NOTUM and RSPO3 in Bone

WNT signaling modulators regulating the skeleton

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Whole body DXA image of adult female mouse.

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Till Björn och Olle.

Nothing shocks me – I'm a scientist
Dr. Henry Walton Jones

There's nothing we can't do if we work hard, never sleep, and shirk all other responsibilities in our life
Leslie Knope

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ABSTRACT

Osteoporosis is a skeletal disease affecting millions of people worldwide, often leading to fragility fractures and a decreased quality of life. There is an unmet medical need for new anabolic, bone building, treatments. WNT proteins, signaling via the WNT canonical or non-canonical pathway, are known to regulate cortical bone, trabecular bone, or both. There is currently a knowledge gap on the mechanisms behind this regulation, and it would be of great interest to find targets to specifically manipulate the different compartments. The aim of this thesis is to study the effect that WNT signaling modulators NOTUM and RSPO3 have on bone, using both *in vivo* and *in vitro* methods. In mouse studies, we inactivated NOTUM and RSPO3 globally and in specific cells using the Cre-*loxP* system, enabling us to, in detail, study these modulators mechanistically. First, we found that endogenous NOTUM, a WNT inhibiting secreted lipase, is a specific regulator of cortical bone, and that inactivation of NOTUM in all osteoblast-lineage cells increased cortical bone quality and thickness by increasing periosteal bone formation. Cell culture experiment showed that this effect is via enhanced osteoblast differentiation, and further studies concluded that it is the NOTUM expressed by late osteoblasts/osteocytes that is the main source of NOTUM. RSPO3, part of the R-spondin family, enhances WNT signaling and inactivation of RSPO3 in osteoblast-lineage cells specifically decreases trabecular bone in the vertebral column, with no effect on cortical bone. Osteoblast cultures revealed that inhibition of RSPO3 decreased proliferation and differentiation of osteoblasts through inhibition of WNT canonical signaling. In ovariectomized mice, a model for postmenopausal osteoporosis, RSPO3 was nonessential for the beneficial effects of estrogens on trabecular bone, but it was, surprisingly, needed for a full effect of estrogen on cortical bone. Together, the results presented in this thesis provide important new knowledge about the WNT signaling modulators NOTUM and RSPO3 and reveals them to be new possible targets for anabolic drugs against osteoporosis.

Keywords: NOTUM, RSPO3, WNT signaling, bone

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NOTUM och RSPO3 i ben
WNT-signaleringsmodifierare reglerar skelettet

Benskörhet, eller osteoporos, är en skelettsjukdom där benet över tid bryts ned. Det drabbar miljoner av människor världen över, och leder ofta till frakturer med en minskad livskvalitet som följd. I dagsläget används främst läkemedel som hämmar nedbrytningen av ben, men det finns ett stort behov av läkemedel som främjar uppbyggnaden av benet, så kallade anabola läkemedel. Det är sedan tidigare känt att WNT-proteiner kan reglera trabekulärt ben, kortikalt ben, eller båda två. Det finns dock inte så mycket kunskap om hur den här regleringen går till, och för att i framtiden kunna utveckla bättre osteoporosläkemedel, vore det av stort värde att hitta proteiner som specifikt kan påverka de olika bentyperna. Syftet med den här avhandlingen var att studera betydelsen av två modulatorer av WNT-signalering för skelettet. Den ena, det extracellulära lipaset NOTUM, är en hämmare av WNT-signalering, och den andra, RSPO3, är en förstärkare av WNT-signalering. För att i detalj studera betydelsen av NOTUM och RSPO3 inaktiverade vi dessa modifierare i möss, både i hela musen och i specifika celler. Vi såg att endogent NOTUM specifikt reglerar kortikalt ben, och när vi inaktiverade NOTUM i alla osteoblastceller så ökade kvaliteten på kortikalbenet samt tjockleken, detta genom en ökad benbildning på ytan av kortikalbenet. I odlade osteoblaster såg vi att denna effekt berodde på en ökad mognad av cellerna, och vidare studier visade att det NOTUM som påverkar benet produceras av de sena osteoblasterna och osteocyterna. När vi i stället inaktiverade RSPO3 i alla osteoblastceller ökade det trabekulära benet i ryggkotorna, men vi såg ingen effekt på det kortikala benet. I de odlade osteoblasterna såg vi att hämning av RSPO3 i osteoblaster leder till minskad celldelning och mognad av osteoblasterna genom en hämmad WNT-signalering. I en modell som används för att studera postmenopausal osteoporos såg vi att RSPO3 och östrogen reglerar trabekulärt ben oberoende utav varandra, men att RSPO3 bildat av osteoblaster behövs för att östrogen ska ha sin fulla effekt på kortikala benet.

Resultaten som presenterats i denna avhandling ger oss ny kunskap om hur WNT-signaleringmodifierarna NOTUM och RSPO3 påverkar skelettet, vilket gör dem till möjliga mål för framtida anabola läkemedel mot osteoporos.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Movérare-Skrtic S*, Nilsson KH*, Henning P, Funck-Brentano T, Nethander M, Rivadeneira F, Coletto Nunes G, Koskela A, Tuukkanen J, Tuckermann J, Perret C, Souza PPC, Lerner UH, Ohlsson C. **Osteoblast-derived NOTUM reduces cortical bone mass in mice and the *NOTUM* locus is associated with bone mineral density in humans.** *FASEB J.* 2019 Oct;33(10):11163-11179.

II. Nilsson KH, Henning P, El Shahawy M, Wu J, Koskela A, Tuukkanen J, Perret C, Lerner UH, Ohlsson C, Movérare-Skrtic S. **Osteocyte- and late osteoblast-derived NOTUM reduces cortical bone mass in mice.** *Am J Physiol Endocrinol Metab*. 2021 May 1;320(5):E967-E975.

III. Nilsson KH*****, Henning P*, El Shahawy M, Nethander M, Andersen TL, Ejersted C, Wu J, Gustafsson KL, Koskela A, Tuukkanen J, Souza PPC, Tuckermann J, Lorentzon M, Ruud LE, Lehtimäki T, Tobias JH, Zhou S, Lerner UH*, Richards JB*, Movérare-Skrtic S*, Ohlsson C*. **RSPO3 is important for trabecular bone and fracture risk in mice and humans**. *Nat Commun.* 2021 Aug 13;12(1):4923.

IV. Nilsson KH, Wu J, Gustafsson KL, El Shahawy M, Koskela A, Tuukkanen J, Tuckermann J, Henning P, Lerner UH, Ohlsson C, Movérare-Skrtic S. **Estradiol and RSPO3 regulate vertebral trabecular bone mass independent of each other.** *Am J Physiol Endocrinol Metab.* 2022 Mar 1;322(3):E211-E218.

* Contributed equally.

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CONTENT

ABBREVIATIONS

αMEM	α Minimum essential medium
BMD	Bone mineral density
BMM	Bone marrow macrophages
BV/TV	Bone volume over tissue volume
cDNA	Complementary DNA
CTX	C-terminal type I collagen
DKK1	Dickkopf-1
DMP1	Dentin matrix protein 1
DXA	Dual-energy X-ray absorptiometry
E2	17β-estradiol
LGR	Leucine-rich repeat containing G-coupled receptor
LRP	Low-density lipoprotein receptor-related protein
M-CSF	Macrophage colony-stimulating factor
μCT	High resolution micro-computed tomography
mRNA	Messenger RNA
OPG	Osteoprotegerin
OVX	Ovariectomy
P1NP	Procollagen type I NH_2 terminal propeptide
PCP	Planar cell polarity pathway
PGE2	Prostaglandin E2

PORCN	Porcupine
PTH	Parathyroid hormone
PTHrP	Parathyroid hormone-related protein
pQCT	Peripheral quantitative computed tomography
RANKL	Receptor activator of nuclear factor kappa-B ligand
RNF	Ring finger protein
ROR	Receptor tyrosine kinase-like orphan receptor
RUNX2	Runt-related transcription factor 2
RYK	Receptor tyrosine factor
scRNAseq	Single cell RNA sequencing
SOST	Sclerostin
TCF/LEF	T-cell factor/lymphoid enhancer factor
TRAP	Tartrate-resistant acid phosphatase
ZNRF	Zinc and ring finger

1 INTRODUCTION

Osteoporosis is a common skeletal disease affecting millions of people worldwide, and osteoporotic fractures lead to large personal suffering for the patients as well as great societal costs. Bone consists of two compartments, the spongy trabecular bone, and the hard cortical bone and these two bone types are regulated *via* different mechanisms but there is currently a large knowledge gap on the mechanism behind this. As the world's population is getting increasingly older, the number of fractures will increase dramatically [1, 2]. Today, the most commonly used drugs against osteoporosis are antiresorptive, meaning that they inhibit the degradation of the bone. There is therefore a need for new anabolic drugs that instead builds and restore the lost bone.

WNT proteins are known to affect cortical bone, trabecular bone, or both [3]. In this thesis, we aim to investigate whether, by manipulating WNT signaling, we can specifically improve bone mass, bone quality and bone strength in the different bone compartments to find new druggable targets to be used to develop new drugs against osteoporosis.

1.1 THE SKELETON

The skeleton is an organ with many functions. Consisting of more than 200 bones, it gives stability and structure to the body, protects the inner organs, and acts as a place for hematopoiesis. In the recent years, the skeleton has also been identified as an endocrine organ [4], producing hormones such as osteocalcin and fibroblast growth factor-23 [5]. It can be categorized as the appendicular skeleton, i.e., the collar bones and the extremities, and the axial skeleton, i.e., the vertebral column, the rib cage, and the skull.

The skeleton is made up by two types of bone, about 80% of it consists of the harder cortical bone, and the rest is the spongier cancellous bone, also called trabecular bone [6]. The bone itself is mainly composed by an inorganic component (called hydroxyapatite), but also water and organic components (bone matrix proteins, mostly type I collagen) [7]. Cortical bone is a highly organized structured, where osteons, divided into primary and secondary osteons, are the functional unit, running longitudinally in bone. In primary osteons, the blood vessels are surrounded by lamellae. Secondary osteons, also called Haversian system, are more matured than primary osteons. They have a cylindrical structure that, aside from containing blood vessels and

nerve fibers, transports nutrients. Volkmann canals runs perpendicularly between osteons, connecting the Haversian canals [8-11].

The long bones, such as the femur, consist of three distinguishable areas. The diaphysis, i.e., the shaft of the bone, consists mainly of cortical bone whereas the epiphysis, at the ends of the bone, and metaphysis, between the diaphysis and epiphysis, are mostly made up of trabecular bone surrounded by a layer of cortical bone. The epiphysis is covered by cartilage. Bone growth occurs at the growth plate located between the epiphysis and metaphysis, and at puberty, the growth plate normally closes and bone growth stops. As we use mouse models to investigate different ways to manipulate bone formation, it is important to know that in rodents, the growth plate never fully closes, making them able to grow throughout their lifetime [12]. At the center of the long bones is the medullary cavity, where the bone marrow is located, hence making it a place for hematopoiesis, along with blood vessels and nerves.

1.1.1 BONE CELLS

The three bone cell types located in bone are osteoclasts, osteoblasts, and osteocytes.

Osteoclasts are large, multinucleated cells responsible for degrading bone, derived from hematopoietic myeloid-lineage cells [13]. For preosteoclasts to develop into osteoclasts, a process called osteoclastogenesis, different cytokines need to be present, the most important being macrophage colony-stimulating factor, M-CSF, also called CSF1, and receptor activator of nuclear factor-kappa B (NF-κB) ligand, RANKL [14, 15]. Signaling through CSF1R, the receptor of M-CSF, is together with RANKL essential for the function, proliferation, and differentiation of osteoclasts. Activation of CSF1R by binding of M-CSF induces the expression of RANK, the receptor of RANKL. RANKL/RANK binding activate signaling pathways that in the end induce the expression of the master regulator of osteoclastogenesis, NFATc1. The mature osteoclasts can then resorb bone [16, 17]. The glycoprotein osteoprotegerin, OPG, is a secreted decoy receptor for RANKL, and when RANKL binds OPG, there is a reduction in bone resorption due to the osteoclast precursors being unable to differentiate without the binding of RANKL to RANK [18]. Thus, OPG acts as a negative regulator of osteoclastogenesis. The life span of an osteoclast has recently been measured to at least 24 weeks and the osteoclasts have the capacity to form new nuclei when fused with new progenitor cells [19]. They will undergo apoptosis when M-CSF and RANKL are no longer present [20, 21]. Tartrate-resistant acid phosphatase (TRAP) is an enzyme used to measure osteoclast activity, and is

also present in preosteoclasts [20]. TRAP dephosphorylates osteopontin and bone sialoprotein, regulating osteoclast attachment [22], and mice lacking TRAP display mild osteopetrosis [23].

Osteoblasts are the bone forming cells. Osteoblasts are from the mesenchymal lineage and are derived from skeletal stem and progenitor cells. These cells have a trilineage potential to *in vitro* form either chondrocytes, a cell type found in cartilage, osteoblasts, or adipocytes [24]. Runt-related transcription factor 2 (RUNX2) is required for the cells to differentiate into preosteoblasts, to further differentiate into osteoblasts [25]. Osteoblasts produce bone matrix [26] and produce, for example, alkaline phosphatase (APL), needed for mineralization of bone matrix, and osteocalcin, which could be used as a bone turnover marker [27]. They have an estimated life span of roughly three months [28]. When the osteoblasts have performed the bone formation, one of three cell fates will occur; they will become osteocytes, imbedded in the bone matrix, they will undergo apoptosis, or they will become bone lining cells, being inactive on the bone surface [29-31]. Apart from driving the bone formation, osteoblasts also produce cytokines important for osteoclastogenesis, such as RANKL and M-CSF [16].

Osteocytes are the most abundant cell type in the bone, about 90-95% of all cells in bone are osteocytes, in relation to osteoclasts and osteoblasts (~1% and 3-5% of all bone cells, respectively) [32]. When the osteoblast differentiates into an osteocyte, the shape of the cell is changed; from the cuboidal shape of the osteoblast to the dendritic shape of the osteocyte [33]. Osteocytes are part of a network called lacunar-canalicular network, where lacunae are the small cavities in the bone in which the osteocytes are located, and the canaliculi are the small connection in between the lacunae. The osteocytes have different ways of communication, one of them being via cell-cell contacts using gap junctions (coupling between membrane-bound protein complexes on adjacent cells), another being paracrine signaling, either by small molecules (as prostaglandins and ATP), or large molecules (as sclerostin or RANKL) [33]. Osteocytes are also the main cell type expressing Dentin Matrix Protein 1 (DMP1), a non-collagenous protein, important for mineralization [34]. At mechanical loading, there is a fluid flow in the lacunar-canalicular network that osteocytes sense and initiate bone modeling [35]. Osteocytes are long living cells, and it has been proposed that its life can span over decades [32].

1.1.2 BONE REMODELING AND MODELING

The skeleton is in constant change. Osteoclasts are constantly degrading the bone, and osteoblasts are building it. Every ten years, the entire skeleton has been exchanged [28]. There are two mechanisms through which bone can form, bone remodeling and bone modeling.

Bone remodeling (depicted in figure 1) is when osteoclasts and osteoblasts work together to replace old and damaged bone. Bone remodeling participates in calcium homeostasis, where parathyroid hormone (PTH) stimulates bone to release calcium from the bone. There are four stages of bone remodeling. First is the activation, where osteoclasts are recruited. Second is the resorption, where the osteoclasts start to resorb bone. Third is reversal, when osteoclasts undergo apoptosis and osteoblasts are recruited. Finally, the formation stage when osteoblasts form new bone matrix that eventually will be mineralized.

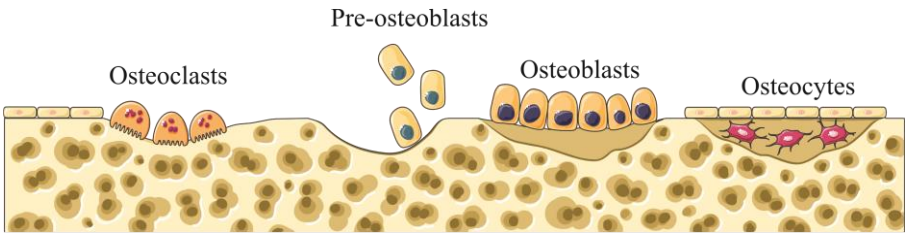

Figure 1: The bone remodeling process where the osteoclasts are degrading bone, the osteoblasts rebuild the bone, and the osteocytes are finally embedded in the bone. Illustration adapted from Servier Medical Art by Servier.

It is believed that it is the osteocytes that recruits the osteoclasts at the start of bone remodeling, either by sensing bone deformation or detecting small damages in the old bone. At the end of bone remodeling, the osteocytes become embedded in the newly formed bone [36-38]. This nonpermanent structure was first described by Harold Frost and is termed basic multicellular units (BMU) [39]. In resorption, the osteoclasts form a ruffled border with the bone, and the osteoclasts are attached to the resorption area by the sealing zone. This creates an isolated resorption compartment, where the osteoclasts dissolve bone, and the degraded products are transported in vesicles through the osteoclasts. The organic part of the skeleton is degraded by cathepsin K, which is used as a marker for bone resorption [40]. When the skeleton has reached peak bone mass, the bone remodeling will be at equilibrium, leading to the bone mass being constant, until age-dependent bone loss [36]. It is estimated that 80% of all remodeling occurs at trabecular bone [41], which is notable since trabecular bone makes up about 20% of all bone. The bone remodeling that occurs at the endocortical or periosteal side of cortical bone is similar to the remodeling occurring in trabecular bone [42].

Bone modeling is when osteoblasts or osteoclasts individually reshape the bone. This does not necessarily happen at the same time or in the vicinity of each other. Bone modeling is occurring even in the elderly and is responsible for the forming of bone. Physical activity can stimulate bone modeling, as seen after tibial loading in mice [43], where the loaded tibia exhibits a distinct bone increase.

1.2 OSTEOPOROSIS

The world's population is getting increasingly older. A recent report by the United Nations predicted that the number of individuals older than 65 years old will triple, compared with adults under the age of 65, by the year 2100 [1, 2]. Osteoporosis is a systemic disease causing the bone to deteriorate, and it mainly affects the elderly. It is estimated that one in two elderly women, and one in four elderly men, will suffer an osteoporotic fracture [44, 45], making osteoporosis a disease affecting a large part of the population, causing suffering for the patients, as well as substantial costs for the society as a whole.

In osteoporosis, the skeleton is unable to maintain bone homeostasis, so the bone resorption, driven by the osteoclasts, are larger than the bone formation, driven by the osteoblasts. There is a decrease in bone mineral density (BMD) and an impairment in the bone structure, leading to an increased risk of fracture.

The most common fracture sites are in the vertebral column, the radius or ulna, or the femoral neck (figure 2) [45]. Where the fracture most often occur is dependent on age, for example, women aged 50-54 most often fracture at distal radius, but for women around 90 years of age, hip fractures are most common [46].

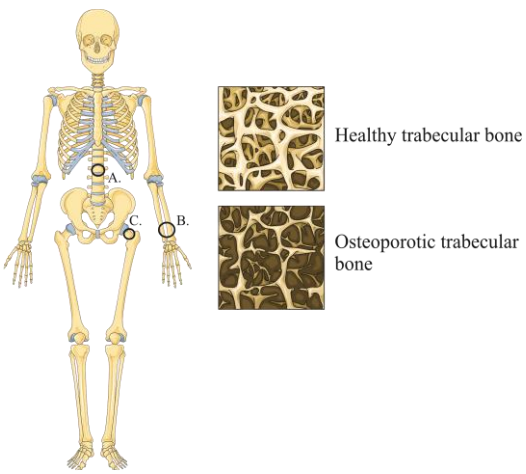

Figure 2: Overview of common fracture sites in an osteoporotic patient (left). A: Vertebral column; B: Distal radius or ulna; C: The femoral neck. Example of healthy trabecular bone (upper right) and osteoporotic trabecular bone (lower right). Illustration adapted from Servier Medical Art by Servier.

Diagnosing osteoporosis is mainly done by DXA (Dual-energy X-ray absorptiometry) analysis, where the assessment is compared with the BMD mean of the healthy, young population. It is defined as osteoporosis if the BMD of the patient differs more than 2.5 standard deviations from the reference BMD [47]. Osteoporosis can be divided into primary osteoporosis, where the bone loss happens over time and that is often dependent on age and decreasing levels of estrogen, and secondary osteoporosis, that is caused by an underlying disease or medical treatment such as glucocorticoids [48].

1.2.1 CURRENT OSTEOPOROSIS TREATMENT

There are two main treatment strategies against osteoporosis, antiresorptive drugs and anabolic drugs [49]. Antiresorptive drugs (i.e., bisphosphonates and the RANKL antibody denosumab) inhibit osteoclast function or differentiation, but they do not rebuild the bone already lost. There have, however, been reports of serious adverse events following bisphosphonate treatment, such as atypical femoral fractures and osteonecrosis of the jaw [50]. Anabolic drugs (i.e., romosozumab and teriparatide), on the other hand, initiate bone modeling. Romosozumab is a sclerostin antibody, and teriparatide is PTH that when given intermittently is anabolic, but when given continuously is destructive for bone [49]. Some but not all studies have indicated that treatments targeting sclerostin may be associated with cardiovascular side effects [51-53].

1.3 WNT SIGNALING PATHWAYS

WNT is a family of hydrophobic, secreted proteins first discovered in Drosophila [54, 55]. The WNT family consists of 19 proteins having many functions throughout the body, amongst other involvement in skeletal patterning during development, and overall bone health [56, 57].

In WNT signaling, there are three major signaling pathways; the β-catenin dependent pathway, also called the canonical signaling pathway, and two non-canonical signaling pathways [56]. For the canonical signaling pathway, the acyl tail of the WNT protein binds to its receptor Frizzled and the coreceptor LRP (low-density lipoprotein receptor-related protein), and in the non-canonical pathways, WNTs bind to Frizzled (Fzd) and the receptor ROR (tyrosine kinase-like orphan receptor) and RYK (receptor tyrosine factor) coreceptors (see figure 3) [3, 56, 58].

1.3.1 CANONICAL WNT SIGNALING PATHWAY

When the WNT receptors are unbound by their ligand, the destruction complex, consisting of Axin, Adenomatous polyposis coli (APC), and the two serine threonine kinases protein kinase glycogen synthase kinase (GSK3α/β), and casein kinase (CK1α/δ), phosphorylates β-catenin, which then becomes ubiquitinated, leading to its degradation [59, 60]. If β-catenin cannot translocate into the nucleus, transcription will not start. At the start of the WNT signaling cascade, porcupine, a membrane-bound O-acetyltransferase located in the endoplasmic reticulum (ER), adds an acyl group from a palmitoleic acid to the tail of the WNT protein [61]. The WNT is then transported out of the cell by the chaperone protein Wntless [62]. The acyl tail of the WNT binds its receptors Frizzled, and the coreceptor LRP5/6. When this ligand-receptor complex is formed, the LRP coreceptor is phosphorylated instead of β-catenin, Dishevelled is bound to the receptors and interacts with Axin in the destruction complex, leading to the disbandment of the destruction complex and a subsequent increase in available β-catenin. The β-catenin then translocates into the nucleus, activates the transcription factors TCF/LEF, and finally, transcription starts.

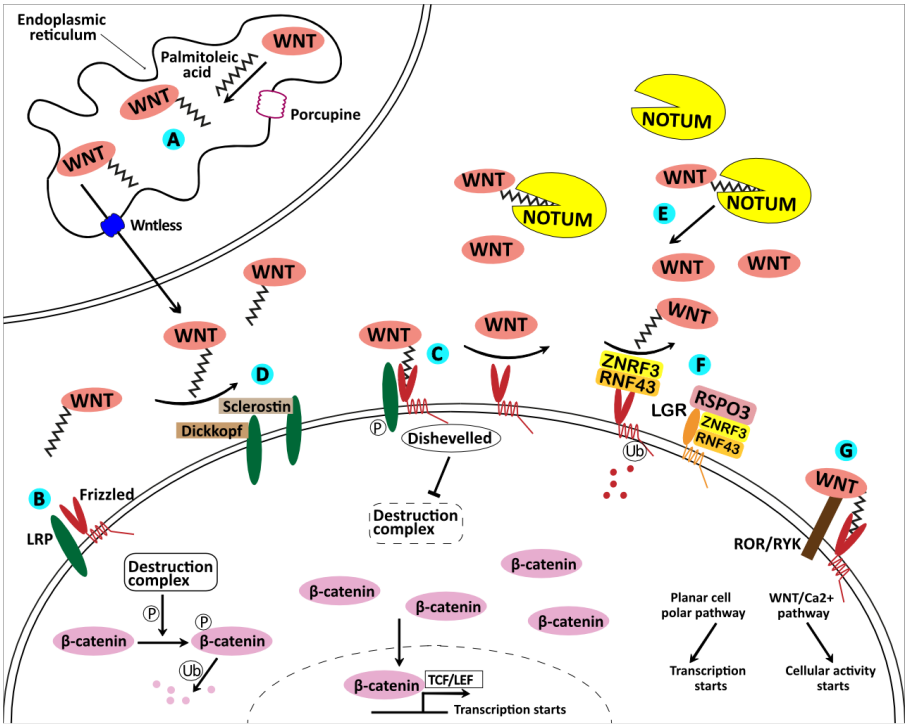

Figure 3: Overview of WNT signaling. In the endoplasmic reticulum (A), Porcupine adds an acyl group from palmitoleic acid to WNT and Wntless transports the WNT protein out of the cell. When the WNT receptors Frizzled and LRP are unbound (B), β-catenin is marked for destruction by the destruction complex. When the WNT protein binds the receptors (C), the destruction complex is disbanded, and β-catenin will translocate into the nucleus to start transcription. Dickkopf and sclerostin (D) can inhibit canonical WNT signaling by binding to the LRP receptor. NOTUM (E) inhibits canonical and non-canonical signaling by removing the acyl tail from the WNT protein, making it unable to bind the Frizzled receptor. RSPO3 (F) enables both signaling pathways by stopping ZNRF3 and RNF43 from blocking the Frizzled receptor. For non-canonical signaling (G), the WNT protein will bind the receptors and either start the planar cell polar pathway or the WNT/Ca^{2+} pathway.

1.3.2 NON-CANONICAL WNT SIGNALING PATHWAYS

The non-canonical signaling pathways are very complex, with several proteins involved, and all of these pathways, or even how many there are, are not yet fully understood [63, 64]. It is not even clear how many pathways are involved. What unites the non-canonical pathways is that they are independent of β-catenin. Two defined non-canonical WNT signaling pathways are the planar cell polar (PCP) pathway, which controls cell and tissue polarity that is important when the embryo undergoes bone and joint formation [65-67], and the WNT/Ca^{2+} pathway, which increases the amount of intracellular Ca^{2+} concentration leading to several cellular responses, including regulation of cytoskeletal reorganizations [68-72]. Although ligands of the non-canonical signaling pathway also activate the Frizzled receptor, the co-receptor differs. Two well-established co-receptors are RYK (Related to Tyrosine Kinase), and ROR (Receptor tyrosine kinase-like Orphan Receptor). Signaling via ROR2 receptor is known to play a role in BMD regulation and bone biology [63].

1.4 WNT PROTEINS

The 19 WNT proteins that have been discovered can signal via the canonical pathway, the non-canonical pathway, or both [54, 56, 73, 74]. Different WNTs can also regulate the bone compartments differently; WNT16, for example, regulates cortical bone [75], WNT10b regulates trabecular bone [76, 77], and WNT7b regulates both trabecular and cortical bone [78]. In table 1, the 19 WNTs identified in mice are listed, together with some important functions. There are several inhibitors of the WNT signaling pathways, Dickkopf (DKK) and sclerostin (SOST), for example, binds to LRP, thereby blocking WNT from binding to its receptors [79], and the RING finger proteins ZNRF3 and RNF43 promote Fzd receptor turnover by binding to the Fzd receptor, causing it to be degraded [80] (figure 3). The secreted Frizzled-related protein 4 (SFRP4) is a soluble decoy receptor for WNT proteins [81], and NOTUM deacetylates WNTs to prohibit WNT binding to its receptors [82].

WNTs have many effects in bone physiology, from embryonal development to homeostasis in the adult individual, but WNTs have many effects aside from the skeleton. Different types of cancers display deviation in WNT pathways [60], and there are for example preclinical studies where the porcupine inhibitors WNT974 (also called LGK974) and Wnt-C59 are evaluated for treatment of hematological diseases, such as Hodgkin Lymphoma and Burkitt Lymphoma [83-85].

Table 1: WNT ligands and important functions in mice

Ligand	***Signaling pathway***	***Important function***
Wnt1	Canonical signaling	Improves cortical thickness in adult mice [18] Mesenchymal-derived Wnt1 regulate subchondral bone [86]
Wnt2 ***Wnt2b***	Canonical signaling	Required for patterning of lung during embryogenesis [87]
Wnt3	Canonical signaling	Expressed in follicles, overexpression reduces hair growth [88]
Wnt3a	Canonical signaling	Regulate embryonic hindgut extension [89]
Wnt4	Canonical signaling Non-canonical signaling	Conditional Wnt4 KO mice display thinning of cortical bone [90] Wnt4 induced osteogenesis through both canonical and non-canonical signaling [91]
Wnt5a	Non-canonical signaling	Upregulates osteoclasts formation [92]
Wnt5b	Non-canonical signaling	Knockout mice display higher bone mass in high throughput screenings [93]
Wnt6	Canonical signaling	Regulate adipogenesis and osteoblastogenesis, and fate of mesenchymal progenitors [94]
Wnt7a	Canonical signaling	Involved in mammalian limb development [95]
Wnt7b	Non-canonical signaling [96]	Reverses loss of bone mass caused by glucocorticoids [97]
Wnt8a	Not known	Not known
Wnt8b	Canonical signaling	Expressed in hippocampus, alters other Wnt expression [98]
Wnt9a	Canonical signaling	Needed for joint integrity [99] Deletion from Prx1-expressing cells leads to osteoarthritis in aging mice [90]
Wnt9b	Non-canonical signaling	Involved in establishing and maintaining the diameter in kidney tubule [100]
Wnt10a	Canonical signaling	Knockout mice showed decreased trabecular bone and adipose tissue in bone marrow [101]
Wnt10b	Canonical signaling	Required for maintenance of bone in adult mice [76, 77]
Wnt11	Canonical signaling Non-canonical signaling	Needed for osteoblast maturation and mineralization [102]
Wnt16	Canonical signaling Non-canonical signaling	Regulates cortical bone thickness and prevent fragility fracture risk via bone formation on the periosteal site and inhibits osteoclasts [75, 103]

1.5 SCLEROSTIN

Sclerostin (SOST) is a glycoprotein secreted by osteocytes that acts as an antagonist to WNT signaling [104]. SOST is secreted by mature osteocytes that are embedded in mineralized bone [105], and it binds to the WNT canonical co-receptors LRP5 and LRP6, thereby making it impossible for the WNT protein to bind to its receptors (see figure 4) [106]. It has also been reported that mutations in LRP4 leads to increased bone mass, this through an interaction between SOST and LRP4 [107]. SOST is known to have clinical implications, where two rare bone diseases, van Buchem disease and sclerosteosis, are associated with lack of sclerostin. In these diseases, the patients have high osteoblastic activity, and they suffer from a very high bone mass [108]. Romosozumab, a SOST antibody, is a novel drug to treat osteoporosis that is acting as an anabolic agent as well as inhibits bone resorption [109], and it has been shown that the SOST antibody can rescue bone loss in both trabecular and cortical bone in mice with glucocorticoid-induced osteoporosis, but it did not prevent the negative effect the glucocorticoid have on linear growth [110]. However, SOST is also expressed in heart, aorta, and coronary and peripheral arteries, especially in vascular smooth muscle cells (VSMCs), and SOST have been believed to act protective against cardiovascular diseases [111]. Therefore, concerns have arisen that treatment with romosozumab may cause cardiovascular side effects, especially in patients with pre-existing cardiovascular diseases, but the clinical data are inconclusive [51-53].

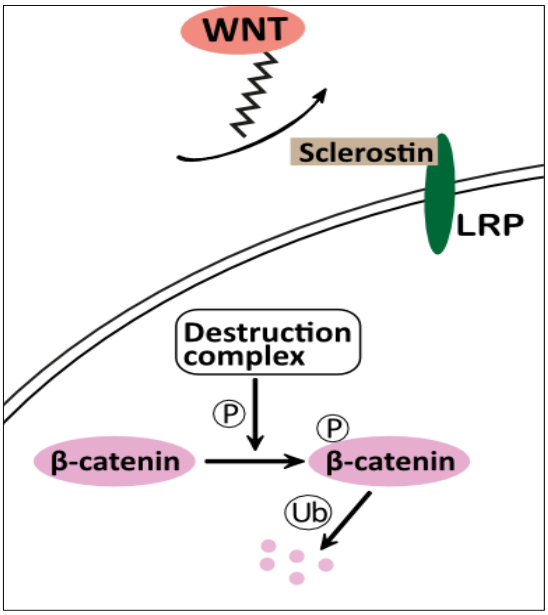

Figure 4: SOST inhibit WNT signaling by binding to the LRP co-receptor, blocking WNT signaling. When Frizzled is unbound, the destruction complex phosphorylates (P) β-catenin, leading to it being ubiquitinated (Ub) and, finally, degraded.

1.6 NOTUM

One of the inhibitors in WNT signaling is NOTUM, a circulating, extracellular enzyme identified in Drosophila [112, 113]. A genetic screen revealed that gain-of-function of NOTUM resulted in loss of wing, and that *Notum* overexpression gave a similar phenotype as WNT inactivation [113]. In fact, the gene is called *Notum* because of the anatomic structure notum on flies, located at the dorsal side of the fly's thoracic segment, and in early reports, *Notum* was called *wingfull* [112].

Since NOTUM is structurally similar to pectin acetylesterases, a theory that NOTUM cleaved the glycosylphosphatidylinositol (GPI) anchor on the Drosophila glypicans Dally and Dally-like protein (Dlp) was proposed, where Dally and Dlp help stabilize WNTs [113]. This theory was however discarded, when later studies showed that NOTUM is a carboxylesterase that have a hydrophobic pocket where palmitoleate are able to bind (see figure 5). When the acyl group on the WNT proteins tail, added by porcupine, binds this pocket, NOTUM deacetylates WNT, rendering the WNT protein unable to bind the receptors [82].

The relationship between NOTUM and bone have been studied, albeit not extensively. Together with researchers from Lexicon Pharmaceuticals, we took part in a study where NOTUM was deleted globally from birth, using a gene trapping technique. In this study, the mice displayed increased bone mass [114]. The NOTUM knockout mice also had dentin dysplasia and unilateral kidney agenesis [114], and there appeared to be a decreased viability of the knockout mice. All *Notum*$^{-/-}$ mice had dentin defects in incisors and molar teeth, and some of the molar roots were shorter and had a deviant shape, compared to wildtype mice. These dental effects can all be due to increased WNT signaling [115].

NOTUM has also been shown to inhibit osteogenic differentiation of human periodontal ligament stem cells, and NOTUM was highly expressed in periodontal tissue in mice with periodontitis which could make NOTUM a pharmacological target against periodontitis [116].

Mice with a conditional inactivation of NOTUM in the liver was shown to not change architecture or zonation of the liver, but male mice with deletion of NOTUM in liver had a significantly increased risk of obesity and they had high plasma insulin concentration as well as fasting blood glucose levels, indicating that NOTUM might be involved in metabolic disorders in men [117].

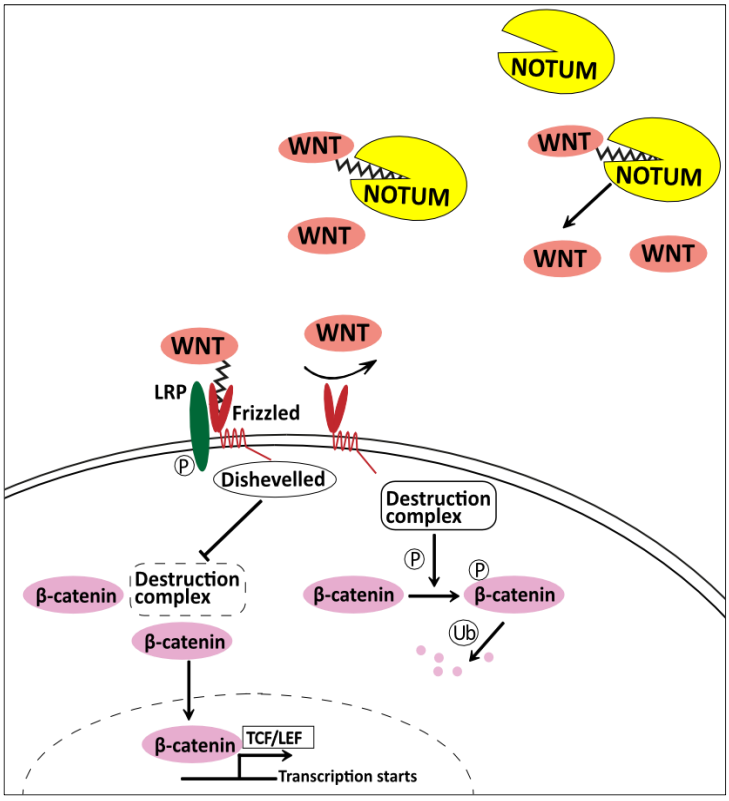

Figure 5: NOTUM inhibits WNT signaling by deacetylating the protein, making the protein unable to bind its receptors.

1.7 R-SPONDIN 3

As opposed to NOTUM, R-spondins (roof-plate specific spondins) are not inhibitors of WNT signaling, but enhancers of WNT signaling. The R-spondin superfamily consists of four proteins (RSPO 1-4), all having a similar homology [118]. The four RSPOs have different functions; mice lacking *Rspo1* show decreased fertility and are unable to lactate, loss of *Rspo2* in mice cause kidney dysfunction and laryngeal-tracheal malformation, *Rspo3* is important regarding vascularization of the placenta, and *Rspo4*

deficiency leads to loss of fingernails and toenails and is highly expressed in developing heart and limb buds [119].

The RSPOs bind to the receptors leucine-rich repeat containing G-protein coupled receptor (LGR) 4 and 5 (see figure 6). These receptors are similar to the receptors for follicle stimulating hormone, luteinizing hormone and thyroid-stimulating hormone [120], and they were long considered to be orphan receptors [121]. The RSPOs binds the LGR receptor and the E3 ubiquitin ligases RNF43/ZNRF3, forming a ternary complex. When RNF43/ZNRF3 are free, they bind to the WNT receptor Frizzled, causing Frizzled to be ubiquitinated and degraded, so the RSPO-RNF43/ZNRF3-LGR4/5 complex is thereby boosting WNT signaling [122].

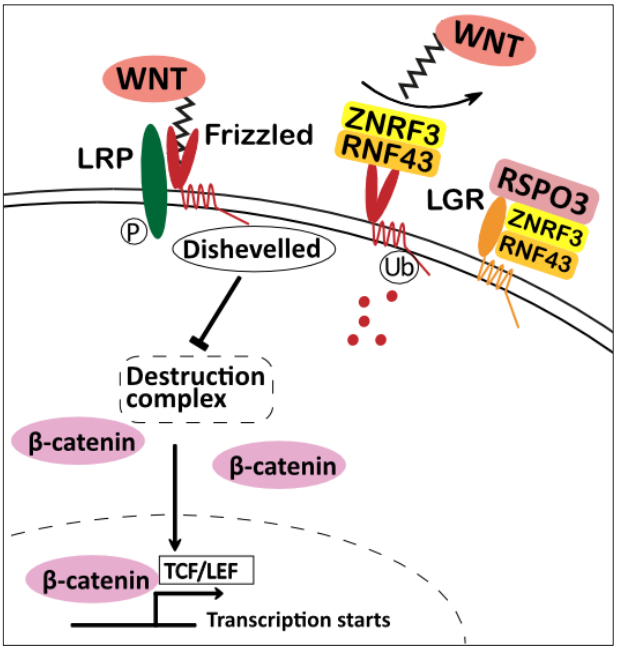

Figure 6: RSPO3 forms a complex with ZNFR3 and RNF43 and binds to the RSPO receptor LGR, allowing WNT to bind to Frizzled.

Murine studies regarding RSPO3 have been limited due to *Rspo3* knockout mice are embryonically lethal [123-126]. In *Rspo3*$^{-/-}$ mice, the mouse embryo has placental defects, as RSPO3 is needed for vascularization of the placenta, especially the labyrinth, where the nutrient exchange between the mother and the embryo occurs [123, 124]. A study using conditional inactivation of *Rspo3* in limb mesenchymal cells demonstrated that it is not required for development of limbs in mice [126], otherwise there have been few mechanistic studies showing RSPO3s function in bone.

1.8 ESTROGEN AND BONE

Through development, estrogen is very important for the skeleton, both the trabecular and the cortical bone. It is involved in regulation of growth in children and young adult, by closing the growth plate and regulating growth hormone [127]. After menopause, the estrogen levels in women are drastically reduced [128], and already in the 1940s, Fuller Albright concluded that estrogen deficiency in elderly women is associated with postmenopausal osteoporosis and bone loss. Loss of estrogen increases both bone resorption and bone formation, however, the bone resorption increases more [129].

2 AIM

There is a medical need for new, safe pharmacological treatments against osteoporosis, especially for anabolic, bone building treatments. The overall aim of this thesis is therefore to study how bone mass can be regulated using the WNT signaling modifiers NOTUM and RSPO3, as a way to find new, safe druggable targets that can modulate specific bone compartments, to create better osteoporosis treatments.

The specific aims of this thesis are:

I. To characterize the possible role of endogenous NOTUM as a physiological modulator of bone mass.
II. To determine if NOTUM increasing cortical bone is derived from osteoblast precursors/early osteoblasts or from late osteoblasts/osteocytes.
III. To determine what effect the WNT signaling enhancer RSPO3 has on bone mass.
IV. To determine if RSPO3 is involved in the bone-sparing effect of estrogen.

3 METHODS

3.1 MOUSE MODELS

Mice are very convenient to use in research since they are physiologically similar to humans, are easy to breed and keep, and are inbred to ensure that they are as genetically identical individuals as possible. The mouse strain we use, C57BL/6N, undergo an age-dependent bone loss that resembles that of humans. However, the growth plate never fully closes in mice, meaning they can continue to grow throughout its lifespan, in contrast to humans where the growth plate closes at puberty followed with a stop in growth [12]. Mice and other rodents also lack osteons and Haversian canals in cortical bone, as opposed to humans [130]. All experiments performed in this thesis are approved by the Ethics committee in Gothenburg and housed in a standard animal facility at the University of Gothenburg.

3.1.1 KNOCKOUT MOUSE MODELS

In this thesis, to functionally study the role of NOTUM and RSPO3 *in vivo*, we used global or conditional gene knockout mouse models to inactivate our genes of interest in all cells, in specific cells, or at a specific time.

In the Cre-*loxP* system [131, 132], the targeted gene is flanked by two recombinase recognition (*loxP*) sites (figure 7), that are placed in non-coding regions where a small change in the genome does not have any effect. The *loxP* sites have a fixed size of 34 base pairs for the Cre recombinase to

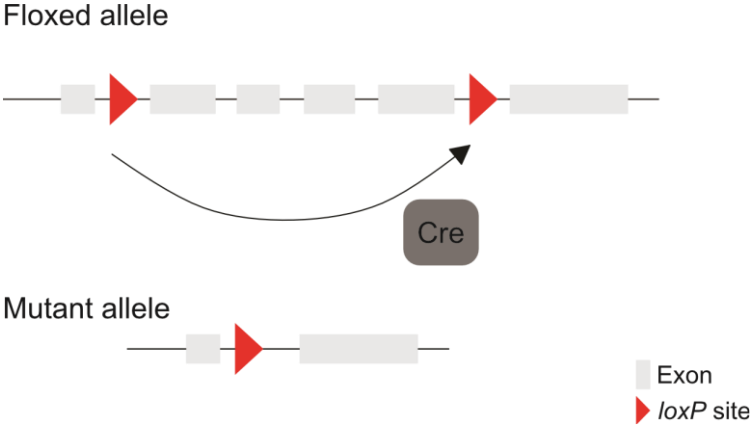

Figure 7: Schematic of a Cre-loxP model. In the presence of a Cre recombinase, the introns and exons between the loxP sites are cleaved, leaving a mutant allele.

recognize. In the presence of a Cre, Cre mediated excision or inversion of the *loxP* flanked sequence occurs and the gene can be cleaved and deleted or cleaved and inverted, depending on the direction of the *loxP*. Depending on the promotor for the Cre recombinase, the gene can be floxed out in different cells or tissues, from birth or by activation. The Cre recombinases discussed in this thesis is listed in table 2.

Table 2: Cre recombinases used in thesis

Cre recombinase	***Gene deleted***	***Paper***
PGK-cre [133]	Globally from birth	I
Runx2-cre [134]	In all osteoblast-lineage cells	I, III, IV
CAGGCre-ER [135]	Globally by activation	I, III
Dmp1-cre [136]	In late osteoblasts and osteocytes	II, III

In paper I and paper II, we combined the Cre recombinase with *Notum*$^{flox/flox}$ mice, where the *loxP* sites are located upstream from exon 2 and downstream of exon 8 [117], and in paper III and IV, we used *Rspo3*$^{flox/flox}$ mice, where the *loxP* sites are located upstream from exon 2 and downstream from exon 4 [126].

In our hands, the global *Notum*$^{-/-}$ mice are embryonically lethal, with only a few surviving null mice, why we instead used the heterozygotes in our analyses. *Notum*$^{+/-}$ mice used in paper I were obtained by crossing female mice expressing both *PKG*-cre and one *Notum*^{flox} allele with male mice expressing *Notum*^{flox}. Since *PGK*-cre is active in the zygote [133], the flox allele will be deleted in all offspring, regardless of simultaneous expression of the Cre. Since *Rspo3*$^{-/-}$ are embryonically lethal [123, 124], we did not attempt to generate a true *Rspo3* knockout mouse.

In paper I, III and IV, we used the *Runx2*-cre (Tg(Runx2-icre)1Jtuc) [134] mouse to inactivate *Notum* (paper I) and *Rspo3* (paper III and IV) early in osteoblast differentiation. No recombination takes place in osteoclasts [75, 134].

In paper II and paper III, we inactivated *Notum* and *Rspo3* in a subset of late osteoblasts expressing *Dmp1* and osteocytes, respectively, using the

Dmp1-cre [136]. In this model, the gene of interest is still present in early osteoblasts.

To overcome the challenge that *Notum*$^{-/-}$ and *Rspo3*$^{-/-}$ mice are embryonically lethal, we used the B6.Cg-Tg(CAG-cre/Esr1*)5Amc/J, hereafter called *CAGGCre-ER*, model [135] to study the effect of global *Notum* and *Rspo3* deletion in all cells. Here, the Cre is driven by a mutated estrogen receptor that are present in the cytosol of the cell. When a mouse with a *loxP*-flanked sequence is treated with the selective estrogen receptor modulator tamoxifen, the Cre along with the receptor is translocated into the nucleus, where the floxed sequence is recombined in presence of the Cre. Using this model, we can inactivate *Notum* in all cells without the risk of embryonal lethality. In paper IV, we used this model both *in vivo* and *in vitro* to delete *Rspo3* globally in adult mice and in cultured cells, respectively. Apart from circumventing the issues with embryonical lethality, the *CAGGCre-ER* model is useful to ensure that the effect seen is truly due to inactivation of the gene, and not a result of developmental effects.

3.1.2 OVARIECTOMY AND ESTROGEN TREATMENT

In paper IV, we wanted to study if RSPO3 is needed for the beneficial effect of estrogen on bone. In mice, ovariectomy is a well-known model of postmenopausal osteoporosis. 13-week-old *Runx2-creRspo3*$^{flox/flox}$ and *Rspo3*$^{flox/flox}$ were ovariectomized (OVX) or sham-operated, and after one week of rest, we started treating the mice with either subcutaneous estradiol (30µg/kg/day, Sigma-Aldrich, USA) or vehicle (Miglyol 812, OmyaPeralta, Germany) for five days a week for three weeks. When the ovaries are removed, the endogenous estrogen in the mice is ablated. We can then evaluate if the response to estradiol differs in the mice where *Rspo3* is deleted in all osteoblast-linage cells.

3.2 GENE EXPRESSION ANALYSES

To analyze gene expression in different tissues, real-time quantitative polymerase chain reaction (RT-qPCR) are performed. RT-qPCR is a quick and sensitive method. Total mRNA were extracted using TRIzol (Thermo Fisher Scientific) for bone and lipids, RNeasy Micro Kit (Qiagen) for cultured cells, and RNeasy Mini Kit (Qiagen) for soft tissues. The mRNA was reversed transcribed into cDNA using High-capacity cDNA reverse transcription kit (Applied Biosystems, Thermo Fisher Scientific), and the PCR analyses were performed using the StepOnePlus Real-Time PCR system (Applied Biosystems, Thermo Fisher Scientific). Assay-on-demand primer and probe

sets (Thermo Fisher Scientific) were used to detect the gene-of-interest. The gene expression was normalized to the ribosomal subunit 18S, a housekeeping gene that are considered to have a constant expression throughout the cell cycle [137], and the relative gene expression was calculated by $2^{-\Delta\Delta Ct}$.

These analyses may have its limitations. When extracting RNA, multiple cell types are being lysed, so we cannot be certain of which cells express the gene-of-interest. Additionally, if the ratio of cells is changed in our transgenic mice, i.e., there might be more cells in our transgenic mouse models compared with control mice, but that each cell might express the same amount of the gene.

3.3 CELL CULTURES

Cell cultures are a method to investigate mechanisms that are difficult to study *in vivo*. The method has its limitations, as it is impossible to mimic the complete physiological conditions that are present *in vivo*, but it is still very helpful to study cells in order to get a detailed view of what happens in the cell following e.g., inactivation of the gene of interest.

3.3.1 CELL CULTURE MEDIA

The cells, both osteoblasts and osteoclasts were cultured in complete α-minimum essential medium (αMEM; Gibco) that have been supplemented with heat-inactivated fetal bovine serum (Sigma), GlutaMAX, i.e., L-glutamine (Gibco), gentamicin (Gibco), an aminoglycoside antibiotic, and the two antibiotics penicillin and streptomycin (Gibco). To study osteoblast differentiation and mineralization, the osteoblasts were cultured in medium supplemented as above with an addition of β-glycerophosphate disodium salt (BGP; Sigma) as well as L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate (Asc-2P; Sigma). To study osteoclast differentiation the bone marrow macrophages were cultured in complete αMEM together with M-CSF and RANKL (both R&D Systems).

3.3.2 MC3T3-E1 CELLS

MC3T3-E1 cells are an osteoblast precursor cell line derived from mouse calvaria, used for studying osteoblast differentiation. In paper I, MC3T3-E1 cells were used to study the effect of decreased *Notum* expression. MC3T3-E1 cells were kept in αMEM as described above. The cells were seeded and treated with an osteogenic medium to induce osteogenic differentiation. To silence *Notum*, the cells were transfected twice using specific small

interfering RNA (siRNA) sequence (Invitrogen), using lipofectamine RNAiMax (Thermo Fisher Scientific). As a negative control, we used a scrambled sequence. The silenced cells were either used for gene expression analyses or stained for alkaline phosphatase (ALP) activity.

3.3.3 PRIMARY CALVARIAL PERIOSTEAL BONE CELL CULTURES

In paper I and III, primary calvarial periosteal bone cells were isolated from 3-5 day old *Notum*$^{flox/flox}$ and *Runx2-creNotum*$^{flox/flox}$ mice (paper I), and 4-6 day old *Rspo3*$^{flox/flox}$ and *Runx2-creRspo3*$^{flox/flox}$ mice (paper III). The cells were detached from dissected calvaria by sequential collagenase treatment [138]. The osteoblasts were cultured in 3-5 days in αMEM, before the experiments started. The cells were thereafter re-seeded into 48-well plates at the start of experiment followed by incubation in osteogenic media that were being replenished after 3-4 days. Cells were harvested after 4-7 days.

3.3.4 PRIMARY OSTEOCLAST CELL CULTURES

In paper I, bone marrow cells from 8- to 12-week-old *Notum*$^{flox/flox}$ and *Runx2-creNotum*$^{flox/flox}$ mice were isolated and cultured in suspension culture discs (Corning) in complete αMEM with M-CSF (R&D Systems) [139]. The bone marrow derived macrophages (BMM) growing on the discs were used as osteoclast progenitors. BMM were detached from the disc and re-seeded into wells for experiments. The cells were cultured in complete αMEM with M-CSF. In some of the wells, RANKL is added to induce osteoclast differentiation. In paper I, osteoclasts were treated with RANKL with or without the addition of recombinant NOTUM, and in paper III, with recombinant RSPO3 (both from R&D Systems). The cells were stained for TRAP (Sigma) and were defined as osteoclasts if they were TRAP positive and had more than three nuclei. Alternatively, the cells were lysed for RNA analyses. In paper I, osteoclast formation and activity on bone was also studied by seeding the BMMs on bovine bone discs (IDS Nordic), the media was changed and saved for analyses, and after 10 days, the cells were stained for TRAP. To assess osteoclastogenesis, TRAP5b (IDS Nordic) was analyzed in the cultured media. CTX ELISA (IDS Nordic) was used to measure release of CTX (C-terminal type I collagen) from the bone slices.

3.3.5 IN VITRO ABLATION OF RSPO3

In paper III, primary calvarial osteoblasts from *CAGGCre-ER-Rspo3*$^{flox/flox}$ and *Rspo3*$^{flox/flox}$ mice were isolated and cultured in αMEM and osteogenic media as described above. After seeding, the plates were cultured for 24 h followed with 24 h treatment of tamoxifen (Sigma). After media change and

further culturing without tamoxifen, osteoblastic differentiation was analyzed as described above. Day 0 was categorized as the time of tamoxifen removal. To study the expression of osteoclastogenic RANKL, PTH (Bachem) and PGE2 (R&D Systems) were added day 1 and continued through day 6, as cells were harvested for gene expression analysis.

3.3.6 PROTEIN PREPARATIONS AND ANALYSES

Cells with in vitro deletion of RSPO3 were used for protein analyses. Protein lysates were prepared 24 hours after removal of tamoxifen by washing with PBS. The cells were lysed in RIPA buffer (Sigma) with protease inhibitor (Roche diagnostics) and phosphate inhibitors (Roche diagnostics). After the lysates been centrifuged, the protein concentration of the supernatant was quantified using DC Protein Assay Kit (Bio-Rad). We used the JESS Protein Simple system with the Compass for SW software to analyze the protein by capillary-based electrophoresis and immunodetection. The proteins are separated by molecular weight through a matrix with a detection range of 12-230 kDa and by UV light becomes immobilized to the capillary walls. Antibodies against LRP6 or pLRP6 (Cell Signaling Technology) are bound to the target protein and detected using an anti-rabbit chemiluminescent detection module (Protein Simple). To normalize the data, a fluorescent protein normalization reagent (Protein Simple) was loaded onto each capillary, which can be multiplexed within the JESS Protein Simple system together with the chemiluminescent signal from the antibodies. When the run was completed, the proteins of interest are sized by comparing with a set of internal standards included in each run and quantified in relation to total amount of protein. A charged-coupled device camera is used to detect the chemiluminescent reaction. This is a new, faster technique compared to traditional Western Blot, albeit has not been used in a great extent yet. The results are presented in accordance to guidelines set by Journal of Biological Chemistry [140].

3.4 BONE ANALYSES

3.4.1 DUAL-ENERGY X-RAY ABSORPTIOMETRY

Dual-energy X-ray absorptiometry (DXA) is used to assess bone mineral density both in clinics and in research. It emits X-rays at two energy levels, and since bone and soft tissue absorbs the energies differently, it is possible to separately evaluate these tissues. Being a non-invasive method, where the mouse is sedated by either gas or injected anesthetics, it can with ease be used for longitudinal studies. Lunar PIXImus densitometer (Wipro GE Healthcare,

Chicago, IL, USA) was used in paper I, and the UltraFocus^{DXA} (Faxitron Bioptics, Tucson, AZ, USA) was used in paper I, II, and IV.

3.4.2 PERIPHERAL QUANTITATIVE COMPUTED TOMOGRAPHY

While DXA measures the BMD dependent on bone area, peripheral quantitative computed tomography (pQCT) measures volumetric BMD. The pQCT can separate between cortical bone and trabecular bone, and by scanning the dissected bone in the mid-diaphyseal region and the metaphyseal region, we obtain cortical bone parameters and trabecular bone parameters, respectively. In paper I, II, and III, the XCT Research M (v4.5B; Norland Stratec) was used for pQCT scans, at a voxel size of 70 µm.

3.4.3 HIGH-RESOLUTION MICRO-COMPUTED TOMOGRAPHY

To assess the microstructure of the bone, high-resolution micro-computed tomography (µCT) was used (figure 8). With this method, we can with a high resolution further analyze the cortical and trabecular region of lumbar vertebra L5, distal femur, and proximal tibia.

The dissected sample is scanned using the SkyScan 1172 (Bruker MicroCT) at 50 kV voltage and a current if 201 µA, using a 0.5 mm aluminum filter. After the scan is complete, the images are reconstructed using NRecon and processed using CTan.

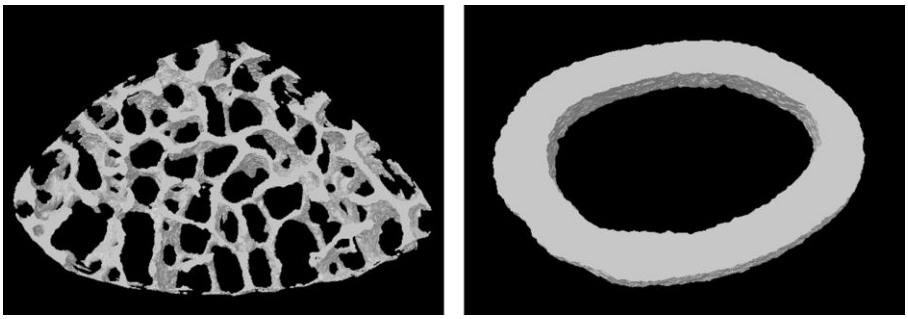

Figure 8: Representative 3D images from µCT analyses. Trabecular region of vertebra L5 to the left, cortical region of the femur to the right. Not to scale. Images done by Jianyao Wu.

3.4.4 MECHANICAL STRENGTH

To test the overall strength and quality of the bone, we performed three-point bending of dissected humerus, or compression test of lumbar vertebra (see schematic in figure 9). In three-point bending, it is very important that every humerus is placed in the same position. Pressure is applied to three points of the humerus, when the top loading point is pushed down. The pressure is gradually increased until the bone breaks.

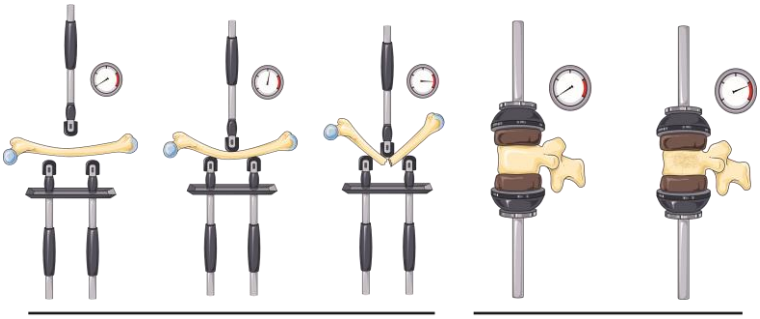

Figure 9: Mechanical strength test of femur (left) by three-point-bending, and vertebra (right) by compression test. Illustration adapted from Servier Medical Art by Servier.

For testing the vertebra, the sample is positioned with pressure from above, and the pressure is increasing until the vertebra fractures. Instron 3366 is used for the testing and the results were calculated from Bluehill 2 software (v.2.6, Instron). In paper I and II, humerus is analyzed and in paper III and IV, lumbar vertebra is analyzed.

3.4.5 BONE HISTOMORPHOMETRY

Bone histomorphometry is used for histological analyses. This gives further insight into bone turnover and bone cells. Histomorphometry is divided into static and dynamic histomorphometry. In static histomorphometry, bone parameters such as cortical bone area and thickness is analyzed, as well as cellular parameters, i.e., number of osteoclasts or osteoblasts per surface area, at a given time. To measure these parameters, OsteoMeasure histomorphometry analysis system was used according to guidelines of the American Society for Bone and Mineral Research [141]. Dynamic histomorphometry is used to assess bone formation rate by measuring mineral apposition rate. To perform dynamic histomorphometry, the mice were labeled with the fluorochromes calcein and alizarin at fixed days prior to end of experiment. Static and dynamic histomorphometry was used in paper I and III, dynamic histomorphometry was used in paper II. As with gene expression

analyses, the limitation to dynamic histomorphometry is again the possibility that a new steady state has been reached. The analyses need to be done at the right age, otherwise the possible difference in for example bone formation rate could be difficult to detect.

3.5 SERUM ANALYSES

To obtain additional information about bone turnover, serum analyses were performed to assess bone resorption and bone formation, using an ELISA RatLaps Kit to measure fragments of C-terminal type I collagen, called CTX, and a Rat/Mouse EIA Kit to assess procollagen type I NH_2-terminal propeptide (P1NP), respectively.

3.6 IN SITU HYBRIDIZATION

In paper II and III, we used chromogenic *in situ* hybridization, also called RNAscope. In this technique, which is very specific with a low risk of false positive signals, the bone is fixated in formaldehyde in phosphate buffered saline, demineralized, and imbedded in paraffin. 3.5 or 6 µm thick sections for human and mouse samples, respectively, were dewaxed and hybridized with a probe [142] to visualize where in the section the gene-of-interest is expressed. It is therefore possible to see in which cell in the tissue the RNA is present. Using consecutive sections, we first stained for *Notum* (paper II) or *Rspo3* (paper III) (Advanced Cell Diagnostics, A Bio-Techne Brand) and subsequent sections with cell specific probes staining osteocytes, osteoblasts, or osteoclasts.

3.7 SINGLE CELL RNA SEQUENCING

In order to study if *Rspo3* is expressed in mesenchymal stem cells in the bone marrow, we, in paper III, performed single cell RNA sequencing (scRNAseq) analysis using published data sets from Cxcl12-eGFP expressing cells [143]. Cells containing less than 1000 genes were filtered out and the data sets were merged and imported into a statistical program. After filtering out cells with a high number of mitochondrial read content, 7332 cells were left to analyze. The data was normalized, and uniform manifold approximation and projection (UMAP) cluster analysis was performed using the first 10 principal components. Eight cell clusters were obtained and in further analyses, the clusters were identified as four osteogenic/adipogenic, one mitotic, one endothelial, and two hematopoietic clusters, and we proceeded to analyze the *Rspo3* expression in these clusters. Further investigations of *Rspo3* expression

were done using the online scRNAseq database nicheExplorer [144] containing data of mouse bone marrow vascular, perivascular, and osteoblast cell populations.

3.8 STATISTICS

The statistical models used in this thesis are listed in table 3.

Table 3: Statistical models used in thesis

Statistical model	***Paper***
Unpaired two-tailed Student's t test	I, II, III, and IV
One-way ANOVA	I (followed by Tukey's multiple comparison test) IV (followed by Dunnett's multiple comparison test)
Two-way ANOVA	II, III, and IV

When only two groups are evaluated, either comparing genotypes or treatment groups, unpaired two-tailed Student's *t* test were used. One-way ANOVA followed by Tukey's multiple comparison test or Dunnett's multiple comparison test was used to evaluate three or more groups. In paper II, III, and IV, two-way ANOVA was used to evaluate the effect of two parameters and their interaction effect.

For all *in vivo* and *in vitro* experiments in this thesis, individual values are presented with the mean value as horizontal line, and standard error as vertical lines. For all *in vitro* results, the experiments were repeated at least two times.

All statistical test used are assuming the data to be normally distributed, and a difference were considered significant when $P < 0.05$.

4 RESULTS

4.1 PAPER I

Osteoblast-derived NOTUM reduces cortical bone mass in mice and the NOTUM locus is associated with bone mineral density in humans

To investigate if the WNT modulator NOTUM affects bone mass, and if this effect is dependent on local or circulating NOTUM, we used two different mouse models inactivating NOTUM in either all cells, or all osteoblast-lineage cells. Further, we determined the effect *Notum* has on a cellular and molecular level using cultured osteoblasts and osteoclasts.

Using two different mouse models where *Notum* is inactivated in either osteoblast cells, using a Cre-*loxP* model where the Cre is under the *Runx2* promoter, or globally, using an inducible Cre-*loxP* model, we found that deleting *Notum* in all osteoblast cells led to an increase in cortical bone mass and bone strength, while the trabecular bone parameters were unchanged, compared with controls. In the inducible, global model, we saw similar results. In the inducible model, we also noted an increase in cortical periosteal bone formation, and that the number of osteoblasts on the periosteal surface was increased, compared to control mice. Using MC3T3-E1 cells, we silenced *Notum* in osteoblasts, and we found that in cells with no *Notum*, osteoblast differentiation was enhanced. No expression of *Notum* was detected in osteoclasts. In human genetic studies, we found genetic variants in the *NOTUM* locus associated with estimated BMD, indicating that *NOTUM* is involved in bone mass regulation in both mice and humans.

In this paper, we established that osteoblast-derived NOTUM is a key regulator of cortical bone. Since the mice with a global inactivation of *Notum* in adult mice displayed a similar phenotype as the cell-specific knockouts, we concluded that local, osteoblast-derived NOTUM is of most importance in regulation of cortical bone mass. Histomorphometric studies showed an increase in the periosteal mineralized surface, reflecting an increase in the number of osteoblasts. Combined with the finding from silencing *Notum* in cultured cells, we concluded that the disadvantageous effect of NOTUM on periosteal bone formation occurs via a local inhibition on osteoblast number and activity.

4.2 PAPER II

Osteocyte-and late osteoblast-derived NOTUM reduces cortical bone mass in mice

To further deepen our knowledge of NOTUM in relation to bone mass, we, in this follow-up study to paper I, wanted to determine whether it is the early osteoblasts or the late osteoblasts and osteocytes that are the principal source of NOTUM. To this end, we created a mouse model with a conditional deletion of NOTUM in late osteoblasts and osteocytes. Using *in situ* hybridization and gene expression analyses we determined where in the bone *Notum* is expressed.

First, we wanted to establish whether *Notum* is expressed in late osteoblasts and osteocytes. We used *in situ* hybridization and found a signal for *Notum* in cells also expressing *Dmp1*, a marker for osteocytes and late osteoblasts. To continue with functional studies, we inactivated *Notum* using a Cre-*loxP* model with Cre under the *Dmp1* promotor, deleting *Notum* in late osteoblasts and osteocytes. Mice with no expression of *Notum* in late osteoblasts and osteocytes had an increased cortical bone mass and an overall improvement of cortical bone quality. Again, the trabecular bone was unaffected in the transgenic mice. The phenotype in mice with no expression of *Notum* in late osteoblasts and osteocytes were similar to the phenotype found in mice with *Notum* deleted in all osteoblast-lineage cells, presented in paper I.

Herein, we hypothesized that *Notum* is mainly expressed by late osteoblasts, and osteocytes since *in situ* hybridization proved *Notum* and *Dmp1* to be co-expressed. We generated a mouse model with conditional inactivation of *Notum* in *Dmp1*-expressing cells, and upon evaluating the skeletal phenotype of these mice, we saw increased cortical bone mass and unchanged trabecular bone mass, as in paper I. These data suggests that NOTUM expressed by osteocytes and late osteoblasts is of most importance to cortical bone.

4.3 PAPER III

RSPO3 is important for trabecular bone and fracture risk in mice and humans

R-spondins are a group of four secreted proteins that increase WNT signaling. In this paper, we aimed to characterize the role of RSPO3 in bone homeostasis, using different mouse models where RSPO3 is inactivated. We used *in situ* hybridization and single cell RNA sequencing to determine which cells specifically express RSPO3.

We determined that RSPO3 is highly expressed in both trabecular and cortical bone, and further analyses in mouse vertebrae using *in situ* hybridization revealed that RSPO3 is expressed in osteoblasts in different stages of maturation, with no expression in osteocytes or osteoclasts. Using previously published dataset, we aimed to map if *Rspo3* is expressed in mesenchymal stem cells using single cell RNA sequencing and noted that *Rspo3* is expressed in stromal cells expressing both osteoblastic and adipogenic markers, and that these cell clusters also express markers for mesenchymal stem cells. In functional studies, mouse with RSPO3-inactivation in early osteoblast cells displayed an ablation of *Rspo3* in the trabecular-rich vertebrae. These mice had a clear bone phenotype, where trabecular bone mass and bone strength in vertebrae were significantly lower compared to control, with no effect on cortical bone. Mice with RSPO3 inactivated in late osteoblasts and osteocytes had a mild reduction in *Rspo3* expression in vertebrae, and a much milder trabecular bone phenotype. Using an inducible model, we inactivated *Rspo3* in *in vitro* cultured calvarial osteoblasts and saw that cells lacking RSPO3 showed decreased proliferation and differentiation, compared with WT osteoblasts. Finally, we saw that inactivation of *Rspo3* decreased canonical WNT signaling by downregulation of the WNT receptor LRP6 leading to a decrease in WNT canonical signaling target genes *Tcf7* and *Lef1*. In human genetic studies, we observed that increased genetically determined circulating RSPO3 levels associated with increased trabecular BMD and reduced risk of fracture in the distal forearm.

In conclusion, in this paper, we demonstrated using multiple mouse models inactivating *Rspo3* globally, in early osteoblasts, or in late osteoblasts and osteocytes, that osteoblast-derived RSPO3 is a modulator of trabecular bone mass in vertebrae. In addition, by studying the effect of *Rspo3* inactivation in cultured osteoblasts we demonstrated that RSPO3 is important for osteoblast differentiation and proliferation, and that this effect is mediated through WNT canonical signaling.

4.4 PAPER IV

Estradiol and RSPO3 regulate vertebral trabecular bone mass independent of each other

To follow up our previous study, we wanted to investigate if the beneficial effect of estrogen on trabecular bone is dependent on RSPO3. To this end, we treated ovariectomized mice lacking *Rspo3* in all osteoblast-lineage cells with estradiol and compared to control mice.

The *Rspo3* deficient female mice and their control littermates were ovariectomized, or remained gonadally intact, and treated with either estradiol or vehicle. We saw that the expected estrogen response in trabecular bone volume was similar both in the presence and absence of osteoblast-derived RSPO3. However, we did notice that osteoblast-derived RSPO3 is necessary for a complete estrogenic response on cortical bone thickness. In the cortical region of the femur, the increase of both cortical thickness and area was smaller in the knockouts than the control littermates, following estradiol treatment. When analyzing gene response, we saw an interaction for genotype and treatment in the expression of the WNT signaling inhibitors *Dkk1* and *Sost* in cortical bone.

Here, we continued to investigate the importance of local RSPO3 in bone homeostasis by evaluating its role in estrogen deficiency. Although osteoblast-derived RSPO3 is dispensable for estrogens osteogenic response in trabecular bone, it is surprisingly needed for a full effect by estrogen on cortical bone. It has previously been shown that trabecular bone and cortical bone are regulated differently, and we determined that osteoblast-derived RSPO3 can regulate both trabecular and cortical bone, depending on different physiological and pharmacological conditions.

5 DISCUSSION

There is a need for safer osteoporotic treatments. Different WNT proteins can regulate cortical bone, trabecular bone, or both [75-78], and these proteins are of high interest when it comes to developing new, anabolic antiosteoporosis drugs, with the possible ability to target specific bone sites to achieve a more precise treatment for osteoporosis. In this thesis, we aimed to study two regulators of WNT signaling, the inhibitor NOTUM and the enhancer RSPO3, and their importance for regulation of bone mass.

5.1 ENDOGENOUS NOTUM IS A SPECIFIC REGULATOR FOR CORTICAL BONE

NOTUM is a newly discovered modulator of WNT signaling [112, 113], with its main functions mapped in *Drosophila*. First thought to cleave glycosylphosphatidylinositol (GPI) anchors [113], it was later shown that NOTUM is a carboxylesterase, with a hydrophobic pocket cleaving the tail of WNT proteins [82]. NOTUM was shown to be of importance in *Xenopus*, especially for the neural induction [145], but few studies had been conducted in mouse until one group designed a *loxP* mouse to study the effect of *Notum* deletion in the liver [117]. Mechanistic studies of NOTUM proved difficult, since global *Notum* inactivation are lethal [146], or at least born with decreased viability [115]. In paper I, we attempted to globally delete NOTUM by breeding the previously described $Notum^{flox/flox}$ mouse [117] with *PGK*-cre mouse [133], inactivating *Notum* in all cells early on in development. About 90% of the expected $Notum^{-/-}$ mice died during embryonal development, and the few mice surviving to the end of experiment at 13 weeks-of-age were smaller than their $Notum^{+/-}$ and wildtype littermates. The female $Notum^{+/-}$ mice displayed an increase in cortical thickness, while the trabecular bone was unaffected. We saw in our experiment that NOTUM is highly expressed in liver, followed by high expression levels in cortical bone and trabecular-rich bone. To determine the cellular source of the NOTUM important for bone homeostasis, we went on to inactivate *Notum* in all osteoblast-derived cells using a Cre recombinase under the *Runx2* promotor (see figure 7) [134]. We saw a clear bone phenotype in *Runx2-cre*$Notum^{flox/flox}$ mice where cortical bone mass and quality were increased, compared to littermate $Notum^{flox/flox}$ mice. These mice were born healthy and in the expected rate, leading to the conclusion that there is a different source of NOTUM giving the developmental effects, most likely related to the kidney agenesis observed by others [115, 146], or the defected trachea observed by Gerhardt *et al*. The

mice with NOTUM inactivation in hepatocytes were born at an expected rate [117], however, it is important to note that the Cre recombinase used by Canal *et al* is active between embryonic day 9.5 and 10.5 [147], raising the possibility that if the Cre was active earlier, the liver bud might not be formed.

As the osteoblast-derived NOTUM in *Runx2-creNotum*$^{flox/flox}$ mice is knocked out from birth, we did not know if the effects we saw on cortical bone was a developmental effect, or if there is an effect on adult bone homeostasis. We therefore inactivated *Notum* globally in adult mice, using the *CAGGCre-ER* mouse model [135], showing that the osteoblast-derived NOTUM has an effect on adult bone, not only during development. Using this model, we could also study dynamic effects of NOTUM inactivation, since with a lifelong inactivation, a new steady state in bone remodeling had been reached when the bones were harvested. In the inducible model, we could conclude that the knockouts had increased periosteal mineralized surface as well as increased periosteal mineral apposition rate, while we found no evidence for an effect on osteoclastogenesis. WNT16 is a specific modulator of cortical bone, and adult *Wnt16*$^{-/-}$ mice has a decreased periosteal cortical bone formation [75] so a possible mechanism is that osteoblast-derived NOTUM inhibits periosteal bone formation by inactivating WNT16.

One setback in using the *Runx2*-cre is that the floxed gene is inactivated very early in the osteoblast lineage (see figure 10). RUNX2 is involved in mesenchymal stem cell differentiation, making it possible for *Runx2*-cre to inactivate the gene of interest in not only osteoblasts, but also in some chondrocytes and adipocytes [25, 148, 149], so when using this model, we cannot be sure that it is only the NOTUM secreted by osteoblasts that affect cortical bone. So, to continue our investigation regarding the source of NOTUM with an effect on cortical bone, we wanted to study at which stage of osteoblast differentiation the NOTUM is produced, and to make sure it is only in the osteoblastic lineage the inactivation occurs.

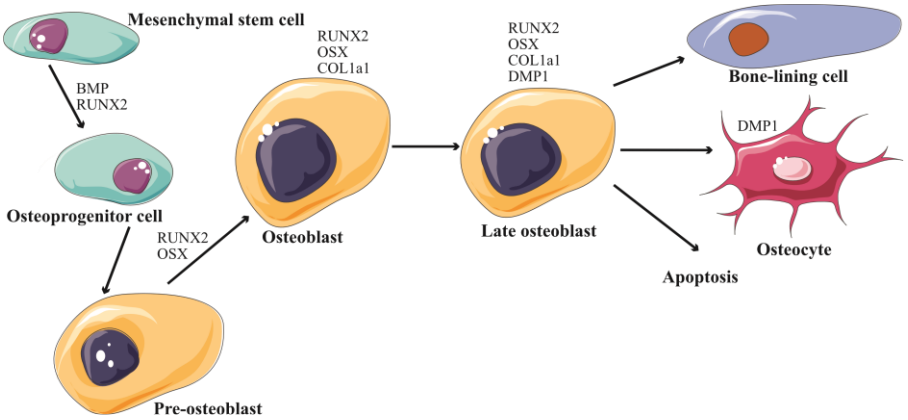

Figure 10: Schematic view of osteoblast differentiation. Illustration adapted from Servier Medical Art by Servier.

To this end, we used *Dmp1*-cre [136] where the Cre recombinase is under the *Dmp1* promotor, where *Dmp1* is expressed by osteocytes and late osteoblasts (see figure 10). In this experiment, the *Dmp1-creNotum*$^{flox/flox}$ mice had a phenotype matching the one we saw in *Runx2-creNotum*$^{flox/flox}$ experiment, leading to the conclusion that it is the NOTUM expressed by late osteoblasts/osteocytes that is most important for regulation of cortical bone. These results are in line with the results later reported by Choi *et al* [150]. This group used a different *loxP* mouse, where exon 8 through 12 were replaced with a LacZ cassette following recombination. Choi *et al* also created a global knockout mouse, and when on the C57BL/6 background, the knockout was non-viable. Switching to a 50/50 mixed background of B6/129, knockout mice were born as expected and were healthy [150], showing the importance of the strain of the modified mouse. There are known differences between mouse strains, for example, the C57BL/6 strain has lower BMD compared to other inbred mouse strains [151].

There are still large knowledge-gaps regarding regulation of cortical bone [152], so there is a great need for studies evaluating ways to increase cortical bone. Osteocytes are highly involved in keeping a healthy bone homeostasis, and several studies of osteocytes and its produced proteins show that they can be used to manipulate bone [153]. In a recent study, NOTUM was revealed to be highly expressed in osteocytes [154], making osteocytes interesting target cells in osteoporosis research.

Further, NOTUM has several emerging clinical implications. For example, *Notum* is a possible pharmacodynamic biomarker for diseases involving the WNT pathway, as a recent study has shown that in cell lines where porcupine

is inhibited, *Notum* expression is significantly downregulated, as it was in mouse models carrying a transgene mimicking WNT dependent cancer models [155]. And, as a first step in developing a new anabolic drug targeting NOTUM, Lexicon Pharmaceuticals tested the possibility to treat mice with a small, orally active molecule that inhibits NOTUM, in the paper published in collaboration with us [114]. When treating wildtype mice with this molecule, cortical bone thickness as well as cortical bone strength increased in femur, with no effect on the trabecular bone parameter bone volume over tissue volume (BV/TV) in the spine, whereas no differences in bone parameters were observed in *Notum*$^{-/-}$ mice.

5.2 OSTEOBLAST-DERIVED RSPO3 REGULATES TRABECULAR BONE AND IS REQUIRED FOR A FULL ESTROGENIC EFFECT ON CORTICAL BONE

Genetic studies had revealed that a fracture-reducing allele at the *RSPO3* locus was associated with an increase in BMD [156], but the mechanism behind this was unknown. The human genetic studies performed in paper III showed that increasing circulating levels of genetically determined RSPO3 are associated with increased trabecular BMD and decreased fracture risk. We then aimed to characterize the expression pattern of RSPO3, and, by using three Cre-*loxP* mouse models, determine the *in vivo* role of RSPO3.

First, we saw that *Rspo3* is expressed in both trabecular-rich bone and cortical bone, and using chromogenic *in situ* hybridization, single cell RNA sequencing, and cell cultures we could establish that *Rspo3* is expressed by osteoblasts and osteoprogenitor cells, but not by osteocytes or osteoclasts. As *Rspo3*$^{-/-}$ mice are embryonically lethal [123, 124, 126], a global knockout was impossible. To determine the source of the bone modulating RSPO3, we again used the *Runx2*-cre, inactivating *Rspo3* in all osteoblast-lineage cells, and the *Dmp1*-cre, inactivating *Rspo3* in late osteoblasts and osteocytes. When analyzing the *Rspo3* mRNA level in bone, the expression was almost completely abolished in the *Runx2*-cre mice, but only slightly downregulated in *Dmp1*-cre mice, leading to the conclusion that it is the early osteoblasts and not the RSPO3 produced by the late osteoblast/osteocyte that is the main source of RSPO3 in bone.

We went on to preform extensive mechanistic studies to characterize the skeletal effect of deleting osteoblast-derived RSPO3, and we concluded that

early osteoblast-derived RSPO3 regulates trabecular bone in the axial skeleton, and that trabecular BV/TV in the vertebrae is correlated with *Rspo3* mRNA levels in vertebrate. In the *Dmp1*-cre mice, the trabecular bone phenotype was much less pronounced, indicating that RSPO3 produced by earlier osteoblasts is the most important source. No effect on cortical bone was observed in these mouse models. The inducible mouse model confirmed previous findings, and the result from that study showed that RSPO3 can modulate trabecular bone in adult mice and not only during development.

The result from our *in vivo* mouse studies confirmed the findings from the human genetic studies, leading to our hypothesis that osteoblast-derived RSPO3 regulates trabecular bone in the axial skeleton.

Recently, another group published a study where they inactivated RSPO3 in osteoblast-lineage cells [157]. This group used the same *Runx2*-cre mouse model as we did [134], but they used different *loxP* mice [124]. Nagano *et al* confirmed our finding that inhibition of osteoblast-derived RSPO3 leads to a robust decrease of trabecular BMD in the lumbar spine and they proposed a mechanism involving the canonical WNT signaling pathway and ERK, i.e., extracellular signal-regulated kinase, as others previously have shown [158]. They also confirmed the lack of cortical bone phenotype in this mouse model. In contrast to a decrease in LRP phosphorylation following RSPO3 inactivation as we demonstrated in cultured primary osteoblasts, Nagano *et al* saw an increase in phosphorylation when investigating mouse embryonic fibroblasts, supporting cellular context-dependent effects of RSPO3. In addition, Nagano *et al* also noted an unexpected increase in metaphyseal trabecular bone formation close to the growth plate in long bones. It is previously known that trabecular bone and cortical bone can be regulated differently, and that different WNT proteins can regulate either one of the compartments, or both [75, 76, 159]. As discussed by Nagano *et al*, the axial skeleton originates from paraxial mesoderm, and appendicular skeleton originates from later plate mesoderm [160] which in theory could mean that since these bone sites have different embryonic origins, they could respond differently to the same stimuli, which in this case is *Rspo3* inactivation.

Loss of estrogen after menopause is a large risk factor for fractures, and supplementation with estrogen has proven to reduce this risk and increase bone mass [128, 161, 162]. The importance of WNT signaling for estrogenic effect on bone has previously been explored, as in a previous study by our group, it was concluded that WNT16 is not needed for the bone-sparing effect or the stimulatory effect of estrogen [163]. Further, there was no interaction

effect between ovariectomized *Notum*$^{-/-}$ mice and wildtype mice, stipulating that NOTUM and estrogen regulate cortical bone mass *via* independent pathways [114]. However, a possible synergy/crosstalk between signaling *via* the estrogen receptor α, (ERα) in mesenchymal progenitor cells and WNT3a induced mineralization and osteogenic markers has been proposed, and the relationship between the WNT signaling system and E2 signaling is highly interesting for further investigations to explore possible druggable targets for postmenopausal osteoporosis [164].

In paper IV, we had in an initial study observed that *Rspo3* mRNA expression is regulated by estrogen in cortical bone and went on to determine the role, if any, RSPO3 has in estrogens beneficial effect on bone. We used the same *Runx2-creRspo3*$^{flox/flox}$ mice as used in our previous study and treated them with estradiol or vehicle following OVX. We again observed the reduction in vertebral trabecular BMD in gonadally intact *Runx2-creRspo3*$^{flox/flox}$ mice, when compared to control littermates, as seen both by us in paper III and by Nagano *et al* [157], confirming our main finding that osteoblast-derived RSPO3 is beneficial for trabecular BMD in the vertebral column. Interestingly, in paper IV, we also found that osteoblast-derived RSPO3 is indispensable for the estrogenic effect on trabecular bone, as there was no difference in response magnitude to E2 treatment in the knockout mice when compared to wildtype littermates, implying that E2 and RSPO3 modulate trabecular bone *via* different pathways. We did, however, notice a difference in estrogenic response in the cortical bone compartment. Neither we nor Nagano *et al* [157] observed any effects in the cortical bone compartments following *Rspo3* inactivation. In paper IV, we found a significant genotype-estrogen interaction effect on cortical area and cortical thickness in the long bones, but this interaction effect was not seen in cortical porosity or cortical volumetric BMD, indicating that RSPO3 is required for a full estrogenic response in cortical bone parameters connected to cortical thickness. We observed a significant genotype-treatment interaction effect in *Sost* and *Dkk1* gene expression, and we speculate that this upregulation could hamper E2s stimulatory effect on bone, but this inhibition is not present when the osteoblasts express *Rspo3*.

Paper III and paper IV show that RSPO3 is an important regulator of bone, needed both in young individuals with intact estrogen levels, and, since RSPO3 is needed for a full estrogenic effect on cortical bone in paper IV, it is conceivable to believe it plays an important role in elderly individuals with postmenopausal osteoporosis. The reports discussed here, together with the study from Nagano *et al* [157], show the complexity of how RSPO3 modify

WNT signaling, and there are still much remaining before we fully understand the action of this WNT signaling modifier.

5.3 CLINICAL IMPLICATIONS

In recent years, there has been an update in available osteoporotic treatments. The sclerostin antibody romosozumab is among the next-generation osteoporotic drugs [165], and this drug is the first targeting the WNT signaling pathway. Since there is a possible risk for cardiovascular side effects [51-53], there is need for new, safer osteoporotic drugs.

We used human genetic studies in two of the projects. In paper I, we identified genetic variants in the *NOTUM* locus that are associated with estimated BMD in the heel, and in paper III, human genetic studies revealed that increased genetically determined RSPO3 levels are associated with decreased fracture risk and increased bone mass. This makes osteoblast-derived NOTUM and RSPO3 possible targets to increase bone mass and reduce fracture risk, either by inhibiting NOTUM or potentiate RSPO3 to enhance WNT signaling. It could be assumed that it would be easier to target NOTUM for osteoporosis treatment, as it is easier to inhibit a protein than increasing it, which would have to be done for RSPO3. In the Lexicon Pharmaceutical paper [114], mice were treated with a NOTUM inhibitor, which resulted in increased cortical bone mass, with no effect on trabecular bone, proving NOTUM to be a potential, druggable target against osteoporosis. However, since the WNT signaling system is also involved in cancer diseases [60] and atherosclerosis/metabolic syndrome [166], it is necessary to target only the bone-specific NOTUM to avoid serious side effects.

In addition, inhibitors of porcupine, that is responsible for adding the palmitoleic acid to the WNT protein and enabling its binding to Frizzled receptor, are in trials to be used for cancer treatments [83-85], so drugs modulating the WNT signaling pathway is highly interesting, for several ailments. It is nevertheless important, when treating serious diseases such as cancer with WNT signaling inhibitors, to take precautions so the patient will not suffer from serious side effects in the skeleton.

6 CONCLUDING REMARKS

Osteoporosis is a disease affecting millions of individuals worldwide, leading to fractures and reduced quality of life. From a societal perspective, an osteoporotic fracture is very costly, often with long treatment periods for the patient. WNT proteins have been studied to understand their involvement in bone homeostasis, and new anti-osteoporotic drugs target the WNT signaling system.

In this thesis, we have studied two different modulators of WNT signaling, the inhibitor NOTUM and the enhancer RSPO3. Through extensive *in vivo* and *in vitro* studies, we have shown that late osteoblast- and osteocyte-derived NOTUM is a specific regulator of cortical bone, by increasing periosteal bone formation *via* increased osteoblast differentiation. Drugs targeting late osteoblast- and osteocyte-derived NOTUM would be very beneficial to increase cortical bone mass and prevent fractures in cortical bone. We also, for the first time, showed that osteoblast-derived RSPO3 is a key regulator for vertebral trabecular bone mass in adult mice, and that although it is dispensable for the protective effect of estrogen on trabecular bone, it is required for a full estrogenic effect on cortical bone.

Altogether, WNT signaling is a very interesting and promising biological system regarding regulation of the skeleton, and this thesis provides further knowledge in mapping the WNT signaling system and its role in bone.

7 FUTURE PERSPECTIVES

Even though there are two types of anabolic drugs against osteoporosis available, there are still a great need for safe, anabolic osteoporotic drugs targeting either trabecular bone, cortical bone, or both that can be used over a longer period of time. For both teriparatide and abaloparatide, the patient is treated for a maximum of two years since there is a risk of osteosarcoma as well as a reduction in the therapeutic effect after 12-24 month of use [50]. Romosozumab treatment is restricted to 12 months, as the possible risk of cardiovascular events have not been fully understood [167], as well as a reduction in efficacy [50].

In this thesis, we investigated two different modulators of the WNT signaling system, one inhibitor, NOTUM, and one enhancer, RSPO3. NOTUM was found to only regulate cortical bone, as other also have seen [114, 150]. RSPO3, on the other hand, is a regulator of trabecular bone, as seen in subsequent studies [157]. Both these two proteins would be interesting targets as osteoporotic treatments. A few *in vivo* studies have been made using small molecule inhibitors of NOTUM, with mixed results [150, 168], but further studies are needed to establish if inhibition of NOTUM is a possible treatment option. No studies as of yet have been made on enhancing osteoblast-derived *Rspo3* expression. The first step towards this could be to produce a transgenic mouse overexpressing RSPO3 to study the physiological responses to increased levels of RSPO3. For further studies, it would be highly interesting to develop molecules able to pharmacologically block ZNRF3/RNF43, to enable WNT signaling.

There are, however, several considerations that need to be addressed in regard to modulating the WNT signaling pathway. This pathway is active throughout development, and is involved in several other tissues and diseases, as previously discussed in this thesis. It is especially WNTs involvement in certain types of cancer and the cardiovascular system that is most troublesome, and thorough studies are needed to modify only the bone specific WNT modulators.

8 RELATED PUBLICATIONS NOT INCLUDED IN THESIS

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