Chapter 1
Using Genetically Engineered Mouse Models to Study Wnt Signaling in Bone Development and Disease

Zhendong A. Zhong, Nicole J. Ethen, and Bart O. Williams

Abstract The skeleton supports the body structure and reserves calcium and other inorganic ions, and more roles played by bone are being proposed. The balance between bone formation (by osteoblasts and osteocytes) and bone resorption (by osteoclasts) controls postnatal bone homeostasis. For the past decade, a vast amount of evidence has shown that Wnt signaling plays a pivotal role in regulating this balance. Therefore, understanding how the Wnt signaling pathway regulates skeletal development and postnatal homeostasis is of great value for human skeletal health. We will review how genetically engineered mouse models (GEMMs) have been and are being used to uncover the mechanisms and etiology of bone diseases in the context of Wnt signaling.

Keywords Wnt signaling • Bone development • Transgenic mice • Conditional knock out • Cre-loxP • Tissue-specific promoter

Abbreviations

CKO	Conditional knockout
Fzd	Frizzled
GEMMs	Genetically engineered mouse models
GOF	Gain of function
KO	Full-body knockout
Lrp	Low-density lipoprotein-related receptor protein
LBM	Low bone mass
LEF	Lymphoid enhancer factor
LOF	Loss of function

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MSC	Mesenchymal stem cell
M-CSF	Macrophage colony-stimulating factor
NA	Not applicable
OE	Overexpression
OMIM	Online Mendelian Inheritance in Man catalog
OPG	Osteoprotegerin
RANKL	Receptor activator of nuclear factor kappa-B ligand
TCF	T-cell factor

1.1 Wnt/β-Catenin Signaling

Wnt signaling is initiated by a conserved Wnt family of secreted glycolipoproteins, through β-catenin-dependent mechanisms (also known as canonical Wnt signaling) or in a β-catenin-independent manner (noncanonical Wnt signaling) [1, 2]. We will focus on the better characterized β-catenin-dependent Wnt signaling pathway, which plays fundamentally important roles in regulating cell fate decision, cell proliferation, and oncogenic events. In brief, without an upstream Wnt engaging the Wnt receptors, a "destruction complex" forms in the cytoplasm, where axin exists in the complex that includes the adenomatous polyposis coli (Apc) protein and the serine/threonine protein kinase GSK3 (glycogen synthase kinase 3). GSK3 phosphorylates β-catenin and targets it for ubiquitin-dependent degradation. When a Wnt engages a receptor complex (containing a member of the frizzled family of seven-transmembrane receptors and either Lrp5 or Lrp6), this induces the phosphorylation of the cytoplasmic tail of Lrp5/6, creating a binding site for axin. The recruitment of axin to the plasma membrane interferes with the ability of the destruction complex to recruit β-TrCP for ubiquitinylation and consequently blocks degradation of β-catenin. Then β-catenin accumulates in the cytoplasm and translocates into the nucleus (possibly due to other signaling events such as Rac1 activation), where it complexes with members of the LEF/TCF family of DNA-binding proteins to activate transcription of target genes (Fig. 1.1). This pathway is being intensively investigated, more components are being discovered, and more details about the pathway regulation are being unraveled.

1.2 Wnt/β-Catenin Signaling in Human Skeletal Diseases

The first line of evidence toward the role of Wnt signaling in bone was the finding that loss-of-function mutations in low-density lipoprotein-related receptor 5 (LRP5) are the cause of osteoporosis-pseudoglioma (OPPG) syndrome, a rare disease characterized by dramatic bone mass reduction and leukocoria. Further analyses showed that LRP5 was expressed in osteoblastic cells, which suggested that LRP5-mediated signaling in those cells might be responsible for this skeletal developmental defect [3]. Shortly after two independent groups reported LRP5 gain-of-function mutations that caused high bone mass of variable severity in two different families [4, 5].

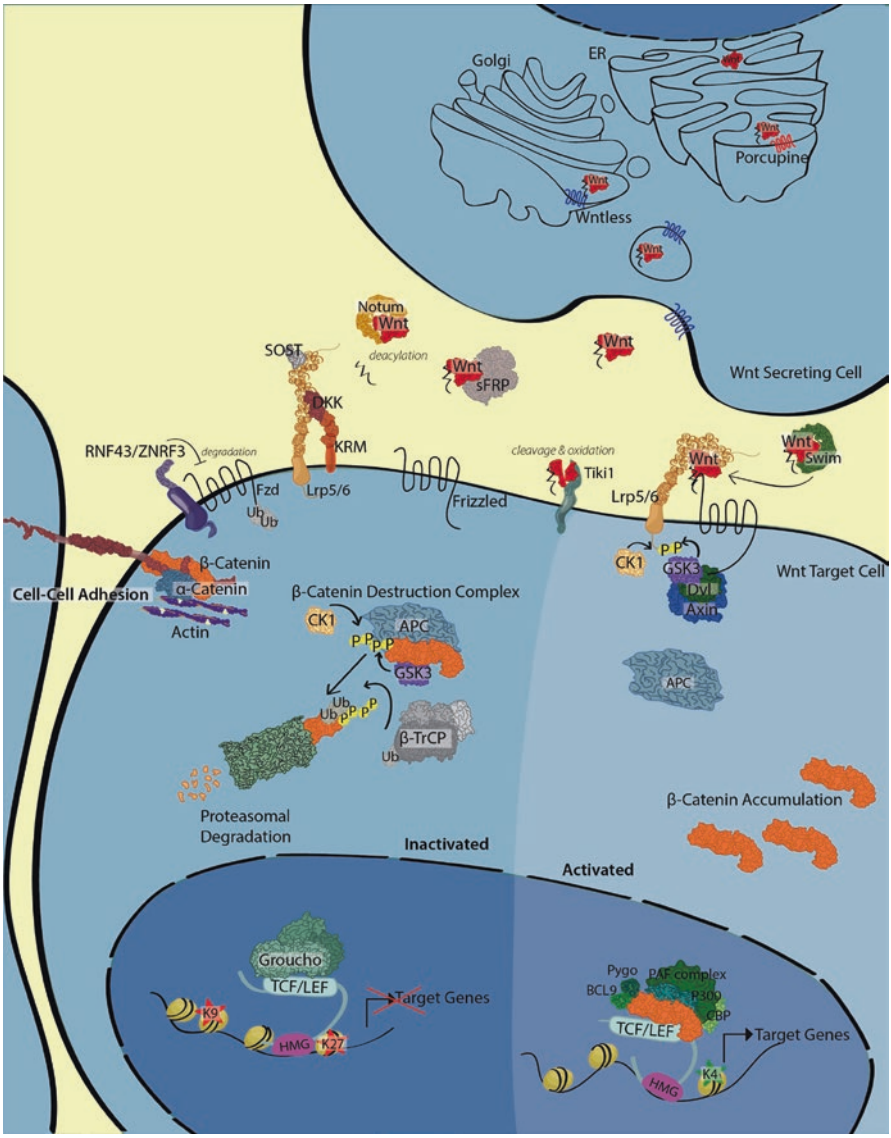

Fig. 1.1 Wnt/β-catenin (canonical) signaling pathway. Wnts are expressed and then lipid-modified by Porcupine in the endoplasmic reticulum (ER) compartment and are secreted out of the cell membrane with Wntless (Wls). Swim is proposed to be an extracellular carrier for Wnts to travel. In the absence of an upstream ligand, the receptor Fzd and coreceptor LRP5/6 are inactive. Cytoplasmic β-catenin will be recruited into a "destruction complex" consisting of axin, APC, CK1, and GSK3. This "destruction complex" facilitates the phosphorylation of β-catenin by GSK3 and subsequent ubiquitinylation (Ub) by β-TrCP, an E3 ligase. Because ubiquitinated β-catenin will be degraded in the proteasome, little β-catenin is accumulated in the cytosol or translocated into the nucleus, and the transcription repressor groucho occupies TCF/LEF. The signaling inactivity could be caused by unavailability of a coreceptor due to DKK1/SOST binding LRP5/6, inaccessibility of the receptor due to RNF43/ZNRF3 binding to Fzd for ubiquitinylation and degradation, or by the absence of active Wnts due to sFRP/Tiki/Notum and others binding to Wnts directly or enzymatically inactivating them.

Other missense mutations in LRP5 were associated with enhanced bone density in ten families and isolated patients [6]. Further, a loss-of-function mutation within an EGF-like domain of human LRP6, which is another important Wnt coreceptor, was identified to be associated with osteoporosis and metabolic syndrome in humans [7]. These findings implied an important role for LRP5 in regulating bone development and homeostasis, presumably through the Wnt/β-catenin signaling pathway. Other mutations in Wnt receptors related to human skeletal diseases are listed in Table 1.1.

Table 1.1 Skeletal phenotypes in mouse strains with germ line/global knockouts (KOs)

Gene		Phenotype(s) and related human skeletal disorders	Ref.
Wnt ligands	*Wnt1*	Swaying mice showed propensity to fractures and severe osteopenia due to defects in osteoblast activity. Wnt1 mutation recently found to be associated with osteogenesis imperfecta (OMIM: 166210)	[55, 56]
	Wnt3a	Homozygotes died by 12.5 dpc. Heterozygote had low bone mass (LBM) phenotype	[20, 57]
	Wnt3	Hypomorphic mutation (Vt) in Wnt3a caused exhibit vertebral abnormalities	[58]
	Wnt4	Homozygotes died at birth. Delayed chondrocyte maturation. Severe joint fusion with concomitant loss of Wnt9a	[59, 60]
	Wnt5a	Homozygotes died at birth. Shortened skeletal elements and loss of digits. Decreased hypertrophic chondrocytes and ossification that was most severe in distal bones. Heterozygotes showed LBM. Robinow syndrome (OMIM: 180700)	[57, 61, 62]
	Wnt7a	KO or inactivating mutation caused frequent loss of posterior digits and ectopic dorsal formation of sesamoid bones in paws. Al-Awadi/Raas-Rothschild/Schinzel phocomelia syndrome (OMIM:276820)	[21] [63]
	Wnt9a/14	Homozygotes died at birth. Decreased size and mineralization of appendicular bones. Ectopic cartilage nodules in cranial sutures and joints. Fusions of wrist and ankle bones	[60]
	Wnt9b	Hypomorphic mutation (clf1) in Wnt9b caused cleft palates. No skeletal assessments	[64]
	Wnt10b	Homozygotes were viable. LBM. Split-hand/split-foot malformation 6 (OMIM: 225300)	[65] [66]
	Wnt16	Homozygotes were viable. Reduced appendicular cortical bone mass and strength. Wnt16 KO mice had reduced cortical but not trabecular bone mass	[67, 68]

Table 1.1 (continued)

Gene		Phenotype(s) and related human skeletal disorders	Ref.
Wnt (co-) receptors	*Fzd2*	Homozygotes have reduced viability and cleft palate. Skeletal elements smaller but normal shape	[69]
	Fzd8	Homozygotes displayed osteopenia with normal bone formation and increased osteoclastogenesis	[70]
	Fzd9	LBM due to decreased bone formation. Williams-Beuren syndrome (OMIM: 194050)	[71]
	Lrp5	Different Lrp5 knockout models have been made by targeting different Lrp5 gene regions. Homozygotes were viable and showed LBM. Osteoporosis-pseudoglioma syndrome (OMIM: 259770)	[31, 72–75]
	Lrp6	Homozygotes died between E14.5 and birth. Numerous abnormalities including truncation of the axial skeleton, limb defects, and urogenital malformation. Heterozygotes had normal skeletogenesis and BMD but reduced BV/TV. Hypomorphic mutations (Cd, Rs) of Lrp6 caused skeletal defects, such as vertebral malformations, delayed ossification of the digits Coronary artery disease, autosomal dominant 2 accompanied by low bone mass (OMIM: 610947)	[75–79]
	Lrp4	Homozygous knockout died at birth. LOF mutations in Lrp4 (dan, mdig, mte, mitt) could cause brachydactyly and syndactyly on all limbs with duplications out of plane. Shortening and fusing of appendicular skeletal elements. Functional Lrp4 deficiency (Lrp4ECD) caused LBM. Cenani-Lenz syndactyly syndrome (OMIM: 212780); Sclerosteosis 2 (OMIM: 614305)	[80–83]
Other secretion factors	*Wls*	Homozygotes could not survive beyond E10.5 and showed defects in embryonic axis formation	[84]
	Dkk1	Homozygotes died at birth. Severe craniofacial malformation as well as fused and ectopic digits. Heterozygotes had no overt phenotype. However, BV/TV, mineral apposition rate, osteoblast surface, and mechanical resistance were increased. There was no change in osteoclasts	[85, 86]
	Dkk2	Homozygotes were viable. Osteopenia with major defects in mineralization rates. Increased osteoclast numbers, but no change in osteoblasts	[87]
	Sost	Homozygotes were viable. High bone mass similar to Lrp5-A214V (HBM mutation) with primary enhancement of cortical bone. Fracture healing was also enhanced. Sclerosteosis 1 (OMIM: 269500)	[88–90]
	Sfrp1	Homozygotes were viable. Skeletal elements and bone accrual normal but reduced age-related trabecular bone loss that was most pronounced in females	[91]
	Sfrp2	Homozygotes were viable. Shortened metacarpals/metatarsals and phalangeal bones with delayed ossification and a reduction of hypertrophic chondrocytes	[92]

(continued)

Table 1.1 (continued)

Gene		Phenotype(s) and related human skeletal disorders	Ref.
Intracellular factors	*Axin1*	Homozygotes died at E9.5. Heterozygotes had tail bifurcation and rib fusion. LOF mutations (Fu, Fu-kb) showed similar phenotypes with incomplete penetrance. Caudal duplication anomaly (OMIM: 607864)	[93] [94]
	Axin2	Homozygotes were viable but commonly developed craniosynostosis due to enhanced mineralization and ossification of the cranial sutures. Protection against age-related decreases in BMD and BV. Homozygotes of LOF mutation in axin2 (canopus, canp) died during gestation with shortened or doubled tails. Oligodontia-colorectal cancer syndrome (OMIM: 608615)	[95–98]
	Gsk3α	Viable mice were recovered with complete loss of Gsk3α in a heterozygous Gsk3β background. These mice were dwarfed with shorter limb bones and vertebrae. Chondrocyte zoning was normal however, there was metachromasia and decreased Col2a1 expression. Cultured chondrocytes had no change in β-catenin protein levels, cellular localization, or signaling ex vivo	[99]
	Gsk3β	Homozygotes died 24 h after birth. Cleft palate. Decreased ossification of the skull, ear bones, and cranial base. In contrast, heterozygous mice had increased cortical and trabecular bone mass, with no change in growth plate morphology. Bone formation rates were increased	[100–102]

Loss-of-function mutations of sclerostin (SOST), a Wnt antagonist, were found to be the causal events in sclerosteosis and Van Buchem's disease, which are both rare high-bone-mass genetic disorders [8]. SOST can bind Lrp5 and Lrp6 to suppress Wnt/β-catenin signaling [9]. LRP4 is an LDL receptor that closely resembles the extracellular domain of Lrp5/6, which was recently identified as a Wnt/β-catenin signaling antagonist presumably serving as a receptor of SOST [10, 11]. Two homozygous missense mutations in LRP4, which lead to LRP4 loss of function, were also identified as causes of sclerosteosis in human patients [12]. A key characteristic that makes the SOST gene particularly important and attractive for therapeutic targeting is that it is primarily expressed in osteocytes [9]. Thus, targeting SOST for osteoporosis treatment may cause fewer undesired side effects in non-skeletal tissues (reviewed in [13]). A number of pharmaceutical companies have generated different kinds of SOST inhibitors or neutralizing antibodies, including a small-molecule SOST inhibitor from OsteoGeneX that is in preclinical development [14]. Romosozumab, an anti-sclerostin antibody from Amgen, was reported to increase bone mineral density in healthy postmenopausal women, presumably and mechanistically due to both enhanced bone formation and repressed bone resorption [15]. Blosozumab, another SOST antibody developed by Eli Lilly, appears to have similar effects [16].

Alterations in Wnt signaling identified in human skeletal diseases indicate a pivotal role for this pathway in bone. With more sophisticated screenings, the revolu-

tion of sequencing technology and an improved understanding of the Wnt signaling pathway, ever more genetic changes within Wnt signaling components, are being identified as related to or causal to human bone mineral density or specific skeletal diseases (Table 1.1). Using genetically engineered animal models, we can not only confirm the importance of these genes in bone development and homeostasis but also explore potential therapeutic interventions targeting the Wnt signaling pathway.

1.3 Transgenic and Germ Line Knockout Models

The first so-called "transgenic" mice that facilitated the expression of exogenous proteins in specific tissues were created by pronuclear injection of appropriately designed segments of DNA into one-cell embryos shortly after fertilization (Fig. 1.2, top left panel). Typically, the DNA segment would contain promoter sequences that drove expression of an included cDNA with the necessary polyadenylation signal in a tissue of interest [17]. These models provided important insights into biological functions for many decades but were limited by the fact that the insertion of the DNA segment is a somewhat random process, so the expression level of the transgene may be heavily influenced by the site of insertion.

The next development was the ability to create mice carrying targeting gene inactivation in the germ line. The techniques that facilitated these approaches were first described in the late 1980s, and their importance in many areas of biomedical research is best illustrated by the fact that the pioneering investigators were awarded the Nobel Prize in Physiology and Medicine in 2007 [18]. These revolutionary techniques exploit the ability to identify clones of mouse embryonic stem cells in which a specific recombination event has occurred. The creation of these genetically engineered mouse models (GEMMs) could take many months, but the biological insights from the resulting GEMMs made these investments of time and resources worthwhile.

Compared with overexpressing a specific gene in a transgenic mouse model, knockout mouse models provide valuable clues about what genes can do in physiological settings. Since mice share many genes with humans, observing the characteristics of a knockout mouse model allows researchers to better understand how similar genes in humans may cause or contribute to diseases. The initial gene function characterization is usually performed on germ line knockout mice. A historical footnote is that one of the first genes chosen for targeted inactivation in the mouse germ line was *Int1* (later called *Wnt1*) [19]. The involvement of the Wnt/β-catenin signaling pathway in skeletal development was first observed in Wnt3a and Wnt7a knockout embryos: Wnt3a-targeted embryos had axial defects and Wnt7a-targeted embryos showed limb-development defects [20, 21]. Although germ line knockouts of important genes would cause embryonic death or serious developmental defects, it is still the most efficient and convenient way

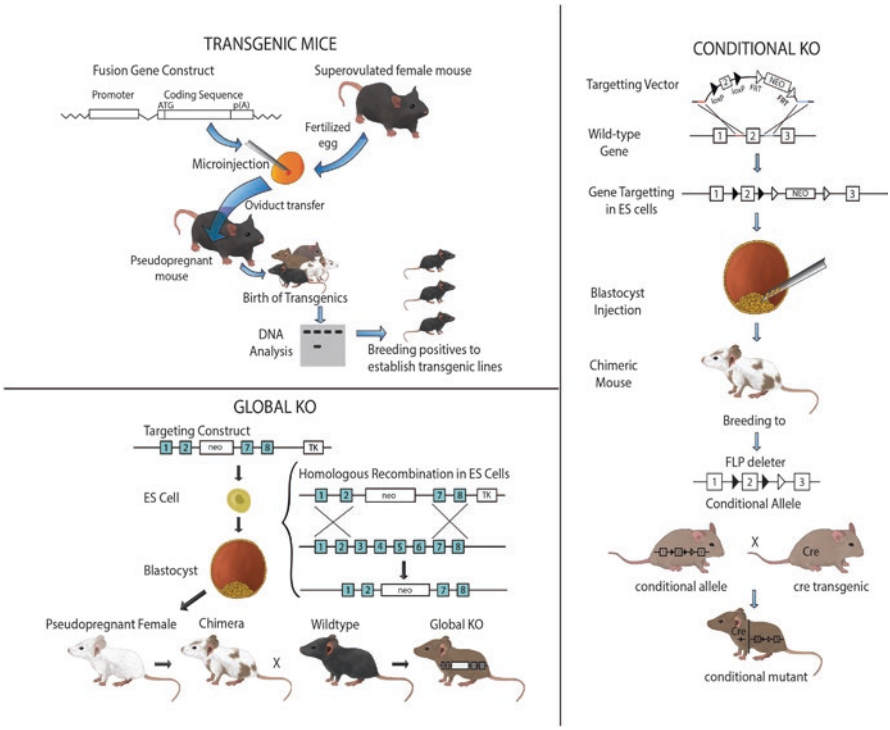

Fig. 1.2 Knockout and transgenic mouse models. *Transgenic mice*: Transgenic mice that facilitate the expression of exogenous proteins in specific tissues are created by pronuclear injection of appropriately designed segments of DNA ("fusion gene") into one-cell embryos shortly after fertilization. The fusion gene contains promoter sequences that drive expression of an included cDNA with the necessary polyadenylation signal (p(A)) in a tissue of interest. Since the insertion of the DNA segment is a somewhat random process, the offspring are analyzed for transgene integration, and then the positive founders are used to establish transgenic lines with stable expression of the transgene. *Global knockout*: A targeting construct contains part of the gene for homologous recombination (exon 1/2 and 7/8), a selectable marker (TK), and a reporter gene (neo, also another selectable marker). This construct is injected into mouse embryonic stem cells in culture, and then the cell clones with correct recombination will be used to contribute to the mouse's tissue via blastocyst injection. The resulting chimeric mice, where the modified cells make up the reproductive organs, are selected for via breeding with wild-type mice. *Conditional knockout*: A conditional targeting vector typically contains a part of critical exons flanked by LoxP sites and a selective marker ("Neo") flanked by FRT sites. Upon gene targeting in ES cells, the vector can change an endogenous gene through homologous recombination and subsequently be screened by treatment with antibiotics (neo) or with PCR. After blastocyst injection, chimeric mice are identified with PCR and further crossed with a FLP deleter mouse to remove the neo gene, subsequently generating an inheritable conditional allele. When bred with a Cre mouse, the "floxed" mouse carrying the conditional allele will permanently remove the floxed exon(s) in the Cre-expressing tissues

to study a novel gene. These approaches generated significant insights into how genes from the Wnt signaling pathway regulate skeletal development (Table 1.1). However their embryonic lethal character often precluded the detailed characterization of bone homeostasis.

1.4 Bone-Specific Conditional Knockout Models

The advances in Cre-lox recombination systems to create enhanced GEMMs have helped to study gene function in specific tissues or cell types. The Cre-lox system was identified in bacteria [22]. A loxP (locus of X-over P1) site is a 34-base-pair consensus sequence containing a core domain of 8 base pairs flanked on each side by a 13-base-pair palindromic sequence [23]. The Cre recombinase is 38 kDa and catalyzes recombination between two of its sequence recognition (loxP) sites, resulting in the elimination of sequences flanked by the loxP sites. This led to the development of numerous mouse strains in which essential portions on a gene are flanked by loxP sites (so-called "floxed" strains). If the Cre gene is expressed in a cell type via the use of well-characterized tissue-specific promoter, Cre-mediated recombination leads to loss of gene function in that particular cell type (Fig. 1.2, right panel). Therefore, the specificity of Cre expression controls where the conditional knockout will occur. To regulate Cre expression spatially and temporally, the Cre recombinase gene is inserted into the genome under the transcriptional control of one of the promoters that would be active in a particular cell type and at a particular stage during skeletal development. Several Cre strains have been created via pronuclear injection followed by random integration of the expression plasmid, while others have been developed using homologous recombination in mouse embryonic stem cells to target Cre expression to occur from endogenous promoters [Fig. 1.3].

1.4.1 Bone-Specific Promoters

In order to accurately evaluate the role of a gene in a particular tissue/cell type using the Cre/lox system, the promoter specificity and penetrance (the percentage of target cells that express Cre) are two major considerations. To characterize the Cre activity, a reporter mouse model that harbors a loxP-flanked DNA STOP sequence in front of a reporter gene (LacZ or EGFP) in the genome is widely used (Fig. 1.4). Upon being crossed with a Cre strain, the STOP codon would be removed to activate the downstream reporter gene expression in those tissues/cells where Cre activity is present. X-gal (an analog of lactose) staining is widely used to locate which cells express a LacZ reporter gene that produces β-galactosidase enzyme [24]. More recently, scientists have developed more sensitive reporter models using similar strategies, such as the mT/mG model with dual-fluorescent protein labeling [25]. Using the mT/mG reporter mouse

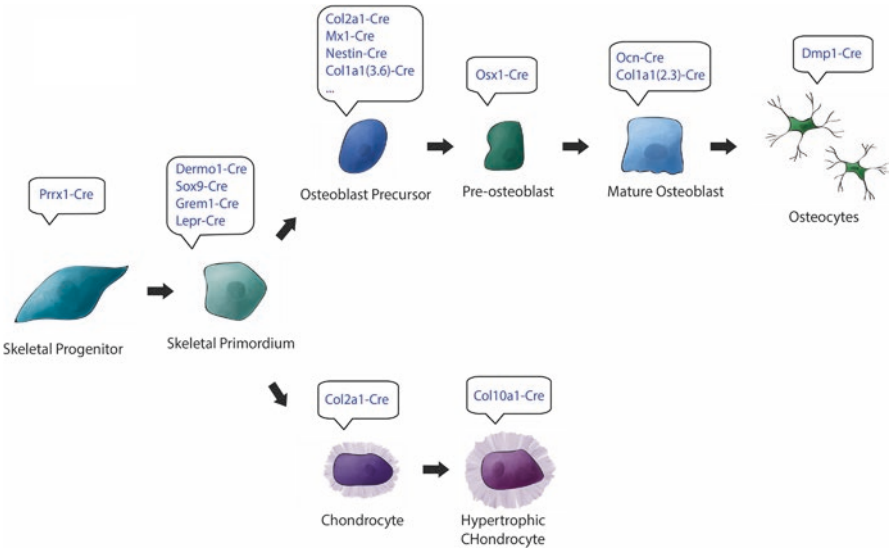

Fig. 1.3 Osteochondral cell differentiation and bone-specific promoters. Chondrocytes, osteoblasts, and some other cell types likely originate from common skeletal progenitor cells. Chondrocytes and osteoblasts express different sets of genes throughout their maturations. The corresponding bone-specific promoters target osteoblasts and chondrocytes at different maturation stages

line, our laboratory was able to detect earlier osteocalcin promoter activity in osteoblasts than could be detected with the LacZ system [26, 27]. Such reporter systems are important in assessing Cre activity, because many promoters have extraskeletal expression patterns that need to be carefully considered in interpreting phenotypes. By comparing the phenotypes of conditional knockout animals with multiple and independent Cre strains, we can often better evaluate and compare the roles of a specific gene at various stages of a lineage or in closely related cell types.

We next will review conditional knockouts that happen in three of the major cell types within the skeleton: osteoblast, chondrocyte, and osteoclast. These three cell types regulate bone development and bone remodeling, and they are involved in the pathogenesis of skeletal diseases such as osteoporosis and osteoarthritis. The interplay between osteoblasts and osteoclasts regulates the balance between bone formation and bone resorption to maintain skeletal homeostasis [Fig. 1.5]. Some promoters are active in the precursors to both osteoblasts and chondrocytes (such as Prrx1-Cre and Dermo1-Cre). More profound phenotypes may be observed in conditional knockout models with Cre drivers that are expressed earlier and in more cell types. GEMMS have demonstrated that Wnt signaling is important for the commitment of mesenchymal stem cells (MSCs) to the osteoblast lineage. However constitutive activation of Wnt signaling may prevent osteoblastic terminal differentiation [28, 29].

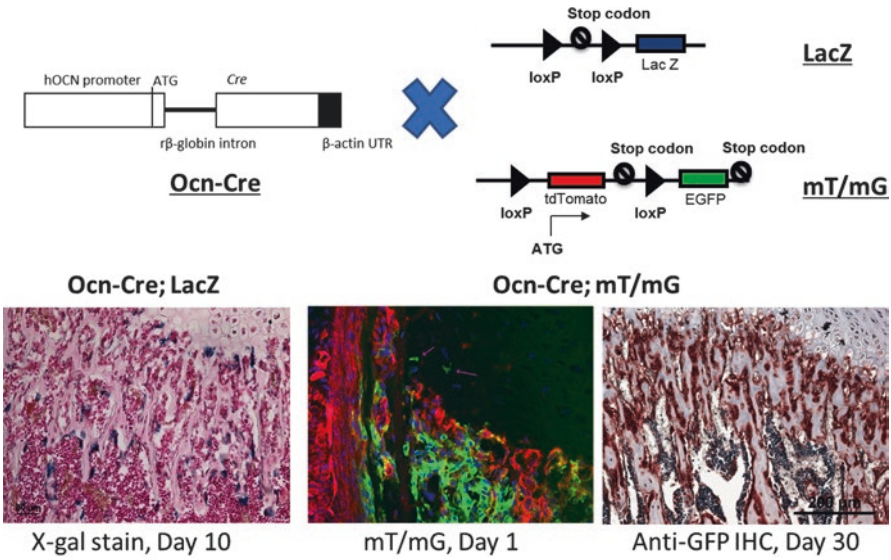

Fig. 1.4 Cre reporter strains. A "LacZ" cassette contains a LacZ gene preceded with a STOP codon. The STOP codon will be removed upon exposure to a Cre recombinase to allow LacZ expression. Similarly, an "mT/mG" cassette contains a tdTomato gene (red fluorescent) preceding a GFP gene, so that it expresses tdTomato until a Cre removes the "tdTomato-STOP" cassette and activates the GFP expression. These transgene cassettes are inserted into ROSA26 genome locus to facilitate ubiquitous expression. Upon being crossed with a tissue-specific Cre driver (the osteocalcin promoter, for example), Cre activity can be detected by X-gal staining, direct fluorescence microscopy examination, or anti-GFP immunohistochemistry

1.4.2 Osteoblast-Specific Knockouts

Osteoblasts are specialized, differentiated products of mesenchymal stem cells (MSCs), and they are terminally differentiated into osteocytes once they are imbedded in bone matrix. Osteoblasts synthesize large amounts of cross-linked collagen and smaller amounts of several other proteins, including osteocalcin and osteopontin, to allow for the formation of the organic matrix of the bone. The osteoblastic lineage is defined at different stages based on biological behavior and transcription markers, and more cell stage-specific markers are being discovered. Using the corresponding promoters, one can delete a gene at a specific maturation stage. So far, most evidence suggests that Wnt/β-catenin signaling in osteoblasts inhibits bone resorption or augments bone formation (Table 1.2). Our group and others have proposed that Wnt/β-catenin signaling in osteoblasts could regulate osteoclast activity by regulating osteoprotegerin (OPG) expression [28, 30]. OPG inhibits osteoclastogenesis by binding to RANKL and preventing it from interacting with RANK on osteoclast precursors, and OPG protects the skeleton from excessive bone

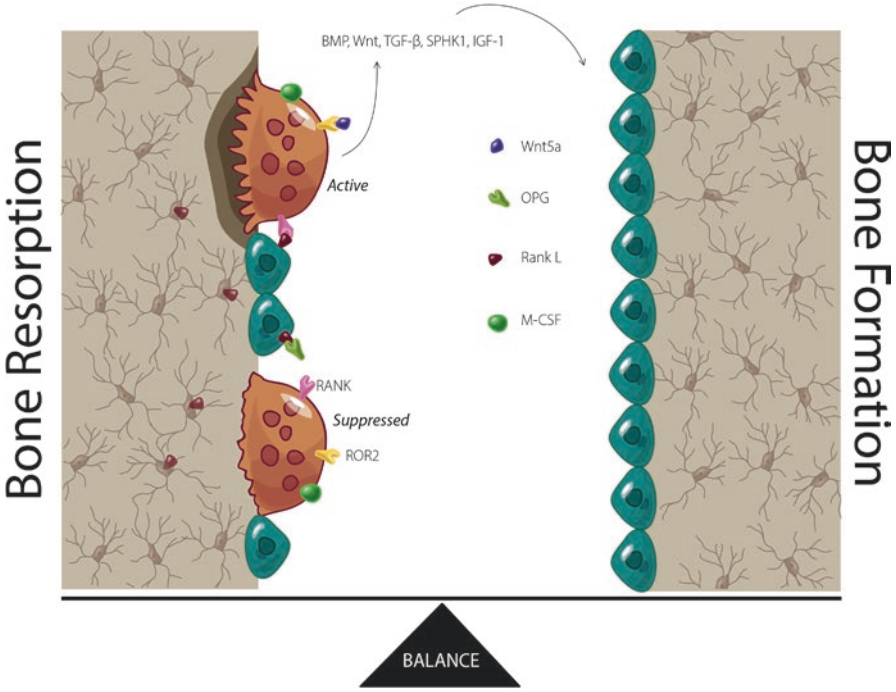

Fig. 1.5 Bone formation and bone resorption. Bone homeostasis is regulated by the balance between osteoblast-mediated bone formation and osteoclast-mediated bone resorption. The activities of osteoblasts and osteoclasts are coupled at least partially through secreted factors. Osteoclasts or osteoclast activity stimulates osteoblast differentiation or homing to bone resorption site by secreting BMP, Wnt, TGF-β, IGF-1, SPHK1, and other factors. RANKL, M-CSF, and Wnt5a are key factors for osteoclast differentiation and maturation, which are expressed by osteoblasts. Another secreted factor from osteoblasts, OPG, acts as RANKL decoy receptor and inhibits RANKL-RANK interaction, therefore impairing osteoclast maturation

Table 1.2 Skeletal phenotypes in mouse strains with conditional knockouts (cKOs)

Gene	Cre line (targeted tissue)	Phenotype(s)	Ref.
Lrp5	CMV-Cre (Germ line)	Similar phenotype to the other Lrp5-KO mice	[103]
	Dermo1-Cre	Normal skeletogenesis. No other skeletal assessments	[103]
	(2.3kb)Col1a1-Cre or Villin-Cre (intestines)	No change in bone mass with Col1a1-Cre, but reduced bone mass with Villin-Cre	[31]
	Dmp1-Cre (osteocytes) or Villin-Cre	No change in bone mass with Villin-Cre, but reduced bone mass with Dmp1-Cre	[34]

Table 1.2 (continued)

Gene	Cre line (targeted tissue)	Phenotype(s)	Ref.
β-Catenin	Col2a1-Cre) or Dermo1-Cre	Homozygotes died shortly after birth. Skeletal defects from Col2a1-Cre crossings include shortened limbs, loss of tarsal synovial joints leading to bone fusion and domed skulls. Dermo1-Cre produced a more severe phenotype	[104]
	Brn4-Cre (neural tube and hind limbs)	Malformation of hind limbs, including truncation or absence of tibia, fibula, and digits	[105]
	(2.3kb) Col1a1-Cre	Homozygotes were viable. Decreased bone mass. Increased osteoclast activity. No change in osteoblasts.	[30]
	Ocn-Cre	Homozygotes died within 1 month of birth with decreased bone mass. Increased osteoclast activity	[28]
	Prrx1-Cre	Mice died at birth. Appendicular bones were shortened, partially fused, with loss of distal structures and mineralization. Growth plate chondrocyte zoning was normal, but bone collars were absent	[40]
	Dermo1-Cre	Homozygotes exhibited an inhibition of commitment to the osteoblast lineage with severe defects in skeletogenesis	[40, 106]
	Osx1-Cre-TetOff	Severe skeletal defects due to a lack of fully differentiated osteoblasts, which resulted in no mineralization or ossification of bone	[107]
	PPARγ-tTA (osteoclast progenitors) + TRE-Cre	Heterozygotes were osteoporotic with decreased trabecular and cortical bone mass. Increased osteoclast number, and surface area with no change in osteoblasts. Interestingly, homozygotes were osteopetrotic, similar to, but not as severe as the β-catenin GOF allele	[42]
	Dmp1-Cre	Homozygotes were viable. Growth retardation and early lethality at 3-5 months. Early onset cortical and cancellous bone loss in both the appendicular and axial skeleton. Osteoclast number and activity were elevated, with no change in osteoblasts	[108]
	Col2a1-Cre-ERT2 (chondro-osteo progenitors-inducible)	Reduced hypertrophic chondrocyte zone, disorganization of prehypertrophic chondrocytes, and a reduction of the primary ossification center. By E18.5, there was a failure of vascularization of the cartilage lacunae	[109]
	Osx1-Cre-ERT2 (tamoxifen inducible)	Tamoxifen induced recombination was performed in 2-month-old mice. Osteopenia developed by day 21 characterized by reduced Tb.N and increased Tb.Sp, but no change in trabecular thickness. Decreased cortical bone by day 49. Osteoclast numbers and activity were elevated at day 21 and beyond. Serum PINP levels and osteoblast numbers were decreased at day 8 but elevated in the subsequent time points	[110]

(continued)

Table 1.2 (continued)

Gene	Cre line (targeted tissue)	Phenotype(s)	Ref.
Porcn	Sox2-Cre (epiblast)	Heterozygous females were viable. Shortened or absent limbs and digits with variable severity. No skeletal or bone specific Cre model assessments. Focal dermal hypoplasia (OMIM: 305600)	[111]
	Msx2-Cre (hind limb ectoderm) or Prrx1-Cre	Shortened hind limbs and syndactyly of digits (Msx2-Cre). Shortened limbs and loss of digits, but no syndactyly (Prrx1-Cre)	[111]
	Chimeras	FRT-Neo cassette created a hypomorphic allele in chimeras. 9/17 resulting male and female chimeras had absent, fused, or shortened digits on limbs and vertebral abnormalities	[112]
	EIIa-Cre or Hprt-Cre (germ line)	Viable male and female mice in low yield with abnormal hair follicle development (Ella-Cre). Hprt-Cre resulted in heterozygous females in low yield. Frequent axial truncations and neural tube malformations in embryos. No skeletal or bone specific Cre model assessments	[112]
	Prrx1-Cre	Similar to the Barrott et al. mouse but with syndactyly of soft tissue and no loss of digits.	[112]
Wls	Ocn-Cre	Progressive decreases in BMD, trabecular, and calvarial bone, as well as cartilage starting at 20 days of age. <20% of mice survived over 2 months. Massive decreases in BV/TV and cortical bone by 7 weeks. Decreased serum osteocalcin. Increased TRAP staining	[47]
	Prrx1-Cre	Homozygotes died at weaning. Limbs were hypoplastic, shortened, with truncated autopods. Ossification, chondrocyte hypertrophy, and osteogenesis were also impaired	[113]
	Msx2-Cre	Truncated autopods in all limbs, but shortened zeugopods only in hind limbs. Defective suture fusion and ossification of the skull	[113]
	Dermo1-Cre	Reduced mineralization, malformation, and/or absence of skeletal elements during skeletogenesis. Loss of hypertrophic chondrocytes	[114]
	(2.3kb)Col1a1-Cre or Osx-Cre	No defects in skeletogenesis or bone mineralization at embryonic stage. Adult bone phenotype was similar to Ocn-Cre-driven wls cKO mice	[114, 115]
	Col2a1-Cre	Shortened long bones, loss of bone collar formation, decreased bone mineralization, and reduced chondrocyte maturation	[114]

resorption by osteoclasts. Some evidence suggested that β-catenin and cofactors could directly bind to the promoter region of the OPG gene and activate its transcription [30]. Currently, several OPG analogs or RANKL antibodies are being evaluated for improving bone homeostasis in clinical trials.

Another model proposes that Lrp5, a Wnt coreceptor, controls osteoblast differentiation by regulating the amount of serotonin that is secreted from intestinal enterochromaffin cells. The varying serotonin levels that bind to their receptors on osteoblasts then regulate differentiation and bone formation [31–33]. However, a great deal of evidence supports the model described above, in which the activity of the Wnt signaling pathway within the osteoblast is regulated to control skeletal development and homeostasis [33–35]. The reasons for the discrepancies between these two models remain unclear.

1.4.3 Chondrocyte-Specific Knockouts

Both chondrocytes and osteoblasts are derived from mesenchymal stem cells (MSCs). Chondrocytes in the growth plate will further differentiate into hypertrophic chondrocytes, which support endochondral ossification. Although we do not focus on osteoarthritis in this review, articular chondrocytes play important roles in maintaining articular cartilage and joint function. The dysregulation of articular cartilage by Wnt/β-catenin signaling in chondrocytes is directly connected to the process of cartilage degeneration in osteoarthritis [36, 37, 41]. It's important to note that a recent fate-mapping study convincingly showed that all tested promoters proposed to have "chondrocyte-specific" activity (including Acan, Col2, and Sox9, all of which are important transcription factors during chondrogenesis) could be chased into osteoblastic and other lineages, which means deleting genes in chondrocytes may also delete genes in the osteoblastic lineage [38]. Higher Wnt/β-catenin signaling in osteoprogenitor cells favors osteogenesis over chondrogenesis, while lower Wnt/β-catenin signaling seems to do the opposite [35, 39, 40]. In committed chondrocytes, Wnt/β-catenin signaling actually promotes chondrocyte proliferation and maturation [29, 37, 41], so it is important to interpret the phenotypes of conditional knockout animals based on the Cre driver's specificity and timing.

1.4.4 Osteoclast-Specific Knockouts

Conditionally activating β-catenin in any stage of the osteoclast lineage could cause osteopetrosis with suppressed bone resorption. However, inactivation of β-catenin in osteoclast precursors (driven by PPARγ or Tie2 promoter) showed dose-dependent effects: β-catenin heterozygosity enhanced osteoclast differentiation, but β-catenin deletion suppressed osteoclast precursor proliferation. Deletion of

β-catenin in more committed stages of osteoclast differentiation (driven by the LyzM or Ctsk promoter) enhanced osteoclast differentiation and bone resorption in vivo [42]. More recent work showed that Wnt3a could inhibit osteoclastogenesis by inactivating NFATc1 in osteoclast progenitors through the β-catenin-independent and Lrp5-/6-dependent signaling pathway, while RANK-Cre-driven Lrp5/6 deletion also suppressed osteoclast precursor proliferation [43].

1.5 Gain-of-Function Mutations and Transgenic Models

In some cases, mutations can cause gene products to become constitutively active. For example, single amino acid mutations (such as A214V or G171V) in the *LRP5* gene, which are found in high-bone-mass human patients, can reduce the ability of the endogenous inhibitor SOST to bind both Lrp5 and Lrp6 and thus increase bone formation caused by mechanical load [4, 5, 34]. Another example is the deletion of exon 3 in the β-catenin gene, causing stabilized β-catenin protein that cannot be phosphorylated, so that β-catenin is constitutively activated and causes profound effects on target tissues (Table 1.3).

As discussed above, transgenic models often contain genes of interest that are driven by an endogenous promoter (by targeted insertion at a selected locus) or an exogenous promoter (inserted into the genome by random or homologous recombination). Although these caveats require some caution in interpreting the resulting phenotypes, the creation of GEMMs remains among the most powerful methods for studying mammalian gene function and regulation, because it can be carried out on the whole organism or in a tissue-specific manner. For example, to understand the novel SOST gene's function in bone, the initial study was performed with a transgenic mouse model with osteoblast-specific expression of mutant SOST (driven by the mouse osteocalcin promoter, OG2) [44], which has been shown to be associated with sclerosteosis [8, 45]. Please refer to Table 1.4 for more examples of transgenic mouse models that manipulate Wnt/β-catenin signaling in the skeleton.

1.6 Detection of Wnt/β-Catenin Signaling Change in Genetically Modified Animals

After a gene linked to regulation of the Wnt pathway has been genetically modified, confirmation that it affects Wnt signaling in target cells is necessary before characterizing the skeletal phenotypes. A reporter mouse strain (BAT-GAL) that expresses β-galactosidase driven by a promoter with multimerized LEF/TCF-binding sites is a sensitive tool to detect Wnt/β-catenin signaling changes. In the presence of activated β-catenin, β-galactosidase expression will be activated and can be detected by its substrate, X-gal [46] (Fig. 1.6). Several other models can detect Wnt signaling

Table 1.3 Skeletal phenotypes in mouse strains with gain-of-function mutations

Gene	Mutations	Cre line (targeted tissue)	Phenotype(s)	Ref.
Lrp5	One amino acid change increases signaling capacity			
	A213V (mimics human Lrp5-A214V)	Germ line	Increased cortical bone mass, BV/TV, Tb.N, and Tb size with a decrease in Tb Sp. Increased mechanical resistance. Endosteal hyperostosis, autosomal dominant (OMIM: 144750)	[34, 90]
	A213V (mimics human Lrp5-A214V)	Dmp1-Cre , Prrx1-Cre, or Villin-Cre (intestine)	Global increases in bone mass with Dmp1-Cre that mimicked global Lrp5-A214V expression. Increased bone mass in limbs only with Prrx1-Cre. No change in bone mass with Villin-Cre. Osteosclerosis (OMIM: 144750)	[34]
	G170V (mimics human Lrp5-G171V)	Germ line	Global increases in cortical bone mass, BV/TV, Tb.N, and trabecular thickness with a decrease in Tb.Sp. Greater enhancement of endosteal bone formation during development and in response to load. Osteopetrosis (OMIM: 607634)	[34, 90]
	G170V (mimics human Lrp5-G171V)	Dmp1-Cre, Prrx1-Cre, (2.3kb) Col1a1-Cre, or Villin-Cre (intestine)	Global increases in bone mass with Dmp1-Cre that mimicked global Lrp5-G171V expression. Increased bone mass in limbs only with Prrx1-Cre. Villin-Cre showed no effect on bone mass in one study (Cui et al., 2011), but resulted in global bone mass enhancement in another (Yadav et al., 2008). Additionally, the latter group showed no enhancement of bone mass when Col1a1-Cre was used to activate Lrp5-G171V in bone. High bone mass (OMIM: 601884)	[31, 34]

(continued)

Table 1.3 (continued)

Gene	Mutations	Cre line (targeted tissue)	Phenotype(s)	Ref.
β-Catenin	Exon 3 deletion causes constitutive β-catenin activation			
		Col2a1-Cre or Dermo1-Cre	Heterozygotes died around E18 with severe and generalized chondrodysplasia. Reduced endochondral bone size	[116]
		Brn4-Cre (neural tube and hind limbs)	Enlarged hind limb buds during development	[105]
		(2.3kb) Col1a1-Cre	Heterozygotes failed to thrive and died shortly after weaning. Skeletal assessments revealed greatly increased bone mass and cartilaginous deposits in long bones and vertebrae. Osteoblast numbers were normal, but Col1a1 expression was increased. Osteoclasts were decreased	[30]
		Prrx1-Cre	Heterozygotes died at birth with loss of limb and the skull bones	[40]
		Osx1-Cre-TetOff (Tetracycline inhibitable)	Heterozygotes died at birth. Shortened long bones with premature ossification and mineralization. Loss of hypertrophic chondrocytes	[107]
		Col2a1-Cre-ERT2 (tamoxifen inducible)	Tamoxifen-induced recombination at 3 or 6 months resulted in a loss of articular cartilage 2 months later	[37]
		Col2a1-Cre-ERT2 (tamoxifen inducible)	Tamoxifen-induced recombination at E13.5 followed by skeletal assessments at several embryonic time points. Disordered chondrocyte zoning. Increased thickness of the perichondrial bone collars in limbs. TRAP staining showed no change in osteoclast activity	[109]
		Axin2-rtTA (Wnt responsive cells) + TRE-Cre [functions as a doxycycline inducible axin2-Cre]	Expansion and increased ossification of cranial sutures. Fully mature osteoblast differentiation was inhibited. No additional skeletal assessments	[117]
		PPARγ-tTA (osteoclast progenitors) + TRE-Cre [functions as a doxycycline inhibitable PPARγ-Cre]	Severe osteopetrosis with a 27-fold increase in the BV/TV ratio. Trabecular bone and cortical bone mass were both increased. Histomorphometry revealed large decreases in osteoclast number and surface area, with no effect on osteoblasts. The osteopetrotic phenotype was already evident in 15-day-old pups. Osteopetrosis could also be induced by removing Dox in adult mice	[42]

Table 1.4 Skeletal phenotypes in transgenic mouse strains

Gene	Transgene	Target tissue	Phenotype(s)	Ref.
Wnt4	R26-Flox-Neo-Wnt4	Col2a1-Cre (osteochondral progenitors)	Dwarfism with increased hypertrophic chondrocytes in growth plates of long bones. Normal BMD	[118]
Wnt5a	Col2a-Wnt5a	Osteochondral progenitors	Shortened skeletal elements and delayed ossification. Increased growth plate cartilage with a large zone of undifferentiated chondrocytes with a low index of proliferation	[62]
Wnt8a	β-Actin-Wnt8a (*Gallus gallus*)	Germ line	Axis duplication during early embryo development	[119]
Wnt9a/14	Col2a-Wnt9a	Osteochondral progenitors	Homozygotes died by 16.5 dpc. Decreased cartilage	[104]
Wnt10b	FABP4-Wnt10b	Adipocytes (adipose tissue and bone marrow)	Mice were viable. Increased Tb.N and decreased Tb.Sp. Increased mechanical resistance. Decreased rate of age-related and ovariectomy-induced bone loss	[65]
Wnt5b	Col2a-Wnt5b	Osteochondral progenitors	Similar phenotype as Col2a-Wnt5a mice, but the undifferentiated chondrocytes were highly proliferative	[62]
Dkk1	Col2a1-Dkk1, Col10a1-Dkk1, or Tie2-Dkk1	Osteochondral progenitors, hypertrophic chondrocytes, or endothelial cells	There was no change in the overall pattern of cartilage or bone development in chondrocyte overexpression of Dkk1 Overexpression in endothelial cells resulted in smaller skeletal elements, abnormal hypertrophic chondrocytes, reduced Tb size but an increase in Tb.N. TRAP staining and osteoclast numbers at the hypertrophic chondrocyte-trabecular bone interface were decreased. The mineral apposition rate was unaffected	[120, 121]

(continued)

Table 1.4 (continued)

Gene	Transgene	Target tissue	Phenotype(s)	Ref.
Dkk1	(2.3kb) Col1a1-Dkk1	Mature osteoblasts	Reduced BMD and trabecular BV. Osteoblast surface area and number were reduced. Osteoclasts were normal	[122]
Dkk2	Col2a1-Dkk2, Col10a1-Dkk2, or Tie2-Dkk2	Osteochondral progenitors, hypertrophic chondrocytes, or endothelial cells	There was no change in the overall pattern of cartilage or bone development in chondrocyte or endothelial overexpression of Dkk2	[120]
SOST	Ocn-SOST	Osteoblasts and osteocytes	Mice were viable but osteopenic with disorganized bone architecture, thin cortices, reduced trabecular bone, and chondrodysplasia. Decreased osteoblast surface and reduced bone formation rate, with no change in resorption markers	[44]
Sfrp4	SAP-Sfrp4	Various	Decreased rate of bone acquisition from 5-15 weeks after birth. Decreased BV/TV and Tb size	[123]
Sfrp4	Transgene (2.3kb) Col1a1-Sfrp4	Mature osteoblasts	Similar phenotype as the SAP-Sfrp4 transgenic mice	[124]
Tcf1 (Tcf7)	Col2a1-Tcf7 Δβ-catenin-binding domain	Osteochondral progenitors	Reduced skeletal element size, endochondral ossification, chondrocyte maturation, and proliferation	[125]
Lef1	(2.3kb) Col1a1-Lef1ΔN isoform	Mature osteoblasts	Increased trabecular BV and osteoblast activity, but no change in osteoblast number. Osteoclasts were normal	[126]
Sfrp4	(2.3kb) Col1a1-Sfrp4	Mature osteoblasts	Osteoblast-targeted expression of Sfrp4 in mice results in low bone mass	[124]

activity in cells and have been widely used with great success. In addition, immunohistochemical analysis of β-catenin or downstream targets is also routinely performed to detect the signaling changes [47](Fig. 1.6).

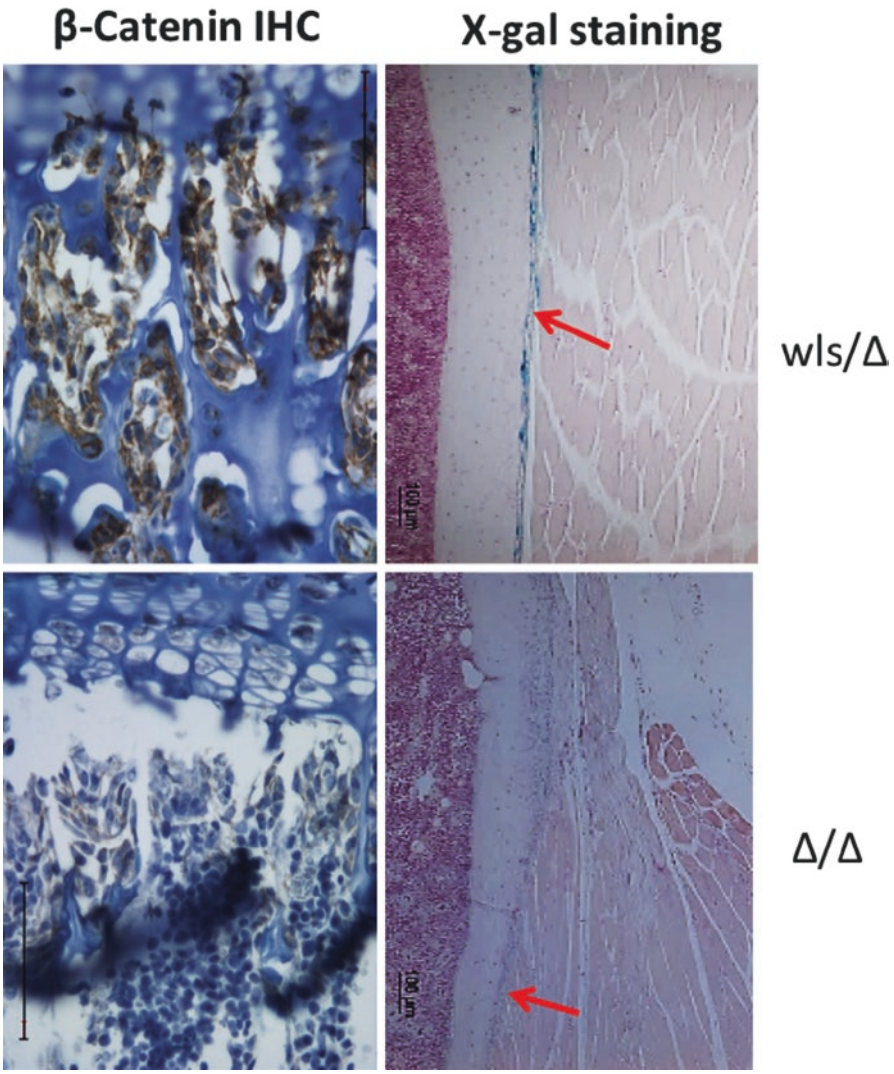

Fig. 1.6 Wnt/β-catenin signaling detection. An example of a conditional mouse model with osteoblast-specific Wntless (wls) knockout is shown. Anti-β-catenin IHC on distal femur is shown to indicate lower β-catenin in the mutant trabecular bone. BAT-GAL transgenic mice (also called β-catenin/TCF/LEF reporter transgenic mice) can express β-galactosidase in the presence of activated β-catenin, so X-gal staining (blue) can identify those cells with activated β-catenin signaling

1.7 Conclusion

A “pipeline” project that aims to knock out every gene in the genome individually has predicted that about 10% of all genes can affect bone strength in one way or another [48]. Further evidence for this assessment was recently provided by Lexicon Genetics, which reported the results of their efforts to screen large numbers of GEMMs to identify new regulators of skeletal development and homeostasis. The fact that the Notum gene, recently linked to direct regulation of the Wnt pathway in other systems [49, 50], was identified as a novel, targetable regulator of bone homeostasis speaks to the power of these types of approaches [51].

We hope that this review has provided a convincing argument for the critical role that GEMMs have played in our understanding of how Wnt signaling regulates skeletal development and homeostasis. While the last three decades of using GEMMs have led to many exciting discoveries, we predict that these insights will grow exponentially in the near future. The recent demonstration that CRISPR/Cas9 technology can quickly and efficiently generate mouse models in weeks that used to take months (if not years) to create will expedite our ability to study gene functions in laboratory animals [52]. We expect to see the technical feasibility of faster generation of genetically modified animals and more studies on gene interactions by simultaneously knocking out multiple genes. It is clearly an exciting time to be contributing to scientific knowledge via the use of GEMMS. Perhaps even more important is the fact that CRISPR/Cas9 (and related [53]) technology will allow the rapid genetic manipulation of many other model systems that will advance our knowledge of normal development and disease. Provided that this powerful system is handled in an ethical manner [54], it is likely to revolutionize methods to gain biological insights. In fact, it already has done so.

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