates Rho-associated protein kinase (ROCK), which regulates cytoskeleton, cell movement and tissue polarity (Baron, R., & Kneissel, M., 2013). Rac1 activates c-Jun amino-terminal kinase (JNK), which causes the activation of the transcription factors c-Jun and activating transcription factor 2 (ATF2) (Jenny, 2010).

The non-canonical Wnt/Ca^{2+} signaling pathway is initiated by binding of a Wnt ligand, such as Wnt5a, to Fzd and its co-receptor ROR1/2 (**Figure 5, B**). The Wnt-Fzd-ROR1/2 complex can activate phospholipase C-β (PLC-β) and subsequently stimulate intracellular Ca^{2+} release from endoplasmic reticulum (ER). The increased

level of intracellular Ca^{2+} leads to activation of Ca^{2+}-dependent signaling proteins, such as calmodulin-dependent protein kinase II (CamKII), protein kinase C (PKC) or calcineurin phosphatase, which activate the transcription factor, the nuclear factor of activated T-cell (NFAT) (Kohn, A. D., & Moon, R. T., 2005). Non-canonical Wnt/Ca^{2+} signaling is involved in many cellular activities, including cell adhesion and migration.

2.5.2.1 Non-canonical Wnt signaling pathway in bone development

Although the canonical Wnt signaling pathway has been known as the main component of Wnt signaling, there are growing evidences for the roles of non-canonical Wnt signaling in bone and the crosstalk between the pathways (Liu, Y., et al., 2007). Osteoblast-lineage cell-specific Wnt5a deficiency in mice exhibit decreased trabecular bone mass, due to impaired bone formation, while osteoclast precursor-specific deletion of Ror2, a well-known receptor of Wnt5a involved in non-canonical Wnt signaling, results in increased trabecular bone mass in mice, due to decreased osteoclastic bone resorption. This suggests that osteoblast lineage-derived Wnt5a activates Ror2 receptor in osteoclast precursors to promote osteoclastogenesis and bone resorption in mice. The authors also demonstrated that Wnt5a did not directly stimulate osteoclasts, even though it upregulated RANK receptor expression in osteoclast precursors through activation of JNK (Maeda, K., et al., 2012).

Wnt7b was proven to induce osteoblast differentiation via non-canonical Wnt/PKCδ signaling. Specific deletion of Wnt7b in skeletal cells have been reported to result in deficient embryonic bone formation (Tu, X., et al., 2007), while Wnt7b overexpression in osteoblast lineage in mice led to increased bone mass (Chen, J., et al., 2014). Mammalian target of rapamycin complex 1 (mTORC1) signaling partially contributes to Wnt7b-induced bone formation through activation of osteoblast function (Chen, J., et al., 2014). In a recent study of a genetic mouse model, Wnt16 was reported to play a key role in cortical thickness, while the effect on trabecular bone was not observed in mice (Movérare-Skrtic, S., et al., 2014). Moreover, the reduced cortical thickness in long bones of Wnt16 deficient mouse was due to increased bone resorption. The authors demonstrated that osteoblast derived Wnt16 inhibits osteoclastogenesis both by directly suppressing osteoclast progenitors and indirectly by inducing the expression of OPG in osteoblasts.

2.5.2.2 Non-canonical Wnt signaling pathway in cartilage development

Unlike canonical Wnt signaling pathway, our current knowledge on the role of non-canonical Wnt signaling pathway in cartilage is still limited. Wnt5a is the main Wnt

ligand that activates Wnt/PCP pathway. Global Wnt5a knockout mice exhibit severe skeletal deformity, while targeted overexpression of Wnt5a in chondrocytes in mice results in delayed chondrocyte hypertrophy and endochondral bone formation (Yang, Y., et al., 2003). These data suggest that Wnt5a is essential for normal development of cartilage and growth plate. Moreover, study from Gao et al. demonstrated that Wnt5a interacts with ROR2 and Van Gogh-like (Vangl), that is a core component in PCP, to regulate limb elongation and chondrocyte differentiation (Gao, B., et al., 2011). Tong et al. recently reported that Col2 promoter-targeted overexpression of Wnt16 in mice significantly suppressed chondrocyte hypertrophy and mineralization during bone development. In addition, Wnt16 inhibits chondrocyte hypertrophy by activating PCP/JNK and crosstalking with mTORC1-PTHrP pathway (Tong, W., et al., 2019).

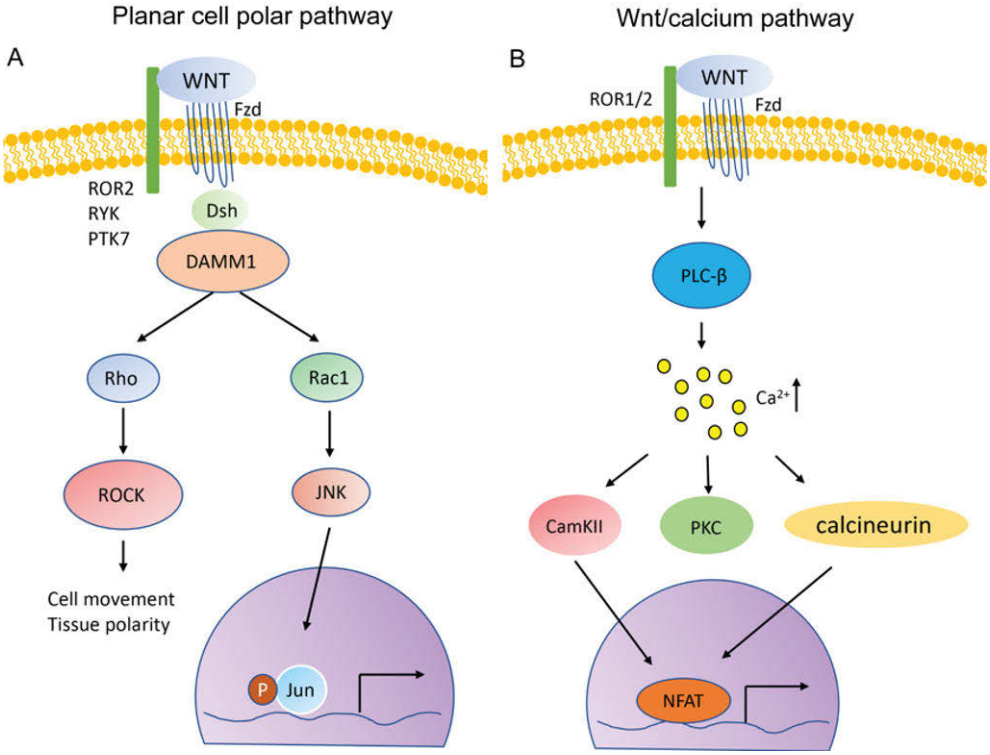

Figure 5. Schematic view of non-canonical Wnt signaling pathway. A. PCP pathway. B. Wnt/$Ca2^{+}$ pathway. Fzd, Frizzled; ROR2, tyrosine kinase-like orphan receptor 2; RYK, receptor tyrosine kinase; PTK7, tyrosine protein kinase-like 7; Dsh, Dishevelled; DAMM1, dishevelled-associated activator of morphogenesis 1; ROCK, Rho-associated protein kinase; Rac1, Rac family small GTPase 1; JNK, Jun amino-terminal kinase; PLC-β, phospholipase C-β; CamKII, calmodulin-dependent protein kinase II; PKC, protein kinase C; NFAT, the nuclear factor of activated T-cell.

2.6 Wnt1 in bone development

Wnt1, the first member of Wnt family, is an important regulator of many developmental decisions. As described in the previous chapter, PORCN and Wls are required for Wnt1 secretion. Before secretion, Wnt1 undergoes a lipid modification in ER by porcupine. After modification, Wnt1 is transported in secretory vesicles and is delivered to specialized microdomains of cell membrane called lipid rafts. Wnt1 then acts as a membrane-anchored protein, being tethered on the cell surface and functioning in a short range to regulate neighboring cells (Bradley, R. S., & Brown, A. M. C., 1990; Zhai, L., et al., 2004).

In mammals, Wnt1 that is important for brain development is expressed in neural tubes during embryogenesis. (McMahon, A. P., & Bradley, A., 1990). However, recent studies have reported that Wnt1 has an important role in bone metabolism. Laine and colleagues identified a heterozygous mutation of Wnt1 (Cys218Gly) in patients with dominantly inherited early-onset osteoporosis. In a separate family, they also identified a homozygous nonsense mutation in Wnt1 (Ser 295*) in the patients with recessive OI (Laine, C. M., et al., 2013). In contrast to wildtype Wnt1, its mutated form did not induce accumulation of stable non-phosphorylated β-catenin in either cytosolic or nuclear fractions in HEK293 cell line. In a superTOPFLASH-luciferase report assay, they found that mutated Wnt1 proteins did not induce Wnt/β-catenin signaling. In addition, expression of mutated Wnt1 resulted in decreased mineralization in MC3T3-E1 osteoblastic cells compared to the wild-type Wnt1. Studies from other groups have also found that Wnt1 mutations in patients cause moderately severe OI or bone fragility (Fahiminiya, S., et al., 2013; Keupp, K., et al., 2013; Pyott, S. M., et al., 2013). These results suggest that Wnt1 regulates bone mass in human via activating Wnt/β-catenin signaling. A mouse genetic study reported that Wnt1 Swaying mouse displayed spontaneous tibia fractures and severe osteopenia (Joeng, K. S., et al., 2014). Moreover, Wnt1 Swaying mouse exhibited reduced bone strength with decreased levels of collagen and mineral in bone matrix. Histomorphometric analyses demonstrated the bone defects in Wnt1 Swaying mouse were caused by impaired osteoblast activity.

The cellular source of Wnt1 in the body that is responsible for its skeletal effects remains unclear. Laine et al. found clear Wnt1 expression in the B-cell lineage of hematopoietic bone marrow as well as in brain tissue, but they did not detect notable Wnt1 expression in mouse calvarial osteoblasts or osteoclasts. On the other hand, Weivoda M.M et al. have demonstrated that osteoclast-derived TGF-β promotes Wnt1 expression in osteoclast, and thereby induces the differentiation of osteoblasts (Weivoda, M. M., et al., 2016). Investigations of cellular source of Wnt1 and its receptors are critical for understanding its role in bone metabolism.

3 Aims

The aim of this study was to investigate the role of Wnt1 and its molecular mechanisms of action in the regulation of bone and cartilage metabolism. Previous work has shown that Wnt1 is a key regulator of bone mass in humans and that Wnt signaling pathway plays an essential role in the skeletal development. However, the cellular source of Wnt1 and the molecular mechanisms, by which Wnt1 regulates bone and cartilage metabolism has been unclear.

The specific objectives of this study were:

I To identify the main cellular source of Wnt1 in bone microenvironment and its mechanisms of action in bone metabolism.

II To explore the role Wnt1 in post-developmental skeleton.

III To identify the role of Wnt1 in the development of articular cartilage, growth plate as well as subchondral bone.

4 Materials and Methods

4.1 Ethics statement (I, II, III)

All mouse studies were approved by the Finnish Ethical Committee for Experimental Animals (Project license number: 5186/04.10.07/2017 and KEK/2010-3003-Kiviranta), and were in compliance with the international guidelines on the care and use of laboratory animals. The protocols of animal experiments followed 3Rs principles (Replace, Reduce and Refine).

4.2 Materials

4.2.1 Primary antibodies (I)

For Western blots, the primary antibodies were rabbit anti-Wnt1 polyclonal antibody (Invitrogen, Carlsbad, CA, USA), rabbit anti-Non-phospho (Active) β-catenin antibody (Cell signaling Technology, Beverly, MA, USA) and rabbit anti-β-catenin antibody (Cell signaling Technology, Beverly, MA, USA).

For immunofluorescence, the primary antibodies were rabbit anti-Wnt1 polyclonal antibody (Abcam, Cambrige, MA, USA) and mouse anti-β-catenin monoclonal antibody (BD Biosciences, San Jose, CA, USA).

4.2.2 Cell lines/primary cells (I, III)

Table 1. Cell lines or primary cells used in the studies. The American Type Culture Collection (ATCC).

Cell lines/primary cells	Obtained from	Modification	Used in study
C3H10T1/2	ATCC	Overexpress Wnt1 or empty vector by retrovirus	I, III
MC3T3-E1	ATCC	Overexpress Wnt1 or empty vector by retrovirus	I
RAW264.7	ATCC	None	I
Primary calvaria osteoblast	Calvaria of newborn mice	None	I
Primary osteoclast (whole bone marrow)	Mouse bone marrow	None	I
Non-adherent bone marrow	Mouse bone marrow	None	I
$CD115^+$ monocyte	Mouse bone marrow	None	III
Primary chondrocyte	Mouse articular cartilage of knee joints	Overexpress Wnt1 or empty vector by retrovirus	III

4.3 Methods

4.3.1 Generation of Wnt1 knockout mice (I)

The targeting vector for the Wnt1 gene, PRPGS00167_B_B10, was obtained from The European Conditional Mouse Mutagenesis program (EUCOMM) (**Figure 6**). The correct targeting vector was further confirmed by PCR, restriction enzyme mapping and sequencing. In the construct, the LacZ-reporter gene and Neo-selection gene are inserted into intron 1 of Wnt1 gene, leading to generation of a truncated protein or nonsense-mediated decay. The G4 embryonic stem (ES) cells, derived from 129S6/C57BL/6Ncr mouse, were electroporated with 30 μg of linearized targeting construct with *AsiSI*. After electroporation, the ES cells were exposed to G418 (300 μg/ml; Sigma). Colonies were picked up after several days selection and DNA were extracted from selected ES cell colonies. Then long-range (LR)-PCR for both 5' and 3' homologous arms was performed to screen targeted ES colonies. The right targeted ES cells were used for blastocyst injection to generate chimeric mice. Germline transmission was achieved by crossbreeding male chimeras with C57BL/6N females. Genotyping of the mice was carried out with DNA extracted from the earmarks of 2-week-old to 3-week-old mice. The primers F1 and R1 were used for detecting mutated and wildtype alleles (**Table 2**).

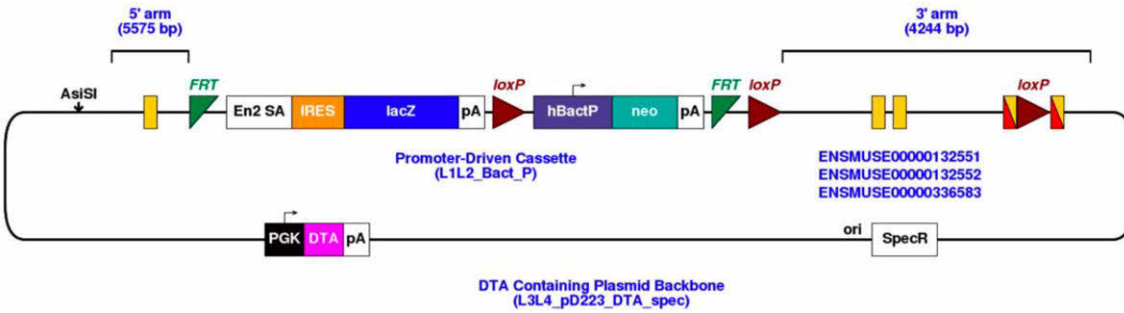

Figure 6: Targeting vector of *Wnt1* gene.

In order to delete Neo cassette, heterozygous $Wnt1^{LacZ/+}$ mice were bred with PGK-Cre mice, in which the expression of Cre recombinase is driven by phosphoglycerate kinase promoter in all tissues, leading to deletion of exons 2 to 4 of the Wnt1 gene and generation of global $Wnt1^{+/-}$ mice. The primers F1 and R1 were used for genotyping wild-type mice. The primers F2 and R2 were used for genotyping $Wnt1^{+/-}$ mice (**Table 2**).

4.3.2 Generation of Wnt1 cell type specific knockout mice (I, II, III)

To generate conditional Wnt1 knockout mice, heterozygous $Wnt1^{lacZ/+}$ females born from the chimera breeding were first bred with males expressing Flp recombinase in order to delete Neo and LacZ cassettes. In their offspring the exons 2-4 of the Wnt1 gene were flanked by Loxp sites ($Wnt1^{flox/+}$ mice). Genotyping of wild-type and $Wnt1^{flox/+}$ alleles was performed by PCR using primer pair F3 and R3 (**Table 2**).

In study I and III, to target Wnt1 knockout to early limb bud mesenchyme, $Wnt1^{flox/flox}$ female mice were crossed with $Wnt1^{flox/+}$ male mice expressing Cre recombinase driven by the Prrx1 promoter specifically expressed in early limb mesenchymal cells (Tg(Prrx-cre)1cjt/J mice) (Logan, M., et al., 2002) acquired from Jackson Laboratory (Bar Harbor, ME, US).

In study II, to target Wnt1 knockout to pre-osteoblasts, $Wnt1^{flox/flox}$ female mice were crossed with $Wnt1^{flox/+}$ male mice expressing Cre recombinase driven by Osx (Sp7) promoter (B6.Cg-Tg(Sp7-tTA,tetO-EGFP/cre)1Amc/J) (Rodda, S. J., & McMahon, A. P., 2006) acquired from Jackson Laboratory (Bar Harbor, ME, US). The presence of the Osx-cre gene was determined using the primer pair. In Osx-cre mouse model, treatment with a tetracycline analog doxycycline (Dox) prevents the expression of GFP-Cre recombinant protein and the recombination of the target gene.

To suppress Osx-Cre:GFP fusion gene transcription during fetal development, designated breeders received 0.2mg/ml Dox in drinking water during pregnancy until

delivery. Lactating dams and $Wnt1_{Osx}^{-/-}$ offspring received 1mg/ml Dox in drinking water until the age of 4 weeks. Drinking water with Dox was changed daily and protected from light. Then the experimental animals (male and female) were analyzed at the ages of 4 weeks, 8 weeks and 12 weeks.

Table 2. PCR primers used to verify correct targeting, knockout and genotype determination.

primers	sequence	Product size (bp)	Used in
F1 **R1**	TTCCACTGGTGCTGCCACGTCA TGGCAAAAGGGTTCGAGCCGAC	333 (mutated) 253 (wildtype)	Detecting mutated and wildtype alleles.
F2 **R2**	GCCATATCACATCTGTAGAG TGGCAAAAGGGTTCGAGCCGAC	442	Verification of cre/loxP recombination mediated deletion ($Wnt1^{+/-}$)
F3 **R3**	TGCATTGTGACTTCACATCC TTAAATGGGAATGGTCTCTG	319	Verification of flp/FRT recombination mediated deletion ($Wnt1^{flox/flox}$)

4.3.3 Microcomputed tomography (μCT) analysis

In studies I and III, μCT analyses were performed on tibias, lumbar L4 vertebrae and knee joint using a Skyscan 1070 μCT scanner (Skyscan, Kontich, Belgium) and imaged with an X-ray tube voltage of 72kV and current of 138 μA, with a 0.25mm aluminum filter. The scanning angular rotation was 182.45 degrees, and the angular increment was 0.45 degrees. Cross-sectional images were reconstructed with NRecon 1.4 software (Bruker, Kontich, Belgium). The regions of interests (ROI) were drawn using CTan 1.4.4 software and the results were then analyzed. BMD values were calibrated by two phantoms (calcium hydroxyapatite, with concentrations of 0.25 and 0.75g/cm^3). For analysis of the trabecular bone in tibia in study I, a ROI excluding the cortical bone was defined at the metaphysis of tibia starting 50 layers (488 μm) below the lower surface of the growth plate and extending 200 layers distally (1954 μm). For the analysis of cortical bone in study I, a slice at the diaphysis of tibia starting 5000 μm below the growth plate and extending for 50 layers was defined. For analysis of the trabecular bone in the 4th lumbar vertebrae in study I, an ROI excluding the cortical bone was defined at the metaphysis of the lumbar L4 vertebrae starting 20 layers (167.4 μm) above the distal growth plate and extending 150 layers proximally (1255.5 μm). For analysis of subchondral trabecular bone in tibia in study III, a ROI excluding subchondral plate was defined at the epiphysis of the tibia starting 50 layers (488 μm) above the lower surface of the growth plate and extending 20 layers (195 μm) proximally.

In study II, μCT analyses on the tibias or femurs were performed using Skyscan 1272 μCT scanner (Skyscan, Kontich, Belgium) and imaged with an X-ray tube voltage of 70 kV and current of 142 μA, with a 0.5 mm aluminum filter. The scanning

angular rotation was 192.8 degree, and the angular increment was 0.4 degree. Cross-sectional images were reconstructed with NRecon 1.4 software (Bruker, Kontich, Belgium). For the analysis of the trabecular bone in tibia, a ROI excluding the cortical bone was defined at the metaphysis of the tibia starting 50 layers (418 μm) below the lower surface of the growth plate and extending 120 layers distally (1004 μm). For the analysis of cortical bone, a cylinder at the diaphysis of the tibia starting 400 layers (3348 μm) below the growth plate and extending for 100 layers (837 μm) was defined. For the analysis of the trabecular bone in femur, a ROI was defined at the metaphysis of distal femur starting 120 layers (600 μm) above the surface of the distal growth plate and extending 240 layers (1200 μm) proximally. For analysis of cortical bone, a cylinder at the diaphysis of the femur starting 1100 layers (5500 μm) above the growth plate and extending for 200 layers (1000 μm) was defined.

4.3.4 Histomorphometric analysis (I, II, III)

For dynamic bone histomorphometry, mice were subcutaneously injected with calcein (Sigma-aldrich, St.Lous, MO, USA) (20 mg/kg body weight) and demeclocycline (40 mg/kg body weight) at 9 and 2 days and at 7 and 2 days prior to euthanasia for 12 and 6-week-old mice, respectively. The right tibias were fixed in 4% formaldehyde overnight and dehydrated in 70% ethanol. The fixed bones were embedded in methyl methacrylate.

For analyses of trabecular bone, undecalcified 5 μm-thick sections were cut with microtome, stained with Von Kossa method for mineralized bone, stained with 2% Toluidine Blue for the analysis of osteoblasts, osteoid, and osteoclasts, or stained with Tracp and counterstained with Toluidine Blue for the analysis of osteoclasts. Consecutive sections were left unstained for the analysis of fluorescence labeling. For analyses of cortical bone in study II, the tibias were sectioned in the transverse plane with a diamond saw and unstained 100 μm-thick sections were analyzed for static and dynamic parameters. Bone histomorphometric analysis was performed using OsteoMeasure software (Osteometrics Inc., Decatur, GA, USA). The structural parameters (bone volume per tissue volume [BV/TV], trabecular thickness [Tb.Th] and trabecular number [Tb.N]) were obtained by taking an average of three different measurements from consecutive sections. The structural, dynamic and cellular parameters were calculated and expressed according to the standardized nomenclature (Dempster, D. W., et al, 2013).

4.3.5 Histology and immunohistochemistry (I, III)

Tibias of adult C57BL/6N mice were fixed in 4% formaldehyde and decalcified in 10% EDTA, and 5 μm-thick paraffin-embedded sections were obtained. The sections

were deparaffinized, rehydrated and stained with hematoxylin & eosin (H/E) in study I and with Safranin O/Fast green for epiphyseal cartilage in study III.

For immunohistochemistry in study III, sections were boiled in 0.01M sodium citrate (pH 6.0) for 2 x 10 min to reveal the epitopes. Endogenic peroxidase activity was quenched by incubation in 0.3% hydrogen peroxide for 30 min. Expression of Wnt1 was investigated using a rabbit polyclonal primary antibody, followed by Invitrogen Histostain-Plus and biotinylated horseradish peroxidase-conjugated streptavidin. Peroxidase activity was visualized using 3'3-diaminobenzidine tetrahydrochloride-plus kit (Invitrogen). Images were captured using Zeiss Axioimager microscope and attached High End Image Analysis XEON workstation.

4.3.6 Cell culture models (I, III)

Primary calvarial osteoblasts were isolated from 3-day-old newborn mice (**Table 1**). Calvariae were isolated, and soft tissues were carefully removed. Osteoblasts were obtained with five consecutive 20-min digestions with 0.1% collagenase and 0.2% dispase in α-MEM at +37ºC. Fractions two to five were pooled and used for experiments. Cells were cultured in osteogenic medium (**Table 3**) and medium was changed every 2 days. Osteoblast differentiation was evaluated by alkaline phosphatase (ALP) staining by using Fast Blue RR Salt (Sigma-Aldrich) (Wang, Y., et al., 2016).

Primary bone marrow cells were flushed from femurs and tibiae of 6-9-week-old mice. For the co-culture of osteoblasts (MC3T3-E1-EV (empty vector)/Wnt1, C3H10T1/2-EV/Wnt1 or primary calvarial osteoblasts) and non-adherent bone marrow cells, the osteoblasts were without any treatment (**Table 1**). In order to exclude the possible effects of enhanced proliferation of Wnt1 overexpressing cells and the effects of Wnt1 on the heterogeneous population of bone marrow hematopoietic cells, we arrested the growth of C3H10T1/2-EV/Wnt1 with 10 μg/ml mitomycin C (Sigma-Aldrich), and then cultured them with Raw264.7 cells or mouse bone marrow monocytes $CD115^+$ cells. Bone marrow monocytic $CD115^+$ cells were isolated according to a standard protocol of the manufacturer (Miltenyi Biotec) (**Table 1**). Osteoclast differentiation of co-culture of growth-arrested C3H10T1/2-EV/Wnt1 and osteoclasts (Raw264.7 or $CD115^+$ cells) were induced with M-CSF and RANKL (**Table 3**). For the experiments with inhibitors, the co-culture of growth-arrested C3H10T1/2-EV/Wnt1 and osteoclasts (Raw264.7 or $CD115^+$ cells) were continuously treated with 10 μM XAV939 (Tocris Bioscience, Bristol) to inhibit canonical Wnt/β-catenin signaling pathway (Huang, S. M. A., et al., 2009), 10 μM KN93 (Tocris) to inhibit calcium-dependent non-canonical Wnt signaling pathway (Sumi, M., et al., 1991) or 5 μM SP600125 (Tocris) to inhibit JNK-dependent non-canonical Wnt signaling pathway (Bennett, B. L., et al., 2001).

Culture medium was changed every 2 days. At the end of experiments, osteoclast differentiation was evaluated by Tracp staining (Sigma-Aldrich) and counting the multinucleated Tracp positive cells.

Primary articular chondrocytes were isolated from 3-5 days-old newborn mice. Mouse hindlimbs were dissected and dipped first in 70% ethanol, then in sterilized phosphate buffered saline (PBS). The skin and soft tissues were removed and femoral head and tibial plateau were isolated by dissection. The cartilage pieces of knee joints were then digested in 3 mg/ml collagenase D (Gibco) for 2 h followed by an overnight digestion in 0.5 mg/ml collagenase D (Gibco). Digested articular cartilage pieces were filtered through a 100 µm Falcon™ cell strainer (Corning Life Sciences) followed by 1,500 rpm centrifugation for 5 min at RT. For micromass culture, freshly isolated primary articular chondrocytes were resuspended at a density of 1 x 10^7 cells/ml in chondrogenic medium, followed by pipetting 20 µl drops of cell suspension into each well of a 6-well plate. The cells were allowed to attach at 37ºC for 3 h and then 2 ml of chondrogenic medium (**Table 3**) was added. Chondrogenesis was evaluated by Alcian blue staining (Sigma-Aldrich).

Table 3. Cell culture models utilized in the studies.

Model	Cell type	Medium
Single culture	Primary calvarial osteoblasts	Osteogenic medium (α-MEM + 10%FBS + 10nM dexamethasone + 50µg/mL ascorbic acid + 5mM β-glycerophosphate)
Single culture	Primary osteoclasts (whole bone marrow)	α-MEM + 10%FBS + 10nM human PTH1-34
Single culture	Non-adherent bone marrow	α-MEM + 10%FBS + 10 ng/ml M-CSF + 50 ng/ml RANKL
Single culture	Primary chondrocytes	Chondrogenic medium (D-MEM + 10%FBS + 2mM Glutamin + Insulin-Transferin-Selenium (ITS-G))
Co-culture	MC3T3-E1-EV/Wnt1 + non-adherent bone marrow	α-MEM + 10%FBS + 10^{-8} M vitamin D +10^{-6} M prostaglandin E2
Co-culture	Primary calvarial osteoblasts + non-adherent bone marrow	α-MEM + 10%FBS + 10 ng/ml M-CSF + 50 ng/ml RANKL
Co-culture	C3H10T1/2-EV/Wnt1 + non-adherent bone marrow	α-MEM + 10%FBS + 10 ng/ml M-CSF + 50 ng/ml RANKL
Co-culture	C3H10T1/2-EV/Wnt1 + Raw 264.7 cell line	α-MEM + 10%FBS + 50 ng/ml RANKL
Co-culture	C3H10T1/2-EV/Wnt1 + mouse bone marrow monocytes $CD115^+$	α-MEM + 10%FBS + 10 ng/ml M-CSF + 50 ng/ml RANKL

4.3.7 Retrovirus-mediated Wnt1 transfection (I, III)

Human Wnt1 gene coding region was cloned to pBabe-puro retrovirus vector. Wnt1 and empty viruses were produced in human embryonic kidney (HEK)293 Phoenix cells according to standard methods. Target cells (C3H10T1/2, MC3T3-E1) were incubated with viral media containing 4 μg/ml polybrene overnight, followed by selection in puromycin at the concentration of 3 μg/ml for 2 to 3 days, after which the puromycin-resistant cell pools were expanded and plated for subsequent experiments.

4.3.8 Western blot (I)

Cells were lysed in RIPA (10mM Tris-HCl pH 7.5, 1%DOC, 0.1%SDS, 1%Triton X 100 and 150mM NaCl) lysis buffer with protease inhibitors (Complete mini; ThermoFisher Scientific). The insoluble material was centrifuged (12,000g, 4°C, 10 min), and supernatants were collected. The lysate was added with 6x SDS loading buffer, boiled and electrophoresed on 10% SDS-PAGE gels and transferred to nitrocellulose membranes (Maine Manufacturing). Membranes were blocked in 5% milk-TBS-Tween 20 for 30 min at RT, and then incubated with primary antibody overnight at 4°C, followed by 1h incubation at RT with secondary antibodies. The protein bands were visualized with WesternBright Quantum (Advansta Inc.) and LAS 4000 Luminescent imager (FUJIFILM). Quantification of the density of each band was performed by Image J software (NIH, Bethesda).

4.3.9 Immunofluorescence staining (I)

C3H10T1/2-Wnt1 cells used for immunofluorescence were seeded on coverslips at the density of 1 x 10^4 cells/well in 12-well plates and treated with 10 μg/ml mitomycin C for 2 h. C3H10T1/2-EV cells were seeded on top of C3H10T1/2-Wnt1 cells at the density of 9 x 10^4 cells/well. Next day, the cells were rinsed with PBS and fixed in 3.7% formaldehyde for 15 min at RT. After blocking in 5% Normal Goat Serum Control (ThermoFisher Scientific) for 30 min, sections were incubated overnight at 4°C with primary antibody. Subsequently, cells were washed in PBS and incubated in secondary antibody for 1 h at RT. Then slides were mounted in mounting medium with DAPI (Vector Laboratories).

4.3.10 Proliferation assay (I, III)

Cells were seeded in 96-well plates at a density of 1,500 cells/well. The CellTiter 96 Non-radioactive Cell Proliferation Assay (Promega, Madison) was used to detect cell viability at indicated time points. Briefly, 20 μl dye solution was added to each well and incubated at 37°C for 4 h. The Solubilization/Stop solution was then added to the culture wells. The absorbance at 570 nm was measured using a 96-well plate reader.

4.3.11 RNA isolation and quantitative real-time PCR (qRT-PCR) analysis (I, II, III)

For mRNA extraction, murine humeri with bone marrow and knee joints including distal femoral and proximal tibial epiphyseal cartilage were snap-frozen in liquid nitrogen and stored in -80 °C. The humeri and knee joints were pulverized in liquid nitrogen and the RNA was isolated using TRIsure reagent (Bioline) followed by RNeasy Mini Kit (Qiagen). For mRNA extraction from cell cultures, total RNA was prepared using RNeasy mini kit according to the manufacturer's instructions. The cDNA was synthesized with SensiFAST cDNA Synthesis Kit (Bioline). RNA expression of the genes of interest (**Table 4**) was determined using Dynamo Flash SYBR Green qPCR kit (ThermoFisher Scientific). mRNA levels were normalized to β-actin and expression was quantified by using $2^{-\Delta\Delta CT}$ method.

Table 4. PCR primers used for qRT-PCR of genes of interest.

Primers	Forward	Reverse
ALP	ACTCAGGGCAATGAGGTCAC	CACCCGAGTGGTAGTCACAA
OPG	ACCCAGAAACTGGTCATCAGC	CTGCAATACACACACTCATCACT
DKK1	GACAACTACCAGCCCTACCC	GATCTGTACACCTCCGACGC
SOST	TCCTGAGAACAACCAGACCA	GCAGCTGTACTCGGACACATC
OSX	GTCCTCTCTGCTTGAGGAAGAA	GGGCTGAAAGGTCAGCGTAT
Runx2	GCCCAGGCGTATTTCAGA	TGCCTGGCTCTTCTTACTGAG
OCN (BGLAP)	TGAGGACCATCTTTCTGCTCA	TGGACATGAAGGCTTTGTCA
OPN	CCCGGTGAAAGTGACTGATT	ATCTGGGTGCAGGCTGTAA
TCF1	GCGCGGGATAACTACGGAAA	ACTGTCATCGGAAGGAACGG
hWnt1	CGCTGGAACTGTCCCACT	AACGCCGTTTCTCGACAG
Col1a1	AGACATGTTCAGCTTTGTGGAC	GCAGCTGACTTCCAGGGA TG
SOX9	GCCACGGAACAGACTCACAT	AGATTGCCCAGAGTGCTCG
Aggrecan	ACAGACCCCAACACCTACAAG	AGCGACAAGAAGACACCATGT
Col10a1	CATCTCCCAGCACCAGAATCTA	CAAGTGGGCCCTTTATGCCT
Comp	ACCGTTGCAATGATACAATCCC	CACACACACCCGTAGCTTGT
Col2a1	GCTGGAAAACCTGGTGACGA	GCCTGGGTAACCTCTGTGAC
PTHrP	CCCGACGCCTATGTAAAACG	CTCTGGGGACTTGTGTAGGA
PTH1R	ACTGTGGCAGATCCAGATGC	AATCTCTGCCTGCACCTCAC
Axin2	AAGCCCCATAGTGCCCAAAG	GGGTCCTGGGTAAATGGGTG
RANKL	AGGCTGGGCCAAGATCTCTA	GTCTGTAGGTACGCTTCCCG
Nfatc1	TCCAAAGTCATTTTCGTGGA	CTTTGCTTCCATCTCCCAGA
Ctsk	CGAAAAGAGCCTAGCGAACA	TGGGTAGCAGCAGAAACTTG
Tracp	CGTCTCTGCACAGATTGCAT	AAGCGCAAACGGTAGTAAGG
ATP6v0d2	AAGCCTTTGTTTGACGCTGT	GCCAGCACATTCATCTGTACC
OSCAR	CGTGCTGACTTCACACCAAC	GTCACGTTGATCCCAGGAG
DC-STAMP	AAATCAGCAGAAGGCTCCAC	ATGAAGCGGTGCAGGAGA
β-actin	CGTGGGCCGCCCTAGGCACCA	TTGGCCTTAGGGTTCAGGGGG

4.3.12 Statistical analyses (I, II, III)

Values are given as mean ± SD. For the comparisons of data following normal distribution, we performed 2-tailed Student's *t* tests with a significance level of 0.05. For the comparisons of data that did not follow normal distribution, we performed Shapiro-Wilk test to detect normality among groups and either applied Steel-Dwass test for the comparing groups with unequal sample sizes, or applied the Mann-Whitney test for the comparing groups with equal size. One-way ANOVA with Bonferroni's comparisons test was used when comparing multiple groups to determine the statistical significance. *P<0.05, **P<0.01, ***P<0.001.

5 Results

5.1 Wnt1 in bone formation and osteoblast differentiation (I, II)

5.1.1 Mesenchymal cell derived Wnt1 regulates bone mass in both trabecular and cortical compartments (I)

To identify the cellular source of Wnt1 and the mechanisms causing severe phenotypes in Wnt1 mutant patients, we generated global and conditional mesenchymal progenitor-targeted Wnt1-deficient mice. Homozygous global knockout ($Wnt1^{-/-}$) embryos were lost in utero. Heterozygous $Wnt1^{+/-}$ mice were viable and developed normally. The body weights of $Wnt1^{+/-}$ mice did not differ from the littermate $Wnt1^{+/+}$ controls. µCT analysis of $Wnt1^{+/-}$ mice revealed mild osteopenia in both proximal tibia bones and fourth lumbar vertebrae at 12 weeks of age (I, Fig.1 A-D). Histomorphometric analysis of proximal tibiae of $Wnt1^{+/-}$ mice did not show any significant differences in any of the parameters analyzed (I, supplemental Fig. 2 A and B). However, nonsignificant decreases in mineral apposition rate (MAR) and bone formation rate (BFR/BV) were observed (I, supplemental Fig. 2 B).

We next targeted Wnt1 knockout into early limb bud mesenchymal cells using Prrx1-Cre mice ($Wnt1_{Prrx1}^{-/-}$). $Wnt1_{Prrx1}^{-/-}$ mice were viable but developed spontaneous fractures in long bones as early as 4 weeks of age (I, Fig. 2A). µCT and histomorphormetric analyses of $Wnt1_{Prrx1}^{-/-}$ mice in proximal tibia showed severe trabecular osteopenia at both 6 and 12 weeks of age (I, Fig. 2F and G). At cortical site, cortical BV/TV and thickness (Cort.Th) in the diaphysis of $Wnt1_{Prrx1}^{-/-}$ mice were significantly decreased (I, Fig. 2F and G).

Interestingly, the osteoblast number (N.Ob) or surface were not altered while osteoblast activity was severely impaired in $Wnt1_{Prrx1}^{-/-}$ mice at 6 weeks of age (I, Fig. 2J and supplemental Fig. 2 C). Similar to µCT analysis, the bone phenotype was milder at 12 weeks, but mineral surface (MS/BS) and BFR/BV were still significantly lower in $Wnt1_{Prrx1}^{-/-}$ mice (I, Fig. 2J and supplemental Fig. 2C). Taken together, $Wnt1_{Prrx1}^{-/-}$ mice presented with an OI phenotype with early spontaneous

fractures and low bone mass in both trabecular and cortical compartments, similar to the patients carrying the homozygous WNT1 mutation.

5.1.2 Mesenchymal cell derived Wnt1 stimulates osteoblast differentiation in a juxtacrine manner (I)

To address the mechanisms how Wnt1 regulates osteoblast differentiation and activity, we analyzed the differentiation of $Wnt1^{+/-}$ primary calvarial osteoblast progenitors. $Wnt1^{+/-}$ calvarial osteoblasts exhibited impaired differentiation potential (I, Fig. 3A). In agreement with this, $Wnt1^{+/-}$ cells expressed lower level of Wnt target genes and osteoblast-specific genes, *DKK1* and *SOST* as well as *OPG* (I, Fig. 3B).

To investigate this using opposite approach, we overexpressed Wnt1 in mesenchymal progenitor cell line C3H10T1/2, which does not express endogenous Wnt1. Wnt1 overexpression robustly induced osteoblast differentiation (I, Fig. 3C). Expectedly, overexpression of Wnt1 increased the expression of Wnt target genes, such as Axin2, DKK1 and SOST (I, Fig. 3D).

We were not able to produce Wnt1 conditional medium by overexpressing Wnt1 in L cell, a widely used cell line for production of medium containing Wnt ligands. But when mixed with control cells Wnt1 overexpressing cells efficiently induced osteoblastic differentiation in the whole culture and increased the expression of active non-phosphorylated β-catenin (I, Fig. 3C and supplemental Fig. 3A and B). Moreover, we found that overexpression of Wnt1 could stimulate the proliferation of C3H10T1/2 cells (I, Fig. 3E). To investigate whether Wnt1 could induce osteoblast differentiation in neighboring control cells and to exclude the enhanced proliferation of C3H10T1/2-Wnt1 cells, we cultured growth-arrested C3H10T1/2-Wnt1 cells with control cells (C3H10T1/2-EV) at different ratios. Reduction of the ratio between C3H10T1/2-EV and C3H10T1/2-Wnt1 cells led to decreased ALP activity (I, Fig. 3F), suggesting that cell-cell contact is necessary for Wnt1 inducing osteoblast differentiation in the neighboring cells.

To further explore this, cell culture well inserts, which allow the passage of humoral factors and small particles between two separated compartments, were used. Indeed, C3H10T1/2-Wnt1 cells in the insert were not able to induce differentiation of the cells at the bottom of the well (I, Fig. 3G and H), suggesting that Wnt1 induces osteoblast differentiation in a juxtacrine manner, requiring immediate proximity to the target cell.

To investigate whether Wnt1 requires cell-cell contact to induce Wnt/β-catenin signaling, we plated C3H10T1/2-EV, C3H10T1/2-Wnt1, or C3H10T1/2-Wnt1 with C3H10T1/2-EV cells in 1:10 ratio in coverslips. The cells were then fixed and stained for Wnt1 and β-catenin. Images by confocal microscope proved that Wnt1 stimulated nuclear accumulation of β-catenin only in the Wnt1-overexpressing

cultures, in which it may work both in an autocrine and juxtacrine manner (I, Fig. 4 A and B). Moreover, we found nuclear accumulation of β-catenin in control cells only when in direct contact with C3H10T1/2-Wnt1 cells, but not in the cells without contact with C3H10T1/2-Wnt1 cells (I, Fig. 4 A and B). These data suggested that Wnt1 functions in a juxtacrine manner, requiring cell-cell contact.

5.1.3 Osteoblastic Wnt1 regulates cortical bone thickness in adult mice by stimulating periosteal bone formation (II)

In study II we generated osteoblast lineage-targeted Wnt1 knockout mouse model using tetracycline-controlled Osx-Cre mouse line to investigate the role of Wnt1 specifically in adult bone metabolism. In this mouse model, the expression of fusion protein EGFP-Cre is prevented by administering of doxycycline (Dox) in drinking water allowing for the temporal control of target gene inactivation (II, Fig. 2A).

$Wnt1_{Osx}^{-/-}$ mice without Dox administration were viable, but exhibited spontaneous fractures in multiple skeletal sites as early as 3 weeks of age (II, Fig. 1A). Moreover, $Wnt1_{Osx}^{-/-}$ mice showed decreased body weight, as well as decreased length of tibia and femur compare to $Wnt1^{fl/+}$, $Wnt1^{fl/fl}$ and $Wnt1_{Osx}^{+/-}$ mice (II, Fig. 1B). Interestingly, the heterozygous knockout $Wnt1_{Osx}^{+/-}$ mice were significantly lighter and their tibias and femurs were shorter than $Wnt1^{fl/fl}$ mice, suggesting that the expression of Osx-Cre had an effect on body weight and length of tibia and femur (II, Fig. 1B). μCT analysis of $Wnt1_{Osx}^{-/-}$ mice in tibia revealed severe trabecular osteopenia (II, Fig. 1C). Furthermore, cortical BV/TV and Cort.Th in the diaphysis of $Wnt1_{Osx}^{-/-}$ mice was significantly decreased compared to $Wnt1^{fl/fl}$ mice (II, Fig. 1C). In line with significantly decreased bone mass, the mRNA expression of bone markers, such as *ALP*, *OSX* and *Runx2* were all downregulated in humeral bones of $Wnt1_{Osx}^{-/-}$ mice compared to $Wnt1^{fl/fl}$ and $Wnt1_{Osx}^{+/-}$ mice (II, Fig. 1D).

Next, we induced osteoblast targeted Wnt1 knockout in adolescent mice. Dox was administrated to $Wnt1_{Osx}^{-/-}$ ($Wnt1_{Osx\text{-}Dox}^{-/-}$) and control mice ($Wnt1^{fl/fl}$ and $Wnt1_{Osx\text{-}Dox}^{+/-}$) in drinking water during pregnancy, lactation and thereof until 4 weeks of age to suppress Wnt1 deletion. Then Dox was withdrawn to induce Wnt1 deletion and mice were analyzed at 8 and 12 weeks of age (II, Fig. 2A). $Wnt1_{Osx\text{-}Dox}^{-/-}$ mice developed normally and their body weight was not altered compared to littermate controls at 8 and 12 weeks of age. However, spontaneous fractures were observed in the distal tibia of $Wnt1_{Osx\text{-}Dox}^{-/-}$. The penetrance of fractures was 25% and 50% in male and female at 8 weeks of age, respectively. At 12 weeks, males did not exhibit spontaneous fractures while 29% of females still displayed tibia fractures (II, Fig. 2B).

μCT analysis of the distal femur revealed a mild trabecular osteopenia phenotype in young female $Wnt1_{Osx\text{-}Dox}^{-/-}$ mice at 8 weeks of age (II, Fig. 2C and D). However,

Wnt1 deletion in osteoblasts of adult mice did not affect trabecular bone structure in young males or older males or females. Histomorphometric analysis of proximal tibia confirmed that there was no significant trabecular bone structure phenotype or alterations in cellular or dynamic parameters in this area in $Wnt1_{Osx\text{-}Dox}^{-/-}$ at 12 weeks of age (II, Table 1).

At the cortical site, μCT analysis of proximal femur showed reduced BV/TV and Cort.Th in $Wnt1_{Osx\text{-}Dox}^{-/-}$ mice (II, Fig. 3B and C). These findings suggested that Wnt1 specifically regulates cortical bone metabolism in adult skeleton. In addition, although reduction of cortical thickness was observed in both genders of $Wnt1_{Osx\text{-}Dox}^{-/-}$ mice, it was generally more pronounced in females compared to males. Moreover, a trend of decreased total tissue volume (TV) and significantly lower cortical bone volume was observed in $Wnt1_{Osx\text{-}Dox}^{-/-}$ mice at both 8 and 12 weeks of age compared to controls, while the volume of bone marrow volume (BMV) was unchanged (II, Fig. 3C). These data suggested that the thinner cortical bone in $Wnt1_{Osx\text{-}Dox}^{-/-}$ mice might be due to impaired periosteal bone apposition.

Histomorphometric analysis of the distal tibia mice confirmed a reduced cortical thickness in 12 weeks-old female $Wnt1_{Osx\text{-}Dox}^{-/-}$ mice when compared to $Wnt1_{Osx\text{-}Dox}^{+/-}$ (II, Fig. 4A). In addition, the periosteal BFR/BV was markedly reduced in $Wnt1_{Osx\text{-}Dox}^{-/-}$ mice due to reduced MS/BS and MAR (II, Fig. 4C). In contrast, at the endosteal surface, MS/BS and BFR/BV were unaffected in $Wnt1_{Osx\text{-}Dox}^{-/-}$ mice (II, Fig. 4C). These results demonstrate that the reduced cortical bone thickness observed in $Wnt1_{Osx\text{-}Dox}^{-/-}$ mice is due to impaired periosteal bone formation.

5.2 Wnt1 in bone resorption and osteoclastogenesis (I)

5.2.1 Wnt1 suppresses bone resorption in vivo (I)

In study I, to further examine the bone homeostasis in $Wnt1^{+/+}$ and $Wnt1^{+/-}$ mice, osteoclast parameters (osteoclast surface (Oc.S), osteoclast number (N.Oc) and eroded surface (ES/BS)) were assessed by histomorphometry. The N.Oc and ES/BS showed a trend towards an increase in $Wnt1^{+/-}$ mice compared to $Wnt1^{+/+}$ mice but the changes were not significant (I, supplemental Fig. 2B). Histomorphometric analysis in tibia of $Wnt1_{Prrx1}^{-/-}$ mice at 12 weeks demonstrated that the N.Oc was increased by 50% $Wnt1_{Prrx1}^{-/-}$ mice compared to the $Wnt1_{Prrx1}^{+/+}$ mice ($p=0.0032$) (I, Fig. 2J). In addition, ES/BS also showed a significant increase in $Wnt1_{Prrx1}^{-/-}$ mice, suggesting that osteoclastic bone resorption was clearly increased in $Wnt1_{Prrx1}^{-/-}$ mice (I, Fig. 2J).

5.2.2 Wnt1 directly suppresses osteoclastogenesis in a juxtacrine manner (I, III)

To investigate how Wnt1 regulates osteoclast differentiation *in vitro*, we performed total bone marrow cultures from $Wnt1^{+/+}$ and $Wnt1^{+/-}$ mice with PTH treatment. Bone marrow cells of $Wnt1^{+/-}$ mice showed a trend towards increased osteoclastogenesis (I, supplemental Fig. 5A and B). However, co-culture experiments of control and $Wnt1^{+/-}$ calvarial cells mixed with control or $Wnt1^{+/-}$ non-adherent bone marrow cells did not indicate any significant osteoclast phenotype in $Wnt1^{+/-}$ cells (I, supplemental Fig. 5C and D).

We also performed co-culture of Wnt1 overexpression in MC3T3-E1 cells and non-adherent bone marrow cells and demonstrated that osteoclastogenesis was completely inhibited by Wnt1 overexpressing MC3T3-E1 cells (I, supplemental Fig. 6A and B). In addition, the expression of RANKL was unchanged, while the expression of OPG, which is a decoy receptor of RANKL inhibiting osteoclastogenesis, was significantly increased in Wnt1 overexpressing E1 cells (I, supplemental Fig. 6 C). This finding provided a potential mechanism, by which Wnt1 could indirectly regulate osteoclastogenesis.

Next, we overexpressed Wnt1 in C3H10T1/2 cells and performed co-culture of Wnt1 overexpressing C3H10T1/2 cells with non-adherent bone marrow cells at different density and induced osteoclast differentiation with M-CSF and RANKL. Again, Wnt1-overexpressing cells suppressed osteoclastogenesis, accompanied with decreased expression of downstream target of RANKL signaling the nuclear factor of activated T-cells, cytoplasmic 1 (*Nfatc1*) and osteoclast markers *Tracp* and *Ctsk* (I, Fig. 5A-C). In contrast to MC3T3-E1 cells, the mRNA expression of *OPG* and *RANKL* were unchanged in Wnt1-overexpressing C3H10T1/2 cells. Interestingly, the suppression of osteoclastogenesis was not observed at low density of Wnt1-overexpressing C3H10T1/2 cells. To test whether Wnt1 could directly suppresses osteoclastogenesis, insert culture of Wnt1-overexpressing or control C3H10T1/2 (in insert) and wild-type non-adherent bone marrow cells (in wells) was performed. No suppression of osteoclastogenesis was observed in this experiment suggesting that Wnt1 directly suppresses osteoclast differentiation in a juxtacrine manner (I, Fig. 5D-F).

To exclude the possible effects of enhanced proliferation of Wnt1-overexpressing cells and the effect of Wnt1 on the heterogenous population of bone marrow hematopoietic cells, we co-cultured growth-arrested Wnt1 or control C3H10T1/2 cells with either murine secondary monocytes/macrophage-like cell line Raw264.7 cells (I, Fig. 5G-I) or purified mouse bone marrow monocytes $CD115^{+}$ cells (III, Fig. 7A) and induced osteoclast differentiation. Wnt1-overexpressing cells suppressed osteoclastogenesis also in these two models, accompanied with decreased mRNA expression of osteoclast markers *Nfatc1*, *Ctsk* and *Tracp* (I, Fig.

5J; III, Fig. 7B). In the co-culture of C3H10T1/2 cells and Raw264.7 cells, we found that the OPG protein levels were actually decreased in the medium of Wnt1-overexpressing cultures suggesting the suppression of osteoclastogenesis by Wnt1 was not due to soluble OPG (I, Fig. 5K). Moreover, in the co-culture of C3H10T1/2 cells and mouse $CD115^+$cells, the mRNA expression of cell-cell fusion markers *ATP6v0d2* and Osteoclast-associated immunoglobulin-like receptor (*OSCAR*) were significantly downregulated in Wnt1-overexpressing cultures, suggesting that Wnt1 inhibits osteoclastogenesis of $CD115^+$ cells by suppressing cell-cell fusion (III, Fig. 7B). Based on our data of *in vitro* experiments, we conclude that mesenchymal cell derived Wnt1 can directly inhibit osteoclastogenesis in a juxtacrine manner, requiring physical contact with the osteoclast progenitors.

5.2.3 Wnt1 suppresses osteoclastogenesis through canonical Wnt signaling (I, III)

Previous studies have reported that different Wnt ligands and pathways of Wnt signaling have different effects on osteoclast differentiation (Maeda, K., et al., 2012; Movérare-Skrtic, S., et al., 2014; Weivoda, M. M., et al., 2016). To investigate the mechanism of Wnt1 action on osteoclastogenesis, we used small molecular inhibitors XAV939 (antagonizes canonical Wnt signaling via stimulation of β-catenin degradation and stabilization of axin) (Huang, S. M. A., et al., 2009), KN93 (inhibitor of calcium-dependent noncanonical Wnt signaling) (Sumi, M., et al., 1991) and SP600125 (inhibitor of JNK-dependent noncanonical Wnt signaling) (Bennett, B. L., et al., 2001) in C3H10T1/2-Raw264.7 and C3H10T1/2-$CD115^+$ co-culture. Administration of XAV939 slightly enhanced osteoclastogenesis in control cultures and completely rescued osteoclastogenesis in Wnt1-overexpressing co-cultures of C3H10T1/2-Raw264.7 and C3H10T1/2-$CD115^+$ cells (I, Fig. 6A and B; **Figure. 7**). In line with this, XAV939 significantly downregulated Wnt1 target genes Axin2 and TCF1 in the Wnt1-overexpressing cultures and partially rescued the expression of osteoclast marker Ctsk (I, Fig. 6C). In co-culture of C3H10T1/2-Raw264.7, inhibition of calcium signaling by KN93 strongly inhibited osteoclastogenesis in both control and Wnt1-overexpressing culture wells and Wnt1 did not modify the effect, while SP600125 partially blocked Wnt1 effect on osteoclastogenesis (I, Fig. 6A-B). Based on these data, we could conclude that Wnt1 effect on osteoclastogenesis mainly through canonical Wnt signaling pathway, however, the suppression of osteoclast differentiation by Wnt1 may also be partly through JNK-dependent noncanonical Wnt signaling pathway.

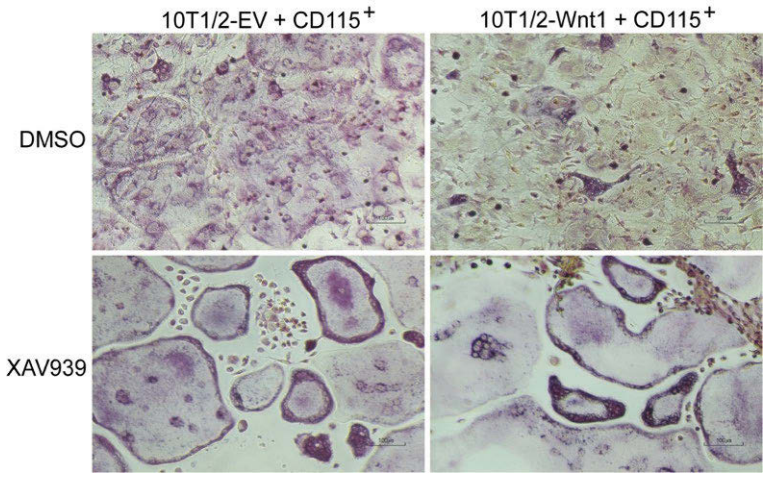

Figure 7. C3H10T1/2 control or Wnt1-overexpressing cells were plated on the bottom of cell culture wells and growth-arrested by mitomycin C. Mouse CD115^{+} monocytes were plated on top of them. Osteoclastogenesis was induced by M-CSF and RANKL. Cells were treated with DMSO or 10μM XAV939. Tracp staining was performed on day 5 to evaluate osteoclastogenesis.

5.3 Wnt1 in cartilage development and chondrocyte differentiation (III)

5.3.1 Wnt1 in articular cartilage

Wnt1 is expressed in cartilage from the earliest obtained epiphyseal cartilage of E16.5 mouse embryos through gestation (III, Fig. 1A). qRT-PCR analysis showed that the expression level of Wnt1 in the articular cartilage increases after birth and reaches a relatively stable level at the age of 3 months (III, Fig. 1A). In addition, Wnt1 expression in murine articular cartilage was studied by immunohistochemistry in sagittal sections of proximal tibia of wild-type mice. Strong staining of Wnt1 protein was observed in the lateral superficial zone of the articular cartilage (III, Fig. 1B).

Chondrocytes are derived from mesenchymal stem cells. In order to assess whether Wnt1 plays a role in the development of articular cartilage, we measured the average thickness and cell density of articular cartilage in Safranin O -stained hind-limb sections of 6-week-old $Wnt1_{prrx}^{-/-}$ and control mice. Neither cartilage thickness nor cell density was altered in $Wnt1_{prrx}^{-/-}$ mice compared to $Wnt1_{prrx}^{+/+}$ control mice (III, Fig. 3A and B). In agreement with this, the mRNA expression of chondrocyte-specific genes, such as *SOX9*, *Aggrecan*, *Col10a1*, *Comp* and *Col2a1* in knee epiphyses of 4-week-old mice was unchanged between the genotypes (III, Fig. 3C).

We isolated and cultured primary chondrocytes from knee epiphyses of $Wnt1_{prrx}^{+/+}$ and $Wnt1_{prrx}^{-/-}$ newborn mice. $Wnt1_{prrx}^{-/-}$ primary chondrocytes exhibited similar *in vitro* differentiation potential as $Wnt1_{prrx}^{+/+}$ primary chondrocytes, as demonstrated by Alcian Blue staining (III, Fig. 4C). Moreover, no significant difference was observed in mRNA expression of chondrocyte-specific genes between the genotypes (III, Fig. 4E). The proliferation potential of primary chondrocytes did not differ between genotypes (III, Fig. 4D). Interestingly, Wnt1 overexpression robustly inhibited chondrogenesis in primary chondrocytes, shown by Alcian Blue staining (**Figure 8A**). The expression of chondrocytes-specific genes was also downregulated in Wnt1 overexpressing chondrocytes (**Figure 8B**).

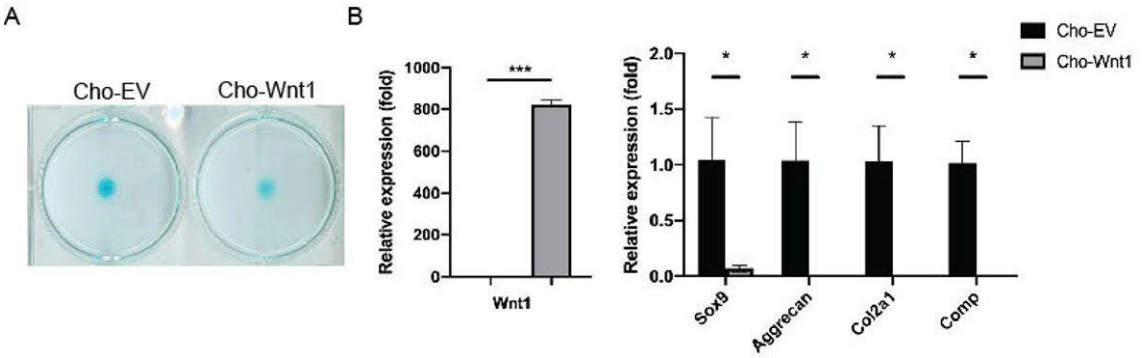

Figure 8. Wnt1 inhibits chondrogenesis. A. Micromass culture of control empty virus (EV) and Wnt1 overexpressing primary chondrocytes isolated from articular cartilage. Alcian blue staining was performed at 7 days of culture. B. mRNA expression of Wnt1 and chondrocytes-specific genes quantified by qRT-PCR at 7 days. * P<0.05, **P<0.01, ***P<0.001.

5.3.2 Wnt1 in growth plate (III)

Immunohistochemistry showed that Wnt1 expression in murine growth plate appears to be concentrated in early proliferative and hypertrophic zones (III, Fig. 1B). In prehypertrophic/hypertrophic and resting chondrocytes, the staining was detected only in nucleus (III, Fig. 1B). In addition, the lengths of femur and tibia in $Wnt1_{prrx}^{-/-}$ mice were decreased by 7.5% and 31.4% (III, Fig. 2B). To investigate whether Wnt1 has function in postnatal bone growth, we measured the average thickness and cell density of proliferative and hypertrophic zones of proximal tibial growth plate in $Wnt1_{prrx}^{+/+}$ and $Wnt1_{prrx}^{-/-}$ mice. No significant difference was found between the genotypes in the thickness or cell density, neither in the proliferative nor hypertrophic zones (III, Fig. 2D-F), suggesting that Wnt1 deletion in limb bud mesenchymal cells does not impair the development of growth plate. The reduced

femur and tibia lengths in Wnt1 deficient mice might be due to fracture caused bending of the long bones (III, Fig. 2C).

5.3.3 Wnt1 in subchondral bone (III)

Wnt signaling has been reported to play an important role in the regulation of subchondral bone (Funck-Brentano, T., et al., 2014). To address whether Wnt1 regulates subchondral bone, we analyzed the subchondral bone mass of $Wnt1_{prrx}^{+/+}$ and $Wnt1_{prrx}^{-/-}$ mice. Safranin O staining of tibia in $Wnt1_{prrx}^{+/+}$ and $Wnt1_{prrx}^{-/-}$ mice showed that the bone mass was significantly decreased in subchondral trabecular bone of $Wnt1_{prrx}^{-/-}$ mice at 6 and 12 weeks of age compared to control mice (III, Fig. 5A). μCT analysis demonstrated that trabecular BV/TV, Tb.N as well as Tb.Th was significantly decreased, while the Tb.Sp was increased in subchondral bone of $Wnt1_{prrx}^{-/-}$ mice at 6 and 12 weeks of age (III, Fig. 5C and D). Both male and female $Wnt1_{prrx}^{-/-}$ mice exhibited similar reduction of bone mass in subchondral trabecular bone compared to $Wnt1_{prrx}^{+/+}$ mice (III, Fig. 5C and D).

Histomorphometric analysis of 12-week-old male mice confirmed the reduction of trabecular BV/TV, Tb.N as well as Tb.Th in tibial subchondral bone of $Wnt1_{prrx}^{-/-}$ mice (III, Fig. 6A). In addition, N.Oc was increased in subchondral trabecular bone of $Wnt1_{prrx}^{-/-}$ mice (III, Fig. 6B and C). For subchondral bone plate (SBP), tissue area (T.Ar), bone area (B.Ar) and bone perimeter (B.Pm) were significantly decreased in $Wnt1_{prrx}^{-/-}$ mice (III, supplemental Table. 2), suggesting that the size of epiphysis in the $Wnt1_{prrx}^{-/-}$ mice is smaller than $Wnt1_{prrx}^{+/+}$ mice. No statistical difference was found in SBP BV/TV, while SBP thickness was slightly but significantly decreased in $Wnt1_{prrx}^{-/-}$ mice at 12 weeks of age (III, supplemental Table. 2) .

6 Discussion

6.1 Wnt1 in bone formation and osteoblast differentiation (I, II)

Wnt1 was recently reported as an important regulator for bone development in humans. Heterozygous Wnt1 mutation in patient has been shown to lead to early onset osteoporosis with high fracture incidence, while patients with homozygous Wnt1 mutation developed OI (Fahiminiya, S., et al., 2013; Keupp, K., et al., 2013; Laine, C. M., et al., 2013; Pyott, S. M., et al., 2013). In order to find out which cell type/types produce Wnt1 in bone microenvironment, global and conditional limb bud mesenchymal progenitor targeted Wnt1 knockout mice were generated. Global heterozygous Wnt1 knockout mice exhibited mild osteopenia, while fractures were not observed. The differences in bone phenotype might be due to species differences, such as body size and life span etc. Full knockout of Wnt1 in mesenchymal progenitor cells resulted in severe osteoporosis and long bone spontaneous fractures in multiple sites. Both trabecular and cortical compartments of long bone were affected by Prrx-Cre driven deletion of Wnt1 and the defects were due to impaired bone formation and increased bone resorption. These data suggested that the mechanism of Wnt1 in regulation of bone metabolism is different from other Wnt proteins. The knockout of Wnt16 results in lower cortical but not trabecular bone, while targeted deletion of Wnt5a in osteoblast lineage in mice leads to lower trabecular bone mass (Maeda, K., et al., 2012; Movérare-Skrtic, S., et al., 2014).

Wnt1 does activate canonical Wnt/β-catenin signaling pathway through Lrp5/6 receptors, although whether Wnt1 induces non-canonical Wnt signaling remains unknown (Ettenberg, S. A., et al., 2010; Laine, C. M., et al., 2013). Abundant evidence have demonstrated the positive role of Wnt/β-catenin signaling in promoting the differentiation of mesenchymal progenitor cells into osteoblast lineage (Day, T. F., et al., 2005; Hill, T. P., et al., 2005). Our histomorphometric data showed that Wnt1 deletion in limb bud mesenchymal progenitor cells did not change osteoblasts number in bone surface, but their activity and function was impaired, leading to overall reduced bone formation. These results suggest that Wnt1 induces osteoblast differentiation and activity via canonical Wnt signaling (**Figure 9**).

Wnt1 activates canonical Wnt/β-catenin signaling by binding to the first propeller of Lrp6, while Wnt antagonist sclerostin suppress it by competitively binding to the same site as Wnt1 on the Lrp6 receptor (Ettenberg, S. A., et al., 2010). Anti-sclerostin antibody romosozumab, which was approved by FDA and EMA to treat osteoporosis in postmenopausal women with high risk of fracture in 2019, functions through stimulating bone formation and suppressing bone resorption (similarly to Wnt1) leading to increased bone mass (Cosman, F., et al., 2016). Thus, romosozumab could have therapeutic potential for patients with Wnt1 mutations. This is supported by recent findings of Joeng and colleagues, who reported that romosozumab treatment efficiently improved bone mass and significantly reduced fracture rates in Swaying mice, a mouse model with spontaneous mutation in Wnt1 gene (Joeng, K. S., et al., 2014).

Our experiments with multiple co-culture systems showed that Wnt1 functions in a juxtacrine manner, requiring cell-cell contact between Wnt1 producing cells and its target cells. In addition, the expression level of Wnt1 mRNA in bone tissue or in calvaria osteoblasts was extremely low. Based on this it is actually very surprising that Wnt1 deletion in limb bud mesenchymal progenitors leads to such a severe bone phenotype affecting both osteoblast and osteoclast activity and function. Before secretion, Wnt1 undergoes a lipid modification by porcupine making it hydrophobic, and subsequently preventing it to diffuse freely in extracellular space (Zhai, L., et al., 2004). However, some membrane-tethered Wnt proteins may be secreted on exosomes to induce Wnt signaling activity in the target cells (Gross, J. C., et al., 2012). In our study, separating Wnt1 overexpressing cells from target cells with porous membrane that allows for diffusion of exosomes resulted in complete blockage of Wnt1 effects. Physical contact between Wnt1 producing and target cells was necessary for activating canonical Wnt/β-catenin signaling. These data indicate that exosome-mediated transfer does not play a role in the transmission of Wnt1 signals, but Wnt1 is likely to act as a membrane-anchored protein on the cell surface of secreting cells. Wls is a newly identified protein, which is necessary for Wnt1 secretion to the cell membrane. Interestingly, mature osteoblast-specific Wls-deficient ($Wls_{Ocn}^{-/-}$) mice exhibited spontaneous fractures and dramatic bone loss in both trabecular and cortical compartments, similarly to the bone phenotype our $Wnt1_{prrx}^{-/-}$ mice (Zhong, Z., et al., 2012). These data suggest that lack of functional Wnt1 likely contributes to the phenotype of $Wls_{Ocn}^{-/-}$ mice.

Recently, Joeng et al. reported that specific deletion of Wnt1 in late osteoblasts and osteocytes using Dmp1-Cre mice caused spontaneous fractures and severe loss of bone. Moreover, they suggested that the bone phenotype was in part due to the activation of mTORC1 signaling (Joeng, K. S., et al., 2017). Another group generated similar mouse model that induced osteoblast-targeted Wnt1 inactivation by Runx2-Cre mice and they have shown that all $Wnt1_{Runx2}^{-/-}$ mice displayed

spontaneous fractures at the age of 24 weeks as well as bone defects in both trabecular and cortical bone compartments (Luther, J., et al., 2018). In addition, they demonstrated that Wnt1 acts independently of Lrp5 co-receptor in bone, indicating that multiple bone-anabolic pathways could be targeted to induce bone formation. Comparing with our $Wnt1_{Prrx}^{-/-}$ mice, Dmp1-driven knockout mice exhibited less severe bone phenotype, showed by fracture rate of 67% in $Wnt1_{Dmp1}^{-/-}$ mice and 100% in male $Wnt1_{Prrx}^{-/-}$ mice. Moreover, $Wnt1_{Dmp1}^{-/-}$ mice exhibited significant decrease in MAR and BFR, but bone resorption parameters, such as osteoclast number and eroded surface per bone volume were unchanged, while bone defects in $Wnt1_{Prrx}^{-/-}$ mice were due to impaired bone formation and enhanced bone resorption. $Wnt1_{Runx2}^{-/-}$ mice displayed similar bone phenotype as $Wnt1_{Prrx}^{-/-}$ mice with 100% fracture rate, decreased trabecular bone volume and cortical thickness. However, the authors did not present the mechanisms causing these bone phenotypes. Taken together, Wnt1 derived from limb bud mesenchymal progenitor cells promotes bone formation and suppresses bone resorption, but Wnt1 produced by late osteoblast and osteocytes only regulates bone formation.

To address whether Wnt1 plays a role in adult bone, an inducible Wnt1 deletion mouse model was generated. Wnt1 deletion in osteoblast in adult mice caused reduced cortical thickness but has no effect on trabecular bone. Moreover, Wnt1 improves cortical thickness in adult by stimulating modeling-based bone formation. However, Wnt1 overexpression in the osteoblast lineage cells in adult mice led to increased trabecular bone and cortical thickness, due to enhanced bone formation, while bone resorption parameters were not altered (Luther, J., et al., 2018). The inconsistent results between the groups might be due to the different mouse models to inactivate endogenous Wnt1 and the overexpression of exogenous Wnt1.

6.2 Wnt1 in bone resorption and osteoclastoogenesis (I, III)

In addition to the regulation of bone formation, Wnt signaling pathway also has an essential role in bone resorption. Fzd8-deficient mice exhibited osteopenia with normal bone formation and enhanced bone resorption, but this phenotype was independent with OPG produced by osteoblast. Moreover, knockout of β-catenin in osteoclast precursors in mice resulted in osteopenia due to increased osteoclast differentiation (Albers, J., et al.). These results indicated that canonical Wnt signaling pathway directly suppresses osteoclastogenesis in an OPG-independent manner. On the other hand, inactivation of Lrp5 and Lrp6 receptors in the early osteoclast lineage using Rank-Cre mice led to reduced trabecular bone mass due to decreased bone resorption and formation. This study also showed that Wnt3a binding to Lrp5/6 receptor in osteoclast precursors inhibits early osteoclast differentiation by

suppressing the activation of Nfatc1 in a β-catenin-independent manner (Weivoda, M. M., et al., 2016). In contrast to canonical Wnt signaling, osteoblast lineage derived Wnt5a promotes differentiation of osteoclast progenitors by inducing noncanonical Wnt signaling via Ror2 receptors (Maeda, K., et al., 2012). A recent study reported that osteoblastic Wnt16 suppresses osteoclastogenesis effecting on the osteoclast progenitors as well as by inducing OPG expression in osteoblast (Movérare-Skrtic, S., et al., 2014).

In our animal study, we found that Wnt1 deletion in mesenchymal progenitor cells resulted in increased bone resorption. *OPG* expression tended to decrease in $Wnt1^{+/-}$ primary calvaria cells, however, in Wnt1 overexpressing cell lines mRNA expression of *OPG* was not always consistent depending on the experimental set up, suggesting that the effects of Wnt1 on osteoclastogenesis could at least in part be mediated via *OPG* (**Figure 9**). In a co-culture and an insert culture system of Wnt1 overexpressing cells and osteoclast cell line, we showed that the suppression requires contact between Wnt1 producing cells and osteoclast progenitors. Moreover, these data also demonstrated that Wnt1 has a direct juxtacrine effect on inhibiting osteoclastogenesis, which is independent of *OPG* (**Figure 9**). It has been reported that *OPG* could be immobilized at the osteoblast cell surface by heparan sulfate to enhance the ability of *OPG* to inhibit *RANKL* and subsequently osteoclastogenesis (Li, M., et al., 2016; Nozawa, S., et al., 2018).

As Wnt1 functions as a membrane-bound molecular, we failed to produce Wnt1-containing conditional medium, but instead used a co-culture system of Wnt1 producing and hematopoietic cells. Thus, it is hard to investigate the exact signaling pathways that mediates Wnt1 effect on osteoclast. By using a panel of small molecule inhibitors for the components of canonical and noncanonical branches, we were able to show that the suppression of osteoclast differentiation by Wnt1 could be rescued by XAV939 (an inhibitor of canonical Wnt/β-catenin signaling) and partially rescued by SP600125 (an inhibitor of JNK dependent noncanonical Wnt signaling), suggesting that canonical Wnt/β-catenin is the main mechanism by which Wnt1 directly suppresses osteoclastogenesis, but JNK-mediated noncanonical Wnt signaling may also contribute.

Recently, Claes Ohlsson and colleagues revealed that Wnt16 deficiency in adult mice led to decreased cortical thickness and strength due to increased bone resorption and impaired periosteal bone formation (Ohlsson, C., et al., 2018). The mRNA expression of *Ctsk* was increased, while the levels of *OPG* were reduced in cortical bone of Wnt16-inactivated mice, suggesting that Wnt16 regulates osteoclast differentiation in adult mice through OPG/RANKL pathway. Inconsistent with Wnt16, we did not observed any changes in bone resorption parameters (Oc.N/B.Pm, ES/BS, etc.) when induced Wnt1 deletion specifically in osteoblast lineage in adult

mice ($Wnt1_{Dox\text{-}Osx}^{-/-}$), indicating that osteoblast lineage derived Wnt1 has rare effect on bone resorption in adult mice.

6.3 Wnt1 in cartilage development and chondrocyte differentiation (III)

Numerous investigations of human genetic diseases and transgenic mouse models have suggested that Wnt signaling should be carefully modulated for normal cartilage development and maintenance (Usami, Y., et al., 2016; Zhu, M., et al., 2009). Both excessive Wnt signaling and inhibition of Wnt signaling result in cartilage degradation and abnormal differentiation of chondrocytes (Diarra, D., et al., 2007; van den Bosch, et al., 2017). Interestingly, we found that Wnt1 strongly suppressed chondrogenesis when overexpressed Wnt1 in primary articular chondrocytes *in vitro* (**Figure 8**). A recent study by Tong et al. demonstrated that chondrocyte-specific activation of Wnt16 in mice significantly suppressed chondrocyte hypertrophy during skeletal development via PCP/JNK and cross-talked with mTORC1-PTHrP pathway (Tong, W., et al., 2019).

Surprisingly, *in vivo*, we did not observe any articular cartilage phenotype of in $Wnt1_{prrx}^{-/-}$ mouse, although we confirmed that Wnt1 was indeed deleted in knee cartilage. Moreover, there were no differences in the proliferation or differentiation of primary articular chondrocytes isolated from $Wnt1_{prrx}^{+/+}$ and $Wnt1_{prrx}^{-/-}$. These data are supported by a recent study of patients with a heterozygous Wnt1 mutation, showing that articular cartilage thickness was unchanged between the mutation positive and mutation negative groups (Lehtovirta, S., et al., 2019). In addition, osteoblasts-specific overexpression of Wnt16 in mice led to thicker subchondral bone plate and increased subchondral trabecular bone, while the thickness of articular cartilage was unchanged (Törnqvist, A. E., et al., 2020). It has been documented that Wnt/β-catenin signaling regulates multiple steps of chondrogenesis in the growth plate, including proliferation, matrix secretion, hypertrophy and mineralization in a direct and indirect manner (Candela, M. E., et al., 2014; Dao, D. Y., et al., 2012; Tamamura, Y., et al., 2005). However, similarly to articular cartilage, Wnt1 does not regulate development of growth plate (**Figure 9**). Taken together, these data indicated that the deletion of Wnt1 in limb bud mesenchymal cells might be compensated for by the other Wnt/s, which could robustly induce Wnt/β-catenin signaling.

Abnormal remodeling of subchondral bone caused by imbalanced activity of bone formation and bone resorption is the hallmark of OA. Wnt16 overexpression in osteoblasts led to increased bone mass in subchondral bone plate and subchondral trabecular bone (Törnqvist, A. E., et al., 2020). Moreover, overexpression of Lrp5 has been shown to result in increased subchondral bone mass, as well as increased

cartilage thickness of mandibular condylar in temporomandibular joint (Utreja, A., et al., 2020). In an OA mice model, the canonical Wnt signaling was activated mainly in osteocytes of subchondral bone, as well as in osteophytes and synovium (Funck-Brentano, T., et al., 2014). The data presented above indicate that Wnt signaling pathway plays a critical role in remodeling of subchondral bone. In our study, we found that Wnt1 deletion in limb bud mesenchymal progenitors resulted in decreased bone mass in both subchondral trabecular bone and subchondral bone plate. The phenotype of subchondral bone was similar as in the trabecular and cortical compartments of long bones in $Wnt1_{prrx}^{-/-}$ mice. Moreover, the reduced subchondral trabecular bone and subchondral bone plate in $Wnt1_{prrx}^{-/-}$ mice were due to impaired bone formation and enhanced bone resorption, which is also consistent with the mechanisms of the reduction in trabecular and cortical bone mass in $Wnt1_{prrx}^{-/-}$ mice. These results suggested that Wnt1 is key regulator of bone remodeling at both primary and secondary ossification centers (**Figure 9**).

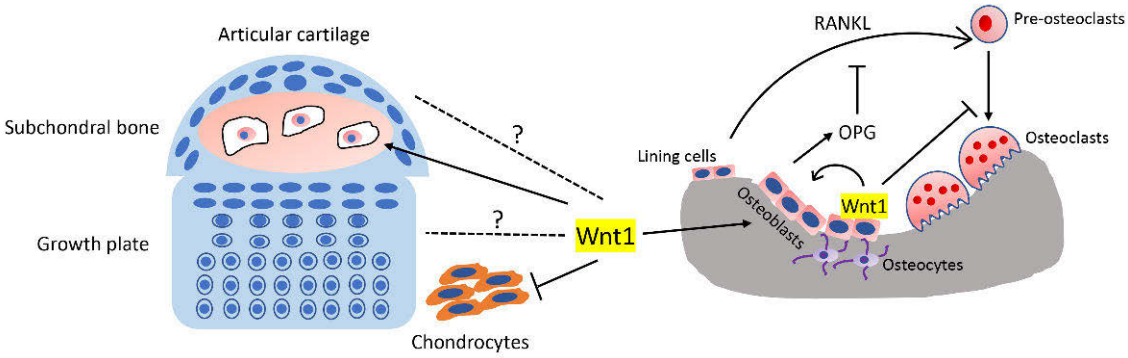

Figure 9. Schematic view of the role of Wnt1 in bone and cartilage. Mesenchymal progenitor derived Wnt1 activates osteoblast differentiation and function, inhibits osteoclastogenesis both directly by acting osteoclast progenitors and indirectly by stimulating OPG expression in osteoblasts. The role of mesenchymal progenitors derived Wnt1 in articular cartilage and growth plate is still unknow, but Wnt1 does inhibit chondrogenesis *in vitro*. RANKL, Receptor activator of nuclear factor kappa-B ligand; OPG, Osteoprotegerin.

6.4 Future perspectives

Although through this study, we have some insights into the regulation of Wnt1 in bone and cartilage metabolism, further investigation needs to be carried out from the following aspects in the future:

In this study, we showed that Wnt1 deletion in osteoblast lineage in adult mice results in thinner cortical thickness and it is more pronounced in female mice compared to males. It is already known that androgens increase cortical bone size via stimulation of longitudinal and radial growth (Vanderschueren, D., et al., 2004).

Although whether androgens stimulate periosteal bone formation in adult rats is still unclear, they indeed stimulate periosteal bone formation in male rats during puberty (Kim, B. T., et al., 2003). Since osteoblastic Wnt1 regulates cortical bone thickness in adult mice by stimulating periosteal bone formation, it is necessary to find out whether Wnt1 deletion in adult can be compensated by androgen in male mice.

Second, the present study has shown that *in vivo* Wnt1 has no impact on cartilage development. However, whether Wnt1 promotes or attenuates progression of OA is still unknown. It would be interesting to investigate the role of Wnt1 in OA progression by inactivating Wnt1 locally in knee joint through intra-articular injection of Wnt1 siRNA in experimental OA mice or using a cartilage specific inducible knockout model. Moreover, as Wnt/β-catenin signaling pathway inhibits chondrogenesis, while promotes chondrocytes hypertrophy (Chun, J. S., et al., 2008; Usami, Y., et al., 2016), further investigation is necessary to address the role of Wnt1 in different stages of chondrocyte differentiation.

Third, our study has shown that limb bud mesenchymal progenitor specific deletion of Wnt1 results in severe bone loss in subchondral bone in mice. Although it is well known that Wnt/β-catenin has an essential role in regulating formation of SOC (Dao, D. Y., et al., 2012; Chen. M., et al., 2008), it is still unclear how does Wnt1 regulate the formation of SOC. It is necessary to further investigate the effect of Wnt1 in the development of vascularized cartilage canals, cartilage hypertrophy and mineralization in SOC by cartilage specific Wnt1 transgenic mouse model (e.g. Col2a-Wnt1).

Multiple genetic animal models have been used in this study. Global heterozygous deletion of Wnt1 ($Wnt1^{+/-}$) led to mild trabecular osteopenia, while the cortical thickness in diaphysis was unchanged between $Wnt1^{+/+}$ and $Wnt1^{+/-}$ mice. Homozygous deletion of Wnt1 in mesenchymal progenitors ($Wnt1_{prrx}^{-/-}$) results in decreased bone mass in both trabecular and cortical compartments. The similar bone phenotypes were observed in homozygous osteoblast lineage-specific deficient mice ($Wnt1_{Osx}^{-/-}$) as $Wnt1_{prrx}^{-/-}$ mice, including spontaneous fractures in multiple sites of long bone, severe osteoporosis, and reduced cortical thickness in diaphysis. In Prrx-Cre driven deletion of Wnt1 mouse model, the control mice, expressing Prrx-Cre gene in wildtype mice ($Wnt1_{prrx}^{+/+}$) exhibited normal skeletal development, suggesting that severe bone phenotypes observed in $Wnt1_{prrx}^{-/-}$ mice was due to the deficiency of Wnt1 in limb bud mesenchymal progenitors, and Prrx-Cre transgene itself had no effect on bone. Unlike $Wnt1_{prrx}^{-/-}$ mice, we observed abnormal skeletal development and deceased body weight of $Wnt1_{Osx}^{+/-}$ mice, expressing both Osx-Cre and heterozygous floxed Wnt1, compared to Osx-Cre-negative control ($Wnt1^{fl/+}$). This finding suggested that Osx-Cre transgene itself had effects on bone and the defects of bone observed in $Wnt1_{Osx}^{-/-}$ mice was partially due to the expression of Osx-Cre transgene. In addition, postnatal deletion of Wnt1 in osteoblast lineage

($Wnt1_{Osx\text{-}Dox}^{-/-}$) led to reduced cortical thickness in adult mice, while the reduction on trabecular bone was mild and transient, suggesting that the effect of Wnt1 on the skeleton, especially on trabecular compartment is mainly caused by early developmental effects. Detailed investigation of effects of Wnt1 on different mouse models could provide a better understanding of the role of Wnt1 in specific cell type/tissue and specific developmental stage in mice.

7 Conclusions

Based on the study presented in this thesis book, the following conclusions can be drawn:

1. Mesenchymal cell lineage derived Wnt1 is a key regulator of bone metabolism regulating both trabecular and cortical compartments.
2. Wnt1 functions on bone cells in a juxtacrine manner, requiring physical contact between the source and its target cells.
3. Wnt1 has dual functions to induce osteoblast activity and differentiation as well as to suppress osteoclastogenesis.
4. Osteoblastic Wnt1 improves cortical thickness in adult mice by stimulating modeling-based bone formation.
5. *In vivo*, Wnt1 deficiency in mesenchymal progenitors does not impact the development of articular cartilage or growth plate, while *in vitro*, Wnt1 inhibits chondrogenesis of primary chondrocytes.
6. Wnt1 regulates subchondral bone mass by inducing bone formation and suppressing bone resorption.

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References

Aarden, E. M.,et al. (1994). Function of osteocytes in bone. *Journal of Cellular Biochemistry, 55*, 287-299 .

Aberle, H., et al. (1997). Canonical Wnt signaling inhibits osteoclastogenesis independent of osteoprotegerin. *Journal of Cell Biology, 200*(4), 537–549.

Albers, J., et al. (n.d.). Canonical Wnt signaling inhibits osteoclastogenesis independent of osteoprotegerin. *Journal of Cell Biology, 200*(4), 537–549.

Alexandre, C., et al. (2014). Patterning and growth control by membrane- tethered Wingless. *Nature, 505*, 180–185.

Alexeeva, L., et al. (1994). *Assessment of fracture risk and its application to screening for postmenopausal osteoporos.* Geneva: World Health Orgnazation.

Alvarez, J., et al. (2005). Expression patterns of matrix metallo-proteinases and vascular endothelial growth factorduring epiphyseal ossification. *Journal of Bone and Mineral Research, 20*(6), 1011-1021.

Andrews, E. B., et al. (2012). The US postmarketing surveillance study of adult osteosarcoma and teriparatide: Study design and findings from the first 7 years. *Journal of Bone and Mineral Research, 27*(12), 2429-2437.

Arlot, M., et al. (2005). Differential effects of teriparatide and alendronate on bone remodeling in postmenopausal women assessed by histomorphometric parameters. *Journal of Bone and Mineral Research., 20*, 1244-1253.

Balemans, W., et al. (2001). Increased bone density in sclerosteosis is due to the deficiency of a novel secreted protein (SOST). *Human Molecular Genetics, 10*(5), 537–544.

Balemans, W., et al. (2002). Identification of a 52 kb deletion downstream of the SOST gene in patients with van Buchem disease. *Journal of Medical Genetics., 39*(2), 91–97.

Baron, R., & Kneissel, M. (2013). WNT signaling in bone homeostasis and disease: from human mutations to treatments. *Nature Medicine, 19*(2), 179–192.

Bartscherer, K., et al. (2006). Secretion of Wnt Ligands Requires Evi, a Conserved Transmembrane Protein. *Cell, 125*, 523–533.

Bennett, B. L., et al. (2001). SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. *Proceedings of the National Academy of Sciences, 98*(24), 13681–13686.

Bennett, C. N., et al. (2005). Regulation of osteoblastogenesis and bone mass by Wnt10b. *Proceedings of the National Academy of Sciences of the United States of America., 102*(9), 3324–3329.

Bennett, C. N., et al. (2007). Wnt10b increases postnatal bone formation by enhancing osteoblast differentiation. *Journal of Bone and Mineral Research., 22*(12), 1924-1932.

Berendsen, A. D., & Olsen, B. R. (2015). Bone development. *Bone, 80*, 14-18.

Black, D. M., & Rosen, C. J. (2016). Clinical Practice. Postmenopausal Osteoporosis. *The New England journal of medicine, 374*, 254-262.

Black, D. M.,et al. (1996). Randomised trial of effect of alendronate on risk of fracture in women with existing vertebral fractures. *Lancet, 348*(9041), 1535-1541.

Blom, A. B., et al. (2009). Involvement of the Wnt signaling pathway in experimental and human osteoarthritis: Prominent role of Wnt-induced signaling protein 1. *Arthritis and Rheumatism., 60*(2), 501-512.

Blumer, M. J. F., et al. (2008). Structure, formation and role of cartilage canals in the developing bone. *Annals of Anatomy., 190*(4), 305-315.

Bonewald, L. F. (2011). The amazing osteocyte. *Journal of Bone and Mineral Research., 26*(2), 229-238.

Bou-Gharios, G.,et al. (2019). Type I collagen structure, synthesis, and regulation. In e. a. John P. Bilezikian, *Principles of Bone Biology.* San Diego: Elsevier.

Bourhis, E., et al. (2011). Wnt antagonists bind through a short peptide to the first β-propeller domain of LRP5/6. *Structure, 19*, 1433–1442.

Bovolenta, P., et al. (2008). Beyond Wnt inhibition: New functions of secreted Frizzled-related proteins in development and disease. *Journal of Cell Science, 121*(6), 737–746.

Boyle W.J., et al. (2003). Osteoclast differentiation and activation. *Nature, 423*(6937), 337-342.

Bradley, R. S., & Brown, A. M. C. (1990). The proto-oncogene int-1 encodes a secreted protein associated with the extracellular matrix. *EMBO Journal., 9*(5), 1569–1575.

Brumsen, C., et al. (2002). Daily oral pamidronate in women and men with osteoporosis: A 3-year randomized placebo-controlled clinical trial with a 2-year open extension. *Journal of Bone and Mineral Research, 17*(6), 1057-1064.

Bänziger, C., et al. (2006). Wntless, a Conserved Membrane Protein Dedicated to the Secretion of Wnt Proteins from Signaling Cells. *Cell, 125*, 509–522.

Candela, M. E., et al. (2014). Distribution of slow-cycling cells in epiphyseal cartilage and requirement of β-catenin signaling for their maintenance in growth plate. *Journal of Orthopaedic Research., 32*(5), 661-668.

Cauley, J. A., et al. (2003). Effects of Estrogen Plus Progestin on Risk of Fracture and Bone Mineral Density: The Women's Health Initiative Randomized Trial. *Journal of the American Medical Association, 290*(13), 1729-1738.

Chan, B. Y., et al. (2011). Increased chondrocyte sclerostin may protect against cartilage degradation in osteoarthritis. *Osteoarthritis and Cartilage, 19*(7), 874-885.

Chang, M. K., et al. (2014). Disruption of Lrp4 function by genetic deletion or pharmacological blockade increases bone mass and serum sclerostin levels. *Proceedings of the National Academy of Sciences of the United States of America., 111*(48), E5187–E5195.

Chen, C. G., et al. (2012). Chondrocyte-intrinsic Smad3 represses Runx2-inducible matrix metalloproteinase 13 expression to maintain articular cartilage and prevent osteoarthritis. *Arthritis and Rheumatism., 64*, 3278–3289.

Chen, J., et al. (2014). WNT7B Promotes Bone Formation in part through mTORC1. *PLoS Genetics., 10*(1), e1004145.

Chen. M., et al. (2008). Inhibition of β-catenin signaling causes defects in postnatal cartilage development. *Journal of Cell Science, 121*, 1455-1465.

Chesnut. C. H., et al. (2004). A randomized trial of nasal spray salmon calcitonin in postmenopausal women with established osteoporosis: the prevent recurrence of osteoporotic fractures study. *The American Journal of Medicine, 109*(4), 267-276.

Choi, S. H., et al. (2013). α-Catenin interacts with APC to regulate β-catenin proteolysis and transcriptional repression of Wnt target genes. *Genes and Development, 27*, 2473-2488.

Chun, J. S., et al. (2008). Wnt signaling in cartilage development and degeneration. *Journal of Biochemistry and Molecular Biology., 41*(7), 485-494.

Church, V., et al. (2002). Wnt regulation of chondrocyte differentiation. *Journal of Cell Science., 115*(24), 4809–4818.

Compston. J. E., et al. (2019). Osteoporosis. *Lancet, 393*(10169), 364-376.

Cosman, F., et al. (2016). Romosozumab treatment in postmenopausal women with osteoporosis. *New England Journal of Medicine, 375*, 1532-1543.

Cruciat, C. M., & Niehrs, C. (2013). Secreted and transmembrane Wnt inhibitors and activators. *Cold Spring Harbor Perspectives in Biology, 13*(4).

Cummings, S. R., et al. (2009). Denosumab for prevention of fractures in postmenopausal women with osteoporosis. *New England Journal of Medicine, 361*, 756-765.

Dallas, S. L.,et al. (2013). The Osteocyte: An Endocrine Cell . . . and More. *Endocrine Reviews., 34*(5), 658–690.

Dao, D. Y., et al. (2012). Cartilage-specific β-catenin signaling regulates chondrocyte maturation, generation of ossification centers, and perichondrial bone formation during skeletal development. *Journal of Bone and Mineral Research., 27*(8), 1680-1694.

Day, T. F., et al. (2005). Wnt/β-catenin signaling in mesenchymal progenitors controls osteoblast and chondrocyte differentiation during vertebrate skeletogenesis. *Developmental Cell, 8*(5), 739–750.

De Gorter, et al. (2018). Signal transduction cascades controlling osteoblast differentiation. In e. a. John P. Bilezikian., *Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism, Ninth Edition.* Hoboken: Wiley.

de Lau, W., et al. (2014). The R-spondin/Lgr5/Rnf43 module: Regulator of Wnt signal strength. *Genes and Development, 28*, 305-316.

Dell'Accio, F., et al. (2008). Identification of the molecular response of articular cartilage to injury, by microarray screening: Wnt-16 expression and signaling after injury and in osteoarthritis. *Arthritis and Rheumatism, 58*(5), 1410-1421.

Dempster, D. W., et al. (2013). tandardized nomenclature, symbols, and units for bone histomorphometry: A 2012 update of the report of the ASBMR Histomorphometry Nomenclature Committee. *Journal of Bone and Mineral Research, 28*(1), 2-17.

Diarra, D., et al. (2007). Dickkopf-1 is a master regulator of joint remodeling. *Nature Medicine, 13*, 156–163.

Duprez, D.,et al. (1999). Expression of Frzb-1 during chick development. *Mechanisms of Development., 89*(1-2), 179-183 .

Enomoto-Iwamoto, M., et al. (2002). The Wnt antagonist Frzb-1 regulates chondrocyte maturation and long bone development during limb skeletogenesis. *Developmental Biology, 251*(1), 142-156 .

Ettenberg, S. A., et al. (2010). Inhibition of tumorigenesis driven by different Wnt proteins requires blockade of distinct ligand-binding regions by LRP6 antibodies. *Proceedings of the National Academy of Sciences of the United States of America., 107*(35), 15473-15478.

Fahiminiya, S., et al. (2013). Mutations in WNT1 are a cause of osteogenesis imperfecta. *Journal of Medical Genetics,, 50*(5), 345–348.

Farin, H. F., et al. (2016). Visualization of a short-range Wnt gradient in the intestinal stem-cell niche. *Nature, 530*, 340–343.

Feng, X., & McDonald, J. M. (2011). Disorders of bone remodeling. *Annual Review of Pathology: Mechanisms of Disease., 6*, 121-145.

Florencio-Silva, R., et al. (2015). Biology of Bone Tissue: Structure, Function, and Factors That Influence Bone Cells. *BioMed Research International, 2015*, 421746.

Funck-Brentano, T., et al. (2014). Dkk-1- mediated inhibition of Wnt signaling in bone ameliorates osteoarthritis in mice. *Arthritis and Rheumatology, 66*(11), 3028-3039.

Gao, B., et al. (2011). Wnt Signaling Gradients Establish Planar Cell Polarity by Inducing Vangl2 Phosphorylation through Ror2. *Developmental Cell, 20*(2), 163-176.

Gao, L., et al. (2013). TAK1 regulates SOX9 expression in chondrocytes and is essential for postnatal development of the growth plate and articular cartilages. *Journal of Cell Science, 126*(24), 5704–5713.

Glass, D. A., et al. (2005). Canonical Wnt signaling in differentiated osteoblasts controls osteoclast differentiation. *Developmental Cell, 8*(5), 751-764.

Gong, Y., et al. (1996). Osteoporosis-pseudoglioma syndrome, a disorder affecting skeletal strength and vision, is assigned to chromosome region 11q12-13. *American Journal of Human Genetics, 59*, 146-151.

Gross, J. C., et al. (2012). Active Wnt proteins are secreted on exosomes. *Nature Cell Biology, 14*(10), 1036–1045.

Guo, X., et al. (2004). Wnt/β-catenin signaling is sufficient and necessary for synovial joint formation. *Genes and Development, 18*(19), 2404-2417.

Hadjidakis, D. J., & Androulakis, I. I. (2006). Bone remodeling. *Annals of the New York Academy of Sciences., 1092*, 385–396.

Hallett, S. A., et al. (2019). Growth plate chondrocytes: Skeletal development, growth and beyond. *International Journal of Molecular Sciences, 20*(23), 6009.

Harada, H., et al. (1999). Cbfa1 isoforms exert functional differences in osteoblast differentiation. *Journal of Biological Chemistry., 274*(11), 6972-6978 .

Hartmann, C., & Tabin, C. J. (2000). Dual roles of Wnt signaling during chondrogenesis in the chicken limb. *Development, 127*(14), 3141-3159.

Hartmann, C., & Tabin, C. J. (2001). Wnt-14 plays a pivotal role in inducing synovial joint formation in the developing appendicular skeleton. *Cell, 104*, 341–351.

Heaney, C., et al. (1998). Human autosomal recessive osteopetrosis maps to 11q13, a position predicted by comparative mapping of the murine osteosclerosis (oc) mutation. *Human Molecular Genetics, 7*(9), 1407–1410.

Hill, T. P., et al. (2005). Canonical Wnt/beta- catenin signaling prevents osteoblasts from differentiating into chondrocytes. *Developmental Cell, 8*(5), 727–738.

Holmen, S. L.,et al. (2005). Essential role of β-catenin in postnatal bone acquisition. *Journal of Biological Chemistry., 280*(22), 21162-21168.

Honsawek, S., et al. (2010). Dickkopf-1 (Dkk-1) in plasma and synovial fluid is inversely correlated with radiographic severity of knee osteoarthritis patients. *BMC Musculoskeletal Disorders, 11*, 257.

Hu, H., et al. (2005). Sequential roles of Hedgehog and Wnt signaling in osteoblast development. *Development, 132*(1), 49–60.

Huang, S. M. A., et al. (2009). Tankyrase inhibition stabilizes axin and antagonizes Wnt signalling. *Nature, 461*, 614–620.

Hunter, D. J. (2011). Pharmacologic therapy for osteoarthritis-the era of disease modification. *Nature Reviews Rheumatology, 7*, 13-22.

Hunter, D. J., & Bierma-Zeinstra, S. (2019). Osteoarthritis. *The Lancet, 393*(10182), 1745-1759.

Janssens, K. et al. (2005). Transforming growth factor-β1 to the bone. *Endocrine Reviews, 26*(6), 743–774.

Jenny, A. (2010). Planar cell polarity signaling in the drosophila eye. *Developmental Biology, 93*, 189-227.

Joeng, K. S., et al. (2014). The swaying mouse as a model of osteogenesis imperfecta caused by WNT1 mutations. *Human Molecular Genetics, 23*(15), 4035–4042.

Joeng, K. S., et al. (2017). Osteocyte-specific WNT1 regulates osteoblast function during bone homeostasis. *Journal of Clinical Investigation, 127*(7), 2678–2688.

Johnson, M. L., et al. (1997). Linkage of a gene causing high bone mass to human chromosome 11 (11q12- 13). *American Journal of Human Genetics, 60*, 1326–1332.

Kadowaki, T., et al. (1996). The segment polarity gene porcupine encodes a putative multitransmembrane protein involved in Wingless processing. *Genes and Development, 10*, 3116-3128.

Kanis, J. A., et al. (2008). Case finding for the management of osteoporosis with FRAX® - Assessment and intervention thresholds for the UK. *Osteoporosis International., 19*, 1395–1408.

Kenkre, J. S., & Bassett, J. H. D. (2018). The bone remodelling cycle. *Annals of Clinical Biochemistry., 55*(3), 308–327.

Kern, B., et al. (2001). Cbfa1 Contributes to the Osteoblast-specific Expression of type I collagen Genes. *Journal of Biological Chemistry, 276*(10), 7101-7107 .

Keupp, K., et al. (2013). Mutations in WNT1 cause different forms of bone fragility. *American Journal of Human Genetics, 92*(4), 565–574.

Kim, B. T., et al. (2003). The structural and hormonal basis of sex differences in peak appendicular bone strength in rats. *Journal of Bone and Mineral Research, 18*(1), 150-155.

Kim, K. A., et al. (2006). R-spondin proteins: A novel link to β-catenin activation. *Cell Cycle, 5*(1), 23-26.

Kitagawa, M., et al. (1999). An F-box protein, FWD1, mediates ubiquitin-dependent proteolysis of β-catenin. *EMBO Journal, 18*, 2401-2410.

Kohn, A. D., & Moon, R. T. (2005). Wnt and calcium signaling: β-Catenin-independent pathways. *Cell Calcium, 38*(3-4), 439-446.

Koide, M., & Kobayashi, Y. (2019). Regulatory mechanisms of sclerostin expression during bone remodeling. *Journal of Bone and Mineral Metabolism, 37*(1), 9-17.

Kramer, I., et al. (2010). Osteocyte Wnt/β-Catenin Signaling Is Required for Normal Bone Homeostasis. *Molecular and Cellular Biology, 30*(12), 3071–3085.

Krishnan, Y., & Grodzinsky, A. J. (2018). Cartilage diseases. *Matrix Biology, 71-72*, 51-69 .

Lacey, D. L., et al. (1998). Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell, 93*(2), 165-176 .

Laine, C. M., et al. (2013). WNT1 mutations in early-onset osteoporosis and osteogenesis imperfecta. *The New England Journal of Medicine, 368*(19), 1809–1816.

Langdahl. B., et al. (2016). Bone modeling and remodeling: potential as therapeutic targets for the treatment of osteoporosis. *Therapeutic Advances in Musculoskeletal Disease, 8*(6), 225–235.

Las Heras, F., et al. (2012). Articular Cartilage Development: A Molecular Perspective. *Orthopedic Clinics of North America., 43*, 155–171.

Lee, S. H., et al. (2006). V-ATPase V0 subunit d2-deficient mice exhibit impaired osteoclast fusion and increased bone formation. *Nature Medicine, 12*.

Lefebvre, V. (2002). Toward understanding the functions of the two highly related Sox5 and Sox6 genes. *Journal of Bone and Mineral Metabolism, 20*, 121–130.

Lefrebvre, V., & de Crombrugghe, B. (1998). Toward understanding S0X9 function in chondrocyte differentiation. *Matrix Biology, 16*(9), 529-540 .

Lehtovirta, S., et al. (2019). Defective WNT signaling may protect from articular cartilage deterioration – a quantitative MRI study on subjects with a 67 heterozygous WNT1 mutation. *Osteoarthritis and Cartilage, 27*(11), 1636-1646.

Li, M., et al. (2016). Heparan sulfate regulates the structure and function of osteoprotegerin in osteoclastogenesis. *Journal of Biological Chemistry., 291*(46), 24160-24171.

Li, X., et al. (2008). Targeted deletion of the sclerostin gene in mice results in increased bone formation and bone strength. *Journal of Bone and Mineral Research., 23*(6), 860-869.

Li, Xiaodong, et al. (2005). Targeted deletion of the sclerostin gene in mice results in increased bone formation and bone strength. *Journal of Bone and Mineral Research, 23*(6), 860-869.

Li, Xiaodong, et al. (2008). Targeted deletion of the sclerostin gene in mice results in increased bone formation and bone strength. *Journal of Bone and Mineral Research., 23*(6), 860-869.

Lindsay, R., et al. (2006). A novel tetracycline labeling schedule for longitudinal evaluation of the short-term effects of anabolic therapy with a single iliac crest bone biopsy: Early actions of teriparatide. *Journal of Bone and Mineral Research., 21*, 366-373.

Liu, Y., et al. (2007). The orphan receptor tyrosine kinase Ror2 promotes osteoblast differentiation and enhances ex vivo bone formation. *Molecular Endocrinology, 21*(2), 376–387.

Loeser, R. F., et al. (2016). Ageing and the pathogenesis of osteoarthritis. *Nature Reviews Rheumatology, 12*, 412-420.

Logan, M., et al. (2002). Expression of Cre Recombinase in the developing mouse limb bud driven by a Prxl enhancer. *Genesis., 33*(2), 77-80.

Long, F., & Ornitz, D. M. (2013). Development of the endochondral skeleton. *Cold Spring Harbor Perspectives in Biology, 5*, a008334.

Luther, J., et al. (2018). Wnt1 is an Lrp5- independent bone-anabolic Wnt ligand. *Science Translational Medicine, 10*(466).

MacDonald, B. T., et al. (2007). Bone mass is inversely proportional to Dkk1 levels in mice. *Bone, 41*(3), 331-339 .

Maeda, K., et al. (2012). Wnt5a-Ror2 signaling between osteoblast-lineage cells and osteoclast precursors enhances osteoclastogenesis. *Nature Medicine, 18*(3), 405–412.

Malinauskas, T., et al. (2011). Modular mechanism of Wnt signaling inhibition by Wnt inhibitory factor 1. *Nature Structural and Molecular Biology, 18*, 886–893.

Mao, B., et al. (2001). LDL-receptor- related protein 6 is a receptor for Dickkopf proteins. *Nature, 411*, 321–325.

Mao, B., et al. (2002). Kremen proteins are Dickkopf receptors that regulate Wnt/β-catenin signalling. *Nature, 417*, 664–667.

Marchetti, B., & Pluchino, S. (2013). Wnt your brain be inflamed? Yes, it Wnt! *Trends in Molecular Medicine, 19*, 144-156.

Martel-Pelletier, J., et al. (2016). Osteoarthritis. *Nature Reviews Disease Primers., 2*, 16072.

McClung, M. R., et al. (2005). Opposite bone remodeling effects of teriparatide and 68 alendronate in increasing bone mass. *Archives of Internal Medicine, 165*(15), 1762-1768.

McMahon, A. P., & Bradley, A. (1990). The Wnt-1 (int-1) proto-oncogene is required for development of a large region of the mouse brain. *Cell, 62*(6), 1073-1085 .

Movérare-Skrtic, S., et al. (2014). Osteoblast-derived WNT16 represses osteoclastogenesis and prevents cortical bone fragility fractures. *Nature Medicine, 20*(11), 1279–1288.

Nakamura, Y., et al. (2005). Expression profiles and functional analyses of Wnt-related genes in human joint disorders. *American Journal of Pathology, 167*(1), 97-105.

Nakashima, K., et al. (2002). The novel zinc finger-containing transcription factor Osterix is required for osteoblast differentiation and bone formation. *Cell, 108*(1), 17-29.

Niemann, S., et al. (2004). Homozygous WNT3 Mutation Causes Tetra-Amelia in a Large Consanguineous Family. *American Journal of Human Genetics., 74*, 558–563.

Nishio, Y., et al. (2006). Runx2-mediated regulation of the zinc finger Osterix/Sp7 gene. *Gene, 372*, 62-70 .

Nozawa, S., et al. (2018). Osteoblastic heparan sulfate regulates osteoprotegerin function and bone mass. *JCI Insight, 3*(3), e89624.

Nusse, R., & Varmus, H. E. (1982). Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome. *Cell, 31*(1), 99-109 .

Nusse, R., & Varmus, H. E. (1992). Wnt genes. *Cell, 69*(7), 1073-1087 .

Ohlsson, C., et al. (2018). Inducible Wnt16 inactivation: WNT16 regulates cortical bone thickness in adult mice. *Journal of Endocrinology, 237*(2), 113-122.

Ott, S. M. (2018). Cortical or Trabecular Bone: What's the Difference? *American Journal of Nephrology, 47*, 373–375.

Otto, F., et al. (1997). Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. *Cell, 89*(5), 765-771 .

Poole, C. A., et al. (1991). Chondrons from articular cartilage. III. Morphologic changes in the cellular microenvironment of chondrons isolated from osteoarthritic cartilage. *Arthritis & Rheumatism., 34*, 22-35.

Pyott, S. M., et al. (2013). WNT1 mutations in families affected by moderately severe and progressive recessive osteogenesis imperfecta. *American Journal of Human Genetics, 92*(4), 590–597.

Qin, L., et al. (2020). Molecular mechanosensors in osteocytes. *Bone Research, 8*, 23.

Raggatt, L. J., & Partridge, N. C. (2010). Cellular and molecular mechanisms of bone remodeling. *Journal of Biological Chemistry., 285*, 25103–25108.

Riddle, R. C., et al. (2013). Lrp5 and Lrp6 Exert Overlapping Functions in Osteoblasts during Postnatal Bone Acquisition. *PLoS ONE, 8*(5), e63323.

Rijsewijk, F., et al. (1987). The Drosophila homology of the mouse mammary oncogene int-1 is identical to the segment 69 polarity gene wingless. *Cell, 50*(4), 649-657 .

Rios-Esteves, J., & Resh, M. D. (2013). Stearoyl CoA Desaturase Is Required to Produce Active, Lipid-Modified Wnt Proteins. *Cell Reports, 4*(6), 1072-1081 .

Rios-Esteves, J.,et al. (2014). Identification of key residues and regions important for porcupine-mediated Wnt acylation. *Journal of Biological Chemistry, 289*(24), 17009-17019 .

Rodda, S. J., & McMahon, A. P. (2006). Distinct roles for Hedgehog and caronical Wnt signaling in specification, differentiation and maintenance of osteoblast progenitors. *Development, 133*(16), 3231–3244.

Rubin, M. R., & Bilezikian, J. P. (2003). New anabolic therapies in osteoporosis. *Endocrinology and Metabolism Clinics of North America, 32*(1), 285-307.

Rudnicki, J. A., & Brown, A. M. C. (1997). Inhibition of chondrogenesis by Wnt gene expression in vivo and in vitro. *Developmental Biology, 185*(1), 104-118 .

Salo, J., et al. (1997). Removal of osteoclast bone resorption products by transcytosis. *Science, 276*(5310), 270-273.

Santos, A., et al. (2009). The role of osteocytes in bone mechanotransduction. *Osteoporosis International, 20*, 1027–1031.

Sato, M., et al. (2013). Osteocytes regulate primary lymphoid organs and fat metabolism. *Cell Metabolism., 18*, 749–758.

Sellam, J., & Berenbaum, F. (2013). Is osteoarthritis a metabolic disease? *Joint Bone Spine, 80*(6), 568-573.

Sharma, R. P., & Chopra, V. L. (1976). Effect of the wingless (wg1) mutation on wing and haltere development in Drosophila melanogaster. *Developmental Biology., 48*(2), 461-465.

Shekaran, A., & García, A. J. (2011). Extracellular matrix-mimetic adhesive biomaterials for bone repair. *Journal of Biomedical Materials Research, 96*(1), 261-272.

Sillence, D. O., et al. (1979). Genetic heterogeneity in osteogenesis imperfecta. *Journal of Medical Genetics, 16*, 101-116.

Simonet, W. S., et al. (1997). Osteoprotegerin: A novel secreted protein involved in the regulation of bone density. *Cell, 89*(2), 309-319 .

Sims, N. A., & Martin, T. J. (2015). Coupling signals between the osteoclast and osteoblast: How are messages transmitted between these temporary visitors to the bone surface? *Frontiers in Endocrinology, 6*, 1-5.

Sokos D, et al. (2015). Role of periodontal ligament fibroblasts in osteoclastogenesis: a review. *Journal of Periodontal Research, 50*(2), 152-159.

Sophia Fox, et al. (2009). The basic science of articular cartilage: Structure, composition, and function. *Sports Health, 1*(6).

Stevens, J. R., et al. (2010). Wnt10b deficiency results in age-dependent loss of bone mass and progressive reduction of mesenchymal progenitor cells. *Journal of Bone and Mineral Research., 25*(10), 2138-2147.

Strom, T. M., et al. (1997). Per gene deletions in Gy and Hyp mice provide mouse models for X-linked hypophosphatemia. *Human Molecular Genetics., 6*(2), 165–171.

Suda T., et al. (1999). Modulation of osteoclast differentiation and function by the new members of the tumor necrosis factor receptor and ligand families. *Endocrine Reviews., 20*(3), 345–357.

Sumi, M., et al. (1991). The newly synthesized selective Ca2+ calmodulin dependent protein kinase II inhibitor KN-93 reduces dopamine contents in PC12h cells. *Biochemical and Biophysical Research Communications, 181*(3), 968-975.

Takada, R., et al. (2006). Monounsaturated Fatty Acid Modification of Wnt Protein: Its Role in Wnt Secretion. *Developmental Cell., 11*(6), 791-801.

Tamamura, Y., et al. (2005). Developmental regulation of Wnt/β-catenin signals is required for growth plate assembly, cartilage integrity, and endochondral ossification. *Journal of Biological Chemistry, 280*(19), 19185-19195.

Tamamura, Y., et al. (2005). Developmental regulation of Wnt/β-catenin signals is required for growth plate assembly, cartilage integrity, and endochondral ossification. *Journal of Biological Chemistry, 280*(19), 19185-19195.

Tatsumi, S., et al. (2007). Targeted Ablation of Osteocytes Induces Osteoporosis with Defective Mechanotransduction. *Cell Metabolism, 5*, 464–475.

Teitelbaum, S. L. (2000). Bone resorption by osteoclasts. *Science, 289*(5484), 1504-1508.

Teitelbaum, Steven L., & Ross, F. P. (2003). Genetic regulation of osteoclast development and function. *Nature Reviews Genetics, 4*, 638-649.

Tong, W., et al. (2019). Wnt16 attenuates osteoarthritis progression through a PCP/JNK-mTORC1-PTHrP cascade. *Annals of the Rheumatic Diseases., 78*(4), 551–561.

Tu, X., et al. (2007). Noncanonical Wnt Signaling through G Protein-Linked PKCδ Activation Promotes Bone Formation. *Developmental Cell, 12*(1), 113-127.

Törnqvist, A. E., et al. (2020). Wnt16 Overexpression in Osteoblasts Increases the Subchondral Bone Mass but has no Impact on Osteoarthritis in Young Adult Female Mice. *Calcified Tissue International., 107*, 31–40.

Urist, M. R. (1965). Bone: Formation by autoinduction. *Science., 150*(3698), 893-899.

Usami, Y., et al. (2016). Wnt signaling in cartilage development and diseases: Lessons from animal studies. *Laboratory Investigation, 96*, 186–196.

Utreja, A., et al. (2020). The Effect of Overexpression of Lrp5 on the Temporomandibular Joint. *Cartilage*, 1-8.

van den Bosch, et al. (2017). Synovial Wnt signaling induces the expression of MMPs and is associated with disease progression in early symptomatic osteoarthritis patients. *Arthritis & Rheumatology., 69*(10), 1978-1983.

Vanderschueren, D., et al. (2004). Androgens and bone. *Endocrine Reviews, 25*(3), 389–425.

Vu, T.H., et al. (1998). MMP-9/Gelatinase B Is a Key Regulator of Growth Plate Angiogenesis and Apoptosis of Hypertrophic Chondrocytes. *Cell, 93*, 411–422.

Wang, M., et al. (2013). MMP13 is a critical target gene during the progression of osteoarthritis. *Arthritis Research and Therapy, 15*, R5.

Wang, Y., et al. (2016). Modulation of osteogenesis in MC3T3-E1 cells by different frequency electrical stimulation. *PLoS ONE, 11*(5), e0154924.

Weivoda, M. M., et al. (2016). Wnt Signaling Inhibits Osteoclast Differentiation by Activating Canonical and Noncanonical cAMP/PKA Pathways. *Journal of Bone and Mineral Research, 31*(1), 65-75.

Willert, K., et al. (2003). Wnt proteins are lipid-modified and can act as stem cell growth factors. *Nature, 423*, 448–452.

Woods, C. G., et al. (2006). Mutations in WNT7A cause a range of limb malformations, including fuhrmann syndrome and Al-Awadi/Raas-Rothschild/Schinzel phocomelia syndrome. *Journal of Human Genetics., 79*, 402–408.

Xiong, L., et al. (2015). Lrp4 in osteoblasts suppresses bone formation and promotes osteoclastogenesis and bone resorption. *Proceedings of the National Academy of Sciences of the United States of America., 112*(11), 3487-3492.

Yagi, M., et al. (2005). DC- STAMP is essential for cell-cell fusion in osteoclasts and foreign body giant cells. *Journal of Experimental Medicine, 202*(3), 345–351.

Yang, Y., et al. (2003). Wnt5a and Wnt5b exhibit distinct activities in coordinating chondrocyte proliferation and differentiation. *Development, 130*(5), 1003–1015.

Yang. L, et al. (2014). Hypertrophic chondrocytes can become osteoblasts and osteocytes in endochondral bone formation. *Proceeding of the National Academy of Sciences of the United States of America, 111*(33), 12097-12102.

Zhai, L., et al. (2004). Drosophila Wnt-1 undergoes a hydrophobic modification and is targeted to lipid rafts, a process that requires porcupine. *Journal of Biological Chemistry, 279*(32), 33220–33227.

Zhong, Z., et al. (2012). Wntless functions in mature osteoblasts to regulate bone mass. *Proceedings of the National Academy of Sciences, 109*(33), E2197–E2204.

Zhu, M., et al. (2009). Activation of β-catenin signaling in articular chondrocytes leads to osteoarthritis-like phenotype in adult β-catenin conditional activation mice. *Journal of Bone and Mineral Research, 24*(1), 12-21.

Zhu, W., et al. (2008). The Regulatory Role of Matrix Proteins in Mineralization of Bone. In R. Marcus, *Fundamentals of Osteoporosis.* Oxford: Elsevier.

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