Modulation of the canonical Wnt signaling pathway in bone and cartilage

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Chapter 1

General introduction

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I. DEVELOPMENT OF THE ENDOCHONDRAL SKELETON

The formation of most of the vertebrate skeleton occurs via endochondral bone formation, a process which begins with the aggregation, proliferation and condensation of mesenchymal cells (MCs) at specific locations within the embryo where the skeletal elements will arise. MCs can be of three origins: the neural crest (forming some craniofacial bones), the sclerotome of the paraxial mesoderm (forming the axial skeleton), or the lateral plate mesoderm (forming the appendicular skeleton). MCs commit to the skeletal lineage once they differentiate into skeletal precursor cells (SPCs), cells from which both chondrocytes and osteoblasts can derive. At the periphery of these condensations, SPCs form a perichondrial layer, while in the core they differentiate into chondrocytes that start producing cartilage-specific extracellular matrix (ECM) proteins and continue to proliferate. Continuous division of chondrocytes and further secretion of ECM together contribute to the elongation of the cartilage template, which prefigures the shape of the future bone. Once the cartilaginous template is formed, the innermost chondrocytes mature, exit from the cell cycle, and become hypertrophic, secreting a progressively calcified ECM. Simultaneously with the onset of hypertrophic chondrocyte differentiation, perichondrial SPCs differentiate into osteoblasts, forming a tight, yet adaptable sheath (later called periosteum), which modulates the final size and shape of the cartilage template. When the cartilage ECM is mineralized, concurrent vascular invasion and apoptosis of terminal hypertrophic chondrocytes together contribute to the formation of the primary ossification centre, the first region of the cartilaginous anlage that will be replaced by bone. This complex differentiation program radiates centrifugally, leading to the development of trabecular bone (the primary spongiosa) (1-6) (Figure 1).

Vascular invasion of the primary spongiosa continues via the so-called “periosteal buds” that provide SPCs (later forming osteoblasts), hematopoietic cells (later forming osteoclasts) and blood vessels, which grow from the periosteum to reach the primary ossification center. Osteoblasts attach to spicules of calcified scaffolds left behind by dying chondrocytes and begin producing osteoid, a gelatinous substance made up of collagen and mucopolysaccharide. Soon after the osteoid is laid down, inorganic salts are deposited in it to form mineralized bone. In turn, osteoclasts break down spongy bone to form the medullary cavity filled with bone marrow, the main site for haematopoiesis in post-natal life (7;8).

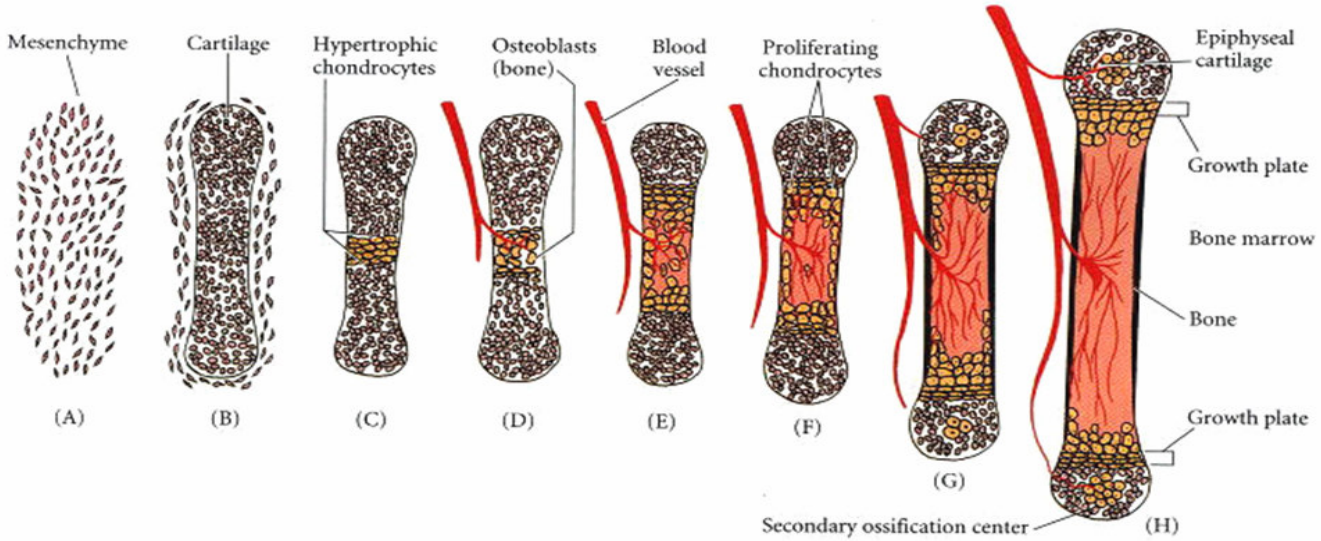

Figure 1. Schematic diagram of endochondral ossification. (A, B) Mesenchymal cells condense and differentiate into chondrocytes to form the cartilaginous model of the bone. (C) Chondrocytes in the center of the shaft undergo hypertrophy and apoptosis while they change and mineralize their extracellular matrix. Their death allows blood vessels to enter. (D, E) Blood vessels bring in osteoblasts, which bind to the degenerating cartilaginous matrix and deposit bone matrix. (F-H) Bone formation and growth consist of ordered arrays of proliferating, hypertrophic, and mineralizing chondrocytes. Secondary ossification centers also form as blood vessels enter near the tips of the bone, physically separating AC from GP. Reprinted with permission from Gilbert SF, Developmental Biology, 6th edition, Sunderland (MA): Sinauer Associates; 2000

The fascinating multi-step process of endochondral bone formation described above, continues postnatally in the growth plates (GP). This highly specialized cartilage structure develops at the distal ends of any growing endochondral bone, secondary to the ordered buildup of the diaphyseal bone from the primary ossification centre. GP activity leads to the persistent formation of chondrocytes and cartilage ECM. This does not, however, lead to a perpetual increase in GP height, since the process of tissue production is balanced by a tightly regulated process of tissue resorption at the epiphyseal/metaphyseal interface (9). GPs are spatially polarized biological structures, comprising several distinct chondrocyte layers: resting, proliferating, prehypertrophic, and hypertrophic, proceeding from the cartilaginous epiphysis to the bony diaphysis (Figure 2). Chondrocyte proliferation, matrix production and hypertrophy in the GP is responsible for the rate of longitudinal growth as well as for the ultimate length of all endochondral bones until the end of puberty, when GPs disappear and bone growth ceases (10;11).

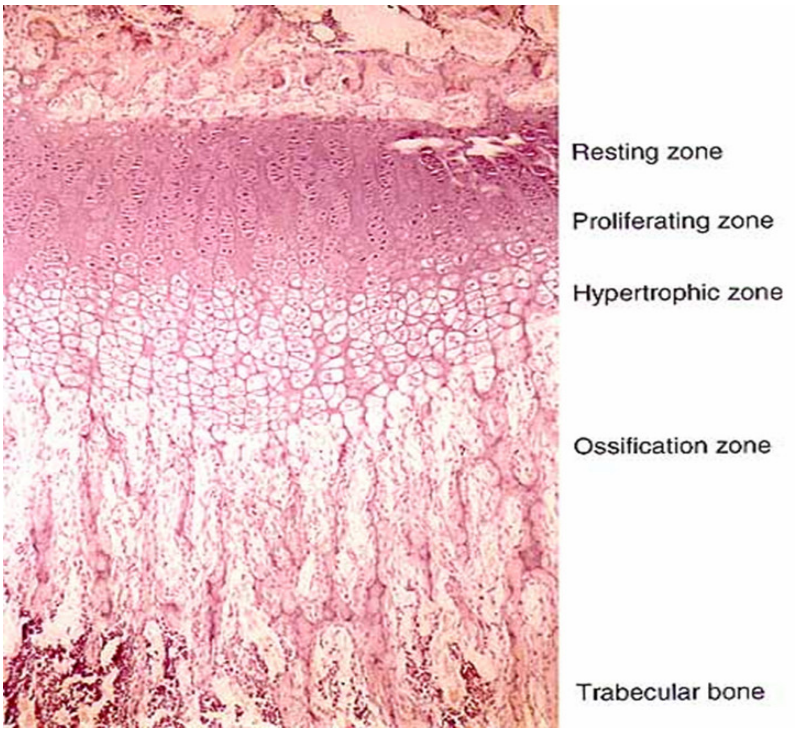

Figure 2. Section of the epiphyseal growth plate from the proximal tibia of a three-week-old mouse depicts the different chondrocyte layers within the growth plate.

Upon a certain trigger, MCs from the resting zone differentiate into chondrocytes assuming a flattened shape and organizing into longitudinal columns. These chondrocytes proliferate at a high rate until they exit the cell cycle and start to mature and increase in size, undergoing prehypertrophy followed by full hypertrophy. Ultimately, hypertrophic chondrocytes undergo cell death allowing primary ossification centers to expand. During the development and growth of endochondral bones, most of the skeletal cartilage is therefore an ephemeral tissue, with two main functions: 1) to compute the size and the shape of the future bone, and 2) to provide the scaffold in which bone will form.

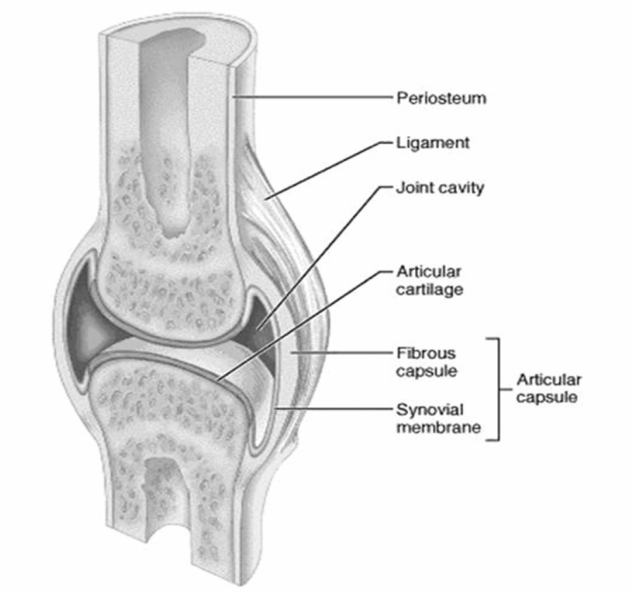

Figure 3. Longitudinal section through a synovial joint depicts its main components.

On the surfaces of diarthrodial joints permanent articular cartilage (AC) maintains joint function throughout life (Figure 3). In marked contrast with the GP, AC retains a stable phenotype, providing the tissue with functional adaptability. During embryogenesis, joint development begins at specific skeletal sites before any chondrocyte differentiation occurs from MCs. At the site of the future joint, condensed MCs do not differentiate into chondrocytes, become highly packed and flattened to form the so-called “interzone”. In the middle of the interzone, a cavity will shape via apoptosis, separating the two skeletal elements to be articulated. On each epiphyseal end of these skeletal elements, chondrocytes start differentiating from a layer of perichondrium-like cells to give rise to AC. These articular chondrocytes are responsible for the initial longitudinal lengthening of the elements through appositional growth, until GPs are fully functional and become the source of self-renewing proliferating chondrocytes and the main mechanism of longitudinal growth. Through a process of endochondral

bone formation, chondrocytes in the centre of the epiphysis will form the scaffold for the second ossification centre, so that only the most epiphyseal among them will survive to become authentic articular chondrocytes (12-14).

II. DEVELOPMENTAL REGULATION OF SKELETOGENESIS

The vertebrate skeleton contains three different cell types spread within the ECM: chondrocytes (cartilage cells), osteoblasts (bone cells) and osteoclasts (cartilage- and bone-resorbing cells). Once differentiated, chondrocytes, osteoblasts and osteoclasts complete one another's functions to accomplish longitudinal bone growth, and maintain skeletal remodeling (formation following resorption), matrix mineralization and bone mass. Regulation of the various steps of skeletal cell differentiation, proliferation and survival is the result of a very complex and formidable interaction between transcription factors, systemic hormones, growth factors, the surrounding matrix, but also environmental and mechanical signals.

II. a. Transcriptional regulation of skeletogenesis

Initially identified due to its inactivating mutations in patients with campomelic dysplasia, Sex determining region Y (SRY)-box 9 (Sox9) is generally accepted as the master transcription factor for the commitment of MCs to the chondrogenic lineage (15;16). *Sox9* can first be detected in MCs condensing at the site of the future endochondral bone and continues to be expressed by chondrocytes throughout their subsequent differentiation steps until they become hypertrophic (17). Although this spatio-temporal expression pattern resembles the one of *α1*(*II*) *collagen* (*Col2α1*), *Sox9* expression begins scarcely earlier (18). Besides stimulating and coordinating the formation of mesenchymal condensations, Sox9 also regulates the expression of *Col2α1*, but also of other chondrocyte markers, like *Aggrecan* (*Acan*), and *α1*(*XI*) *collagen* (*Col11α1*) (19-24). Furthermore, it has been shown that Sox9 not only controls proliferation and differentiation of chondrocytes, but it also prevents them from entering hypertrophy (25-27). L-Sox5 and Sox6, two other high-mobility group (HMG) domain-containing transcription factors, are also expressed in all precartilaginous condensations and in nonhypertrophic chondrocytes (28). Like Sox9, they are essential for chondrogenesis and together promote the expression of chondrocytic genes, like *Col2α1* and *Acan* (22;29).

Hypertrophy, the last chondrocytic differentiation step during endochondral bone formation, is induced by a member of the Runt domain family of transcription factors (RunX2), also known as core binding factor α1 (Cbfa1). *RunX2*, which is transiently expressed by prehypertrophic chondrocytes, is essential for chondrocytes to enter maturation and for the expression of *α1*(*X*) *collagen* (*Col10α1*), a typical marker for hypertrophic chondrocytes (30-33). However, RunX2 is not the sole transcription factor known to stimulate chondrocyte hypertrophy, as genetic studies indicate similar roles

for RunX3, another member of the Runt domain family of transcription factors, and also for Twist-1 (34;35).

Already introduced as a dominant regulator of chondrocyte hypertrophy, RunX2/Cbfa1 was originally identified and described as the critical transcription factor for the commitment of MCs to the osteogenic lineage (36). During development, *RunX2* begins to be expressed in mesenchymal condensations, while later during development it is expressed at high levels in osteoblasts and at much lower levels in prehypertrophic chondrocytes, but never in other cells (37). Osteoblasts do not develop in *RunX2* null mice, while heterozygous *RunX2* mutants display skeletal anomalies similar to those observed in patients with cleidocranial dysplasia: hypoplastic clavicles and delayed closure of the fontanelles (37-40). RunX2 promotes osteogenesis, by positively regulating nearly all osteogenic genes, like *Osteocalcin* (*Ocn*) and bone sialoproteins (36).

Besides Runx2, Osterix (Osx), a zinc finger–containing transcription factor, is also essential for osteoblast differentiation (41). Specifically expressed in osteoblasts, Osx acts downstream of RunX2 during osteoblast differentiation and its expression is regulated by RunX2 (42). Osx inactivation in mice leads to perinatal lethality due to a complete absence of bone formation (41). Unlike Runx2-deficient mice whose skeleton is entirely nonmineralized, the Osx-deficient mice lack a mineralized matrix in intramembranous bones only. This suggests that Osx, unlike Runx2, is not required for chondrocyte hypertrophy, thereby demonstrating that Osx specifically induces osteoblast differentiation and bone formation *in vivo*.

II. b. Paracrine regulation of skeletogenesis

Besides transcription factors, a wide variety of locally produced growth factors play a crucial role during skeletal development and maintenance. Such regulating growth factors are Indian Hedgehog (IHh), parathyroid related hormone (PTHrP), bone morphogenetic proteins (BMPs) and members of the Wnt family of morphogens.

Within the growth plate, chondrocyte proliferation and maturation are tightly regulated by a negative feedback loop between IHh and PTHrP. PTHrP inhibits the rate at which chondrocytes proliferate and are converted to post-proliferative hypertrophic chondrocytes. PTHrP's expression in periarticular chondrocytes is dependent on IHh, which is expressed at the prehypertrophic–hypertrophic boundary so that cells that escape the inhibitory action of PTHrP signaling in the growth plate express Ihh, which in turn will stimulate PTHrP expression (43-46).

BMPs are members of the TGFβ superfamily of growth factors initially isolated from demineralized bone and osteosarcomas. They are best known for their chondro- and osteoinductive effects during skeletal development and patterning (47;48). BMPs bind to type II and type I serine/threonine kinase receptors, thereby initiating intracellular signaling by activating Smad proteins. Early in skeletal development, BMPs promote the condensation step of MCs by stimulating cell–cell interaction through upregulation of N-cadherin function and expression (49). Studies have demonstrated the requirement of BMPs for Sox gene expression in chondrogenesis (50;51) and their stimulatory effect on *Sox9* and *Col2a1* in multipotential mesenchymal C3H10T1/2 cells

and monopotential chondroprogenitor MC615 cells (52). Additionally, BMPs increase the expression of the specific hypertrophic chondrocyte marker *Col10a1* by inducing its promoter activity (53-55). BMPs also induce osteoblastogenesis from MCs to promote osteoblastic maturation and function (56;57), a process that requires interactions of Smad 1/5 and RunX2 (58;59).

Wnts are a family of highly conserved secreted glycoproteins with important roles during cell specification, formation of the body plan, cell growth, differentiation and apoptosis (60). Up to date 19 human Wnt genes have been identified in humans and mice. Wnts can activate a number of different signal transduction pathways, the so-called non-canonical pathways, which include the planar cell polarity and Ca^{2+} pathways, and the canonical Wnt/β-catenin pathway (61). Several members of this growth factor family have inhibitory effects on chondrogenesis (62-66), while their effect on osteoblastogenesis remains heterogeneous. Wnt3a promotes osteoblast proliferation, but suppresses osteoblastogenesis from human mesenchymal stem cells *in vitro* (67;68). Furthermore, Wnt3a and Wnt5a prevent osteoblast apoptosis (69). At the same time, Wnt-10b stimulates osteoblast differentiation from bi-potential skeletal precursor cells (SPCs) by activating RunX2, Osterix and Dlx5 and inhibits adipocyte formation (70).

III. SKELETAL PATHOLOGY

III. a. Growth disorders

Growth is the key characteristic that distinguishes children from adults, and growth disturbances are frequently presented to health personnel at all levels (youth health care, general practitioners, paediatricians, paediatric endocrinologists). Disturbances of longitudinal bone growth occur quite frequently with a high diversity in etiology. Both short and tall stature disorders are divided into primary (skeletal defect), secondary (non-skeletal defect), or idiopathic (cause unknown) (71). Whereas primary growth disorders may have a prenatal onset and may be of chromosomal or genetic origin, secondary growth syndromes are frequently the result of hormonal disturbances. Although growth disorders do not necessarily lead to clinical problems, relatively often they are considered a disability by the affected individuals resulting in psychological, social, educational and professional consequences in childhood, adolescence, but also adulthood.Exposure of patients to gluten prevents healing of gut mucosa, reactivation of specific T cells and reappearance of symptoms. Although not every patient is equally sensitive to gluten exposure, it was reported that exposure to 1 mg of gluten prevented mucosal recovery (35). Therefore, to safeguard patients from gluten exposure, sensitive methods for gluten detection are required and have been developed.

III. b. Osteoarthritis

Osteoarthritis (OA) represents one of the two most frequent chronic skeletal diseases and is undoubtedly by far the most common cause limiting the daily activities of the elderly population (72). OA is characterized by a progressive loss of articular cartilage, synovial proliferation, osteophyte formation and subchondral sclerosis that may culminate in pain, loss of joint function, and disability (73). A variety of risk factors and pathophysiologic processes contribute to the progressive nature of the disease and serve as targets for behavioral and pharmacologic interventions. Risk factors such as age, sex, trauma, overuse, genetics, and obesity can each make contributions to the process of injury in different compartments of the joint (74;75). Although the etiology of OA is not completely understood, it appears to be the result of mechanical, biochemical and enzymatic factors. The final common pathway of these interactions is the failure of the chondrocytes to maintain a homeostatic balance between cartilage formation and resorption (76;77). Loss of articular cartilage is mainly due to proteolytic enzymes that can degrade both proteoglycans (aggrecanases) and collagen (collagenases) (78). Cartilage collagen is cleaved by matrix metalloproteinase (MMP) 1, 8, and 13 (79). Of these three MMPs, MMP13 appears to be the most important in OA because it preferentially degrades type II collagen (80) and its expression is significantly increased in OA (81). Typical phenotypic changes in OA cartilage include the development of the hypertrophic chondrocyte phenotype normally not present in articular cartilage, characterized by increased production of MMP-13, type X collagen, and alkaline phosphatase (ALP) (75).

III. c. Osteoporosis

Osteoporosis is the other most frequent chronic skeletal disease, characterized by low bone mass, concurrent disruption of the bone micro-architecture, and decreased bone strength. Consequently, osteoporotic bones are more fragile and there is increased risk of fracture, particularly of the spine, hip, wrist, humerus, and pelvis (82). Osteoporosis affects an estimated 300 million people worldwide (83). About one in two white women will experience an osteoporotic fracture in her lifetime (84), while older men affected by osteoporosis have a higher mortality from hip fractures and a lower frequency of screening and treatment (85). The risk of fractures increases dramatically with age and most of those affected are over 75 (86). Since the elderly constitute the fastest-growing age group in the world, the number of osteoporotic fractures is predicted to increase considerably with the continued aging of this population in future decades (87;88). Physiological age-related bone loss starts in the 4^{th} or 5^{th} decade of life, as a result of increased bone breakdown by osteoclasts and decreased bone formation by osteoblasts (89). The role of oestrogen deficiency in menopausal bone loss in women is well documented, and bone mass in elderly men is also related to oestrogen levels. Vitamin D insufficiency and secondary hyperparathyroidism are common in elderly people and may also contribute. Other possible factors are reduced physical activity with ageing and decreased production of insulin-like growth factors. As described above, osteoporosis installs due to involutional changes of aging and to

hormonal changes of menopause, being thereby classified as primary. However, osteoporosis can also be caused or worsened by other diseases or medications, when it is referred to as secondary (90).

Bone mineral density (BMD) represents the average concentration of minerals per unit area of bone (measured in g/cm^2). In 1994, the World Health Organization established operational definitions of osteoporosis and osteopenia based on BMD (91). According to this classification normal BMD is defined above -1.0 SD of the young adult reference mean (T-score above –1.0), osteopenia is defined between -1.0 and -2.5 SD of the young adult reference mean (T-score between –1.0 and –2.5), osteoporosis is defined below -2.5 SD of the young adult reference mean (T-score at or below –2.5), while severe osteoporosis requires an osteoporotic BMD in the presence of 1 or more fragility fractures. Different treatments for osteoporosis are available, all aimed at reducing the risk of fractures. Estrogen treatment in post-menopausal women, selective modulators of estrogen receptors (especially raloxifene), calcitonin, a recombinant form of parathormone (teriparatide), strontium ralenate, and especially bisphosphonates, are drugs widely used in clinical practice (92).

IV. CANONICAL WNT SIGNALING DURING SKELETOGENESIS

Increasing amount of evidence points out to the important role of the canonical Wnt/β-catenin signaling in essentially all aspects of skeletal development and maintenance. This pathway is composed of evolutionarily-conserved cellular components, and controls cell proliferation and cell fate determination by inducing changes in gene expression (93). Signaling through this pathway depends on the intracellular levels of its core component, β-catenin. β-catenin is a molecule involved in cell adhesion via its interaction with E-cadherin and α-catenin (94). In the absence of the Wnt ligand, β-catenin is phosphorylated at the NH_2-terminus by glycogen synthase kinase 3 beta (GSK3β) and casein kinase 1 (CK1) in a “destruction” complex brought together by two scaffolding proteins, Axin and Adenomatous polyposis coli (APC). This phosphorylation ultimately results in the ubiquitylation and proteasomal degradation of β-catenin (Figure 4A). When Wnts bind to the 7 transmembrane Frizzled receptors and LDL related protein 5 or 6 (LRP5/6) co-receptors, Dishevelled (Dsh) is activated, leading to suppression of GSK3β activity. As a result, β-catenin will not undergo phosphorylation anymore. Cytoplasmic β-catenin stabilizes and upon reaching a certain level it will translocate into the nucleus where it interacts with transcription factors such as lymphoid enhancer-binding factor 1/T cell-specific transcription factor (LEF/TCF) to initiate the transcription of target genes (95) (Figure 4B). Cells also secrete several Wnt antagonists like secreted frizzled-related proteins (SFRPs), Dickkopf (DKK) and Sclerostin (SOST) (96-98).

APC is involved in a wide variety of cellular processes such as signal transduction, cytoskeletal organization, apoptosis, cell adhesion and motility, cell fate determination and chromosomal stability (99). However, biochemical and genetic evidence was provided showing that APC’s main suppressor activity resides in its ability to bind to β-

catenin and induce its degradation, thereby acting as a strong negative regulator of the canonical Wnt pathway (100). The failure of mutated *APC* to direct cytosolic β-catenin to degradation causes cytoplasmic accumulation of β-catenin and, subsequently, its translocation to the nucleus. *Apc* influences the differentiation capacity of mouse embryonic stem (ES) cells in a quantitative and qualitative fashion depending on the dose of β-catenin signalling (101;102). The differentiation ability and sensitivity of ES cells is inhibited by increasing dosages of β-catenin signaling, ranging from a severe differentiation blockade in severely truncated Apc alleles, to more specific neuroectodermal, dorsal mesodermal and endodermal defects in more hypomorphic alleles. Exclusive levels of APC/β-catenin signaling differentially affect stem cell differentiation (100).

Identified originally as a regulator of glycogen metabolism, GSK3β is now a well-established negative modulator of the canonical Wnt signaling pathway, by inducing degradation of β-catenin (103;104). It also plays important roles in protein synthesis, cell proliferation, cell differentiation, microtubule dynamics and cell motility by phosphorylating initiation factors, components of the cell-division cycle, transcription factors and proteins involved in microtubule function and cell adhesion (105). It is constitutively active and unlike many kinases that are activated following stimulus-dependent phosphorylation, GSK3β is inactivated following phosphorylation (106).

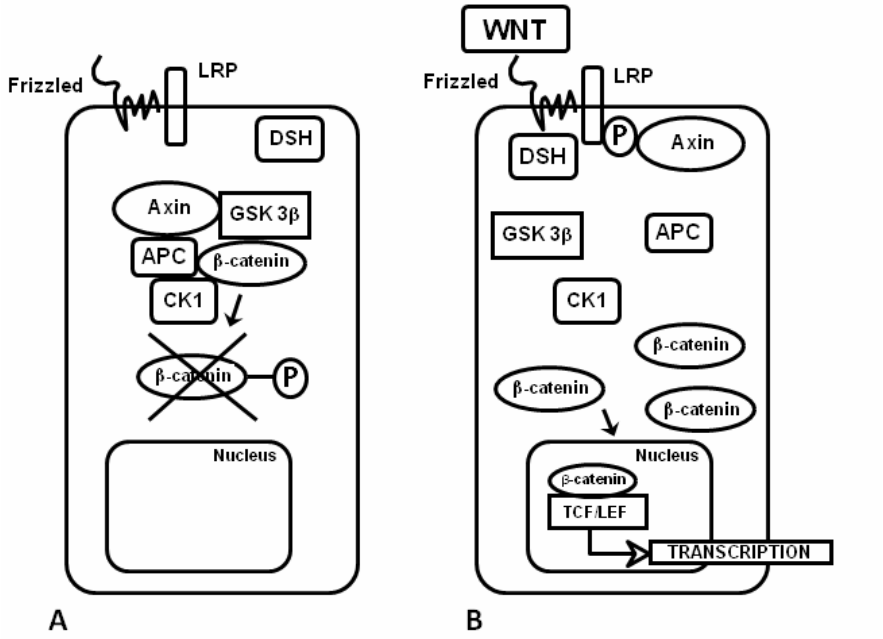

Figure 4. The canonical Wnt/β-catenin pathway. (A) In the absence of a Wnt signal, β-catenin is phosphorylated and targeted for proteasomemediated degradation (details in the text). (B) Upon binding of Wnt to the receptors Fz and LRP, the destruction complex does not form anymore leading to stabilization of β-catenin (details in the text).

The role of canonical Wnt/β-catenin signaling at subsequent stages of skeletogenesis has been suggested based on the expression patterns of many Wnt pathway members, as well as Wnt reporter expression in the mouse (107-113). It is well established that many members of the Wnt family of growth factors, like Wnt1, Wnt3a, Wnt4, Wnt7a, Wnt9a and Wnt 11 inhibit chondrogenesis *ex vivo*, while stabilization of β-catenin has similar effects *in vivo* (62-66;107;110;114-117). However, the detrimental effect of increased canonical Wnt signaling on chondrogenesis is not universal since mouse embryonic fibroblasts (MEFs) lacking the Wnt inhibitor *Sfrp1* display an increased potential to form chondrocytes (118). Besides inhibiting differentiation of MCs into chondrocytes, activation of Wnt/β-catenin signaling also leads to dedifferentiation of chondrocytes, a process associated with downregulation of *Col2a1* and decrease in glycosaminoglycans (GAGs) in the cartilage matrix (64;115;116;119-121). Not only does the canonical Wnt/β-catenin signaling pathway control chondrocyte differentiation and maintenance, it is also highly active in promoting chondrocyte maturation. In this fashion, *in vivo* overexpression of *Wnt4* and *Wnt8* or of a stabilized form of β-catenin accelerates chondrocyte hypertrophy (122;123). Moreover, *Sfrp1*$^{-/-}$ MEFs display increased chondrocyte hypertrophy and mineralization, while Wnt9a positively regulates *Ihh* expression, known for its stimulating role on chondrocyte proliferation and inhibiting role on maturation (116;118;124). Interestingly, overexpression of *Wnt9a*, besides positively regulating chondrocyte hypertrophy, also increases osteoblast differentiation in the surrounding perichondrium, suggesting thereby a stimulating effect of Wnt/β-catenin pathway on this step of endochondral bone formation (108).

In the past decade many studies have identified an important role for the canonical Wnt/β-catenin pathway in joint formation as well. Wnt9a is the molecular marker currently viewed as the earliest "inducer" of joint formation since its expression has been validated *in vivo* at day 5 of embryonic development in a single stripe of the future diartroidal joint (4). Interestingly, *Wnt9a* misexpression *in vivo* induces the formation of ectopic joints through up-regulation of articular chondrocyte markers like *Col3a1*, and joint specific markers such as *Chordin* (*Chdr*), *Autotaxin* (*Atx*), and *growth differentiation factor 5* (*Gdf5*), together with down-regulation of non-articular chondrocyte markers like *Col2a1*, *Col9a1*, *Aggrecan* (*Acan*), *Sox9* and *Bmp4* (4;123). Knockout of both *Wnt9a* and *Wnt4* results in limited joint fusions indicating that possibly *Wnt16* (the 3rd Wnt growth factor known to be expressed in joint interzones) may compensate for the absence of Wnt9a and Wnt4 (116). When no signal is possible through the canonical Wnt/β-catenin signaling pathway at various stages of limb development and joint induction, a severe skeletal phenotype occurs, including chondrodysplasia, ectopic cartilage formation, together with absent or delayed endochondral ossification (107;108;114). Surprisingly, inactivation of β-catenin only in the joints leads to limited fusions in hip joints (125). It is very well possible that joint induction is regulated in a β-catenin-dependent (via Wnt9a and Wnt16) and -independent way (via Wnt4) (62). Alternatively, Wnt/β-catenin signaling might not be exclusively required for the initial induction of joint formation. This observation is sustained by expression of early joint interzone markers in the limbs of conditional mouse embryos lacking β-catenin function in the whole limb mesenchyme (126).

Not less fascinating is the concert regulation of osteoblastogenesis by the multipotent Wnt/β-catenin signaling pathway. LRP5 has a crucial role in BMD accrual and bone metabolism (127). In bone, LRP5 expression is restricted to osteoblasts of the endosteal and trabecular bone surface and regulates osteoblast proliferation, survival and activity (128). Targeted disruption of *Lrp5* in mice leads to a significant reduction in the osteoblast surface density in both primary and secondary spongiosa (129). Surprisingly, although Wnt3a upregulates the levels of canonical Wnt signaling in human mesenchymal stem cells (hMSCs) *in vitro*, it inhibits their osteoblast differentiation, but it stimulates the proliferation of already differentiated osteoblasts (67;68;130). Signaling through Wnt10b induces osteoblast formation from SPCs, while inhibiting adipogenesis; *Wnt10b*$^{-/-}$ mice have a decreased trabecular bone volume and serum osteocalcin levels (70). In agreement with this, *Sfrp*$^{-/-}$ adult mice display enhanced trabecular bone accrual, as a result of increased osteoblast proliferation and differentiation and decreased osteoblast apoptosis (96). Similarly Sfrp4 was shown to be a negative regulator of BMD in mice, by inhibiting Wnt signaling (131). By antagonizing the levels of Wnt/β-catenin transduced signal, Dkk-1 and Dkk-2 are also established regulators of osteoblastogenesis *in vitro* (132). *In vitro* knock-down of *Dkk-1* and *Dkk-2* results in a complete blockade of osteoblast differentiation and matrix mineralization. Another well established antagonist of the Wnt/β-catenin is sclerostin, encoded by the *SOST* gene, whose expression is confined to osteocytes (133). Sclerostin was shown to negatively regulate bone formation both *in vitro* and *in vivo* (133-136).

By far the most investigated component of the canonical Wnt signaling pathway during skeletal development remains its central molecule, β-catenin. Several lines of evidence, especially generated by observation in conditional mouse lines, indicate an indubitable role for β-catenin in the differentiation of osteoblasts from SPCs (107;110-112;117;137;138). Lack of β-catenin in precursor cells impairs osteoblastogenesis and affected SPCs will follow instead the chondrogenic pathway, regardless of the time when β-catenin is inactivated, prior (Prx1-*Cre*) or after (Dermo1-*Cre*, Col2a1-*Cre*) cartilage condensation has occurred (107;110;112). Interestingly, the shift to the chondrogenic lineage of the osteoblast precursors also takes place when β-catenin is deleted in *Osx*-expressing, and therefore committed osteoblasts (137). One would imagine based on these data that activation of β-catenin would have beneficial impact on osteoblast differentiation. This is however not entirely true, since stabilization of β-catenin in *Osx*-positive osteoblast precursors leads to a marked increase in proliferation and an accelerated bone matrix accumulation, yet these osteoblasts fail to express the mature osteoblast marker *Osc* (137). Moreover, the constitutive expression of a stabilized form of β-catenin in the limbs using Prx1-*Cre* mice also negatively affects osteoblastogenesis, leading to the formation of tiny remnants of skeletal elements (110). All these data suggest that β-catenin levels must be finely tuned during subsequent stages of skeletal development for proper osteoblast formation.

V. CANONICAL WNT SIGNALING DURING SKELETAL PATHOLOGY

Initial evidence for a role of the canonical Wnt signaling pathway in skeletal pathology was provided by the identification of mutations in the *LRP5* gene inducing either the Osteoporosis-Pseudoglioma Syndrome (OPPG) or the hereditary High Bone Mass Syndrome (HBMS) in humans. OPPG is a rare autosomal recessive disorder affecting the skeleton and the eye associated with loss-of function mutations in the *LRP5* gene, which prevents Wnt from binding to the receptor (127). Children with the OPPG have a very low BMD and easily develop fractures and deformations. In agreement with this, *Lrp5*$^{-/-}$ mice have a low BMD due to reduced proliferation of precursor cells (129). Interestingly, low bone mass in *Lrp5*$^{-/-}$ mice is further exacerbated by loss of an *Lrp6* allele, suggesting that Wnts signal through both the LRP5 and LRP6 co-receptors to influence bone mass (139). Recently, LRP-6 mutations have been found to cause metabolic syndrome with osteoporosis (140). In contrast, gain-of-function mutations in *LRP5* are associated with increased BMD in the autosomal dominant HBM trait (141-143). These individuals display not only increased BMD, but also increased bone synthesis and excessive bone accrual, yet normal bone resorption, bone architecture, serum calcium, phosphate, PTH and vitamin D levels (128;143;144). These human bone phenotypes were later confirmed by animal models with overexpression of LRP5. For instance, mice that overexpress the HBM LRP5 variant LRP5G171V in osteoblasts have enhanced osteoblast activity, reduced osteoblast apoptosis, and a high BMD supporting the observations in humans with this mutation (145).

Mutations in the *SOST* gene have been shown to result in high bone mass (146). SOST truncation abolishes its inhibitory effect, leading to hyperactivation of canonical Wnt signaling, resulting in the disease sclerosteosis. Furthermore, a 52-kb deletion downstream of the *SOST* gene gives rise to Van Buchem disease. In both these rare and related diseases there is overproduction of bone (147;148). Clinical features of sclerosteosis include: syndactyly as well as very thick and dense bones, particularly in the skull. This can lead to cranial nerve entrapment, resulting in deafness and facial nerve palsy, increased intracranial pressure, and greater risk of stroke (134;149). Patients with Van Buchem disease have similar characteristics, yet in this syndrome syndactyly was not described (134;149). Nevertheless, some Van Buchem patients carry mutations in the *LRP5* gene (149). The HBM LRP5 variant LRP5G171V exhibits reduced SOST binding, suggesting that *LRP5* HBM mutations render LRP5 more resistant to SOST inhibition (147;150). Importantly, this resistance to SOST inhibition may be responsible for most of the pathogenesis associated with increased Wnt signaling in the *LRP5* mutants. The relationship between SOST and LRP5 represents the hope of many researchers in the area of anti-osteoporotic drugs, since the administration of a therapeutic agent that could alter the ability of SOST to bind to LRP5 might lead to increased bone formation (151;152). That this approach is a very attractive basis for developing future osteoporosis therapeutics is proven by the increased bone formation, BMD, and bone strength in several animal models of osteoporosis after administration of sclerostin-neutralizing monoclonal antibodies (153-155).

Another major skeletal disease on which the canonical Wnt signaling lays its fingerprint is osteoarthritis. Recent findings indicate that this pathway responds to mechanical injury to cartilage and is associated with postnatal cartilage matrix degradation, chondrocyte dedifferentiation and apoptosis (119;121;156;157). Upon several whole genome studies, the Wnt antagonist FRZB has emerged as a candidate gene associated with an increased risk for OA (158-161). Although not developing a noteworthy developmental phenotype, *Frzb*$^{-/-}$ mice display greater cartilage loss in comparison to wild-type controls when exposed to factors known to induce OA, like enzymatic treatment (papain-induced osteoarthritis), accelerated instability (collagenase-induced ligament and meniscal damage) or inflammation (mBSA induced monoarthritis) (96;162). Cartilage degradation in the *Frzb*$^{-/-}$ mice is associated with up-regulation of β-catenin and Mmp9. Interestingly, it was also shown *in vitro* that cartilage injury results in increased Wnt activity and lower expression of FRZB (163). While Wnt-7a is associated with cartilage destruction by regulating the maintenance of differentiation status and the apoptosis of articular chondrocytes (119), Wnt-7b expression is upregulated in OA cartilage (164). Mechanical stress resulting in acutely injured cartilage, leads to upregulation of Wnt16, downregulation of FrzB, upregulation of Wnt target genes, and nuclear localization of β-catenin (157). Once canonical Wnt signaling was associated with OA, the question arose whether an animal model with abnormal β-catenin in AC would show an OA phenotype. For this purpose, Zhu and colleagues have generated conditional mice carrying either lower (ICAT) or higher (β-catenin cAct) levels of β-catenin in *Col2α1*-expressing chondrocytes (165;166). Interestingly, both conditional mouse lines displayed OA features, suggesting that precisely regulated canonical Wnt levels are mandatory during AC maintenance. While conditional β-catenin inactivation led to AC destruction and chondrocyte apoptosis, forced expression of a stabilized form of β-catenin resulted in a time-dependent AC degeneration and upregulation of Mmp13. Nevertheless, β-catenin protein expression is upregulated in knee joint samples from patients with OA (165).

VI. OUTLINE OF THIS THESIS

In view of the complex roles of the canonical Wnt signaling during skeletal development and disease, it is important to accurately distinguish the specific roles of this signaling cascade at specific time windows during embryogenesis as well as postnatally in the maintenance of the skeleton. Moreover, a proper understanding of these multifaceted roles will ultimately aid us in identifying new therapeutic targets for the treatment of growth disorders, osteoporosis and osteoarthritis.

Most of the animal models that furnish our knowledge of the effects of canonical Wnt signaling during skeletal development and maintenance use the forced expression of a stabilized and thereby oncogenic β-catenin. The roles of intracellular β-catenin regulators and thereby of wild type β-catenin levels during skeletogenesis, bone mass accrual or AC maintenance are largely unknown. The research described in this thesis aimed at describing the role of two major intracellular regulators of β-catenin, namely Apc and Gsk3β in regulation of SPC differentiation, bone mass accrual and cartilage maintenance.

To investigate whether Apc is involved in lineage commitment of SPCs, we generated conditional knockout mice lacking functional Apc in *Col2a1*-expressing cells (115). Our data presented in **chapter 2** indicate that a tight Apc-mediated control of β-catenin levels is essential for differentiation of skeletal precursors as well as for the maintenance of a chondrocytic phenotype in a spatio-temporal regulated manner. Next we investigated the skeletal development of compound *Apc* mutant embryos with one conditional mutant allele (Apc^{15lox}) and one hypomorphic *Apc* mutant allele (Apc^{1638N} or Apc^{1572T}) resulting in differential levels of transduced canonical Wnt signaling in SPC (167). We show in **chapter 3** that precise dosages of Wnt/β-catenin signaling distinctly influence the differentiation of SPC. In order to reveal the molecular mechanisms by which Apc regulates the differentiation of SPCs *in vitro*, we have knocked down *Apc* in the murine mesenchymal stem cell-like KS483 cells by stable expression of *Apc*-specific small interfering RNA (168). Our results described in **chapter 4** demonstrate that Apc is essential for the proliferation, survival and differentiation of KS483 cells. We next conducted a cross-sectional study evaluating skeletal status in FAP patients with a documented *APC* mutation to determine if *APC* mutations affect bone mass (169). We demonstrate in **chapter 5** that FAP patients display a significantly higher than normal mean BMD compared to age- and sex-matched healthy controls in the presence of a balanced bone turnover. Finally, to investigate the role of Gsk3β in cartilage maintenance we conducted *ex vivo* and *in vivo* experiments in which we treated chondrocytes with GIN, a selective GSK3β inhibitor (170). Our results described in **chapter 6** suggest that, by down-regulating β-catenin, Gsk3β preserves the chondrocytic phenotype, and is involved in maintenance of the cartilage extracellular matrix. In **chapter 7** we summarize the major findings comprised in this thesis. At the same time several possible future research lines are hypothesized, that might help us in more profoundly understanding the function of APC and GSK3β during skeletal development and maintenance.

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