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Contextual Regulation of Skeletal Physiology by Notch Signaling

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Abstract

Purpose of Review This article reviews the past 2 years of research on Notch signaling as it relates to bone physiology, with the goal of reconciling seemingly discrepant findings and identifying fruitful areas of potential future research.

Recent Findings Conditional animal models and high-throughput omics have contributed to a greater understanding of the context-dependent role of Notch signaling in bone. However, significant gaps remain in our understanding of how spatiotemporal context and epigenetic state dictate downstream Notch phenotypes.

Summary Biphasic activation of Notch signaling orchestrates progression of mesenchymal progenitor cells through the osteoblast lineage, but there is a limited understanding of ligand- and receptor-specific functions. Paracrine Notch signaling through non-osteoblastic cell types contributes additional layers of complexity, and we anticipate impactful future work related to the integration of these cell types and signaling mechanisms.

Keywords Notch signaling · Jagged-1 · Bone biology · Musculoskeletal metabolism · Mesenchymal progenitor cells · Osteoblasts

Introduction

Low bone mass and bone trauma impose major public health burdens worldwide, and there is a significant interest in identifying druggable pathways implicated in bone metabolism. Notch, a signaling pathway that regulates a diverse set of developmental functions in metazoa, is one such pathway. However, the function of Notch signaling in bone remains controversial and incompletely understood, in part due to its high degree of temporal and contextual specificity.

In October 1918, Otto L. Mohr discovered "serrated or notched" wing boundary mutations in *Drosophila melanogaster*, caused by a novel hemi/homozygous-lethal mutation in what we now know as the Notch pathway [1]. One hundred years later, Notch signaling remains an alluring subject of evolutionary biology and has been extensively reviewed in the contexts of vertebrate development [2], signal transduction [3], and skeletal disease [4]. In mammals, the Notch family consists of five ligands (Jagged-1 and Jagged-2, Delta-like 1, 3, and 4) and four single-pass transmembrane receptors (Notch1–4). Active binding between ligand and receptor in trans results in proteolytic cleavage of the Notch receptor orchestrated by the γ-secretase complex (with catalytic subunits presenilin-1/2), liberating the intracellular domain (NICD) [5, 6]. The NICD then undergoes nuclear translocation and, together with coactivator Maml1–3 (dominant negative mastermind-like 1–3), activates the otherwise inhibitory Rbpj (recombining binding protein suppressor of hairless), also known as CSL, to induce transcription of canonical Notch target genes [7, 8]. Classically, these genes include the Hes and Hey families of bHLH transcription factors, named from their homology to *Drosophila* hairy and enhancer-of-split [9]. As with many tissues, Notch signaling is indispensible in proper patterning of the skeleton [10].

The purpose of this current review is to summarize advances in our understanding of Notch signaling in skeletal physiology that have occurred over the past 2 years. A PubMed literature search for ("Notch signaling OR Jagged-1") AND ("bone OR osteoblast") between October 2016 and October 2018 returned > 200 results, which were sorted for inclusion by content and

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supplemented with earlier foundational articles. Notch signaling is highly context-dependent, and experimental findings across sexes, anatomical sites, and even bone compartments are difficult to broadly generalize. This article highlights recent additions to our understanding of Notch signaling in bone cells, categorized by cell type.

Skeletal Morphogenesis

The translucent zebrafish embryo is a powerful model of early skeletal development. In these animals, cranial neural crest cells within the mandibular and hyoid arches develop into the pharyngeal cartilages of the lower face [11]. In this context, Notch signaling partitions the uppermost domain of the dorsoventral axis, ultimately specifying the formation of dorsal arch-derived structures including the palatoquadrate. Jag1b (Jagged-1b)-to-Notch2/3 signaling is required for normal development [12]; Jag1b$^{-/-}$ mutants exhibit dorsal mandibular and hyoid cartilage defects and die by 7dpf, although other facial structures develop normally [13]. Jag1b$^{-/-}$ mutants also experience reduced dorsal expression of CD248a (endosialin) and Pou3f3a/b (POU class 3 homeobox 3 a/b), leading to dorsal arch malformation while ventral/intermediate arch gene expression patterns are unaffected [14•]. One function of notch signaling in this dorsal arch is to regionally antagonize the Bmp (bone morphogenetic protein)/Edn1 (endothelin 1) signaling of the ventral/intermediate domains, maintaining the dorsal arches as undifferentiated progenitor cells and delineating a gradient of ventral chondrogenesis that patterns the earliest facial bone structures [12]. Antagonistic Notch/Edn1 signaling in zebrafish pharyngeal arch development is conserved in mice [15].

Constituting a regional positive feedback loop, dorsal Jag1b/Hey1/Grem2 (gremlin 2) signaling is actively excluded from the ventral arches [16]. Misexpression of human Jag1 in zebrafish results in dorsalization of the ventral/intermediate arch domains [13], indicating that Notch is not only necessary, but sufficient for dorsal arch fate determination [12]. Jag1/2-to-Notch1/2/3 signaling is also observed in arch and jaw development among Lake Malawi cichlids [17]. More is currently understood about how Notch signaling restricts early ventral chondrogenesis than how it drives dorsal domain-specific skeletogenesis, though downstream modulation of chondrogenic transcription factors, including Fox proteins, are one potential mechanism [18]. The increasing use of conditional transgenesis in zebrafish may help elucidate the role of Notch signaling in later perichondral and endochondral ossification.

Dysregulation of Notch signaling results in significant limb/fin morphogenic defects [19], due to the pathway's roles both in regulating progenitor cell populations and in the ossification of early cartilage templates. In mice, Notch signaling maintains a proliferative mesenchymal progenitor cell (MPC) phenotype in the developing limbs [20]. Prx1^{+} (paired related homeobox 1) lineage (MPC-targeted) Notch-deficient mice experience an initial pulse of osteoblastogenesis and bone formation, followed by progressive bone loss due to depletion of the MPC pool [21]. However, during zebrafish notochord epithelial sheath segmentation, Notch signaling encodes the transition of Col9a2^{+} (collagen type IX alpha 2) cartilaginous to Entpd5a^{+} (ectonucleoside triphosphate diphosphohydrolase 5a) mineralizing domains in the spine, recruiting early osteoblasts from the paraxial mesenchyme and activating osteoblast transcriptional programs that will form the vertebral bodies [22]. These apparently conflicting roles of Notch signaling in bone formation will remain a consistent and controversial theme throughout this review.

Osteochondral Progenitor Cells

During development, two discrete phases of Notch signaling activity regulate the lineage progression of osteoblastic cells: a pattern that is broadly mirrored in adulthood [23]. The first phase promotes stemness. For example, Notch signaling is correlated with maintenance of cranial suture patency [24], and downregulation of Notch signaling is widely believed to be necessary for initial entrance of MPCs into the osteoblast lineage [25] as well as other lineages [26–28], with significant input from parallel signaling pathways. This initial wave of Notch is then turned off during differentiation: a mechanistic switch that remains the subject ongoing investigation.

As a niche factor, Notch contributes to MPC resilience. Notch-1 intracellular domain (N1ICD) expression was found to protect bone marrow MPCs from cytotoxicity of cigarette smoke extract, promoting proliferation and Cxcr4 (C-X-C motif chemokine receptor 4) expression by activation of the Pi3k (phosphoinositide 3-kinase) cell cycle regulatory pathway [29]. Outside of classical signaling pathways, the response of MPCs to osteogenic stimuli is partially regulated by non-coding RNAs. A concentration-dependent, biphasic activation of lncRNA H19 is one mechanism by which positive feedback of Notch and its associated miRNAs downstream of BMP can drive both early proliferation and late differentiation of osteoblast-lineage cells [30].

Parathyroid hormone (PTH) has long been known for its role in bone homeostasis, and some of this function is due to downstream Notch activity. Transient inhibition of Rbpj binding induced by PTH in the osteoblast lineage may cause entry of MPCs into osteoblasts by switching off Notch [31]. Supporting this, conditional deletion of Jag1 in (Prx1-Cre) MPCs in mice results in premature passage of osteoprogenitor cells into anabolic osteoblasts, increasing femoral and tibial trabecular bone volume fraction and mineral apposition rate, and enhancing bone formation caused by treatment with parathyroid hormone [32•]. Prx1-Cre;Notch2$^{f/f}$ mice have a similar phenotype. Jag1-Notch2 signaling suppresses glycolysis,

and deletion of Notch2 in MPCs triggers metabolic output leading to excessing passage into osteoblasts [33].

There are numerous reports implicating Notch overactivation in osteosarcoma [34] and Notch is linked to malignant features of cancer stem cells [35] including proliferation and migration [36]. Non-coding RNAs regulating Notch are the subject of numerous ongoing investigations as therapeutics [37, 38] or prognostic biomarkers [39]. Jag1 stimulates metastasis, and inhibitory antibody therapy reduces breast cancer metastasis to bone [40]. While many features of Notch signaling may be conserved between healthy and neoplastic stem cells, including its biphasic activation during in vitro osteogenic differentiation of human MG63 osteosarcoma cells [41], the relevance of cancer stem cell physiology to normal bone metabolism is unclear.

Interpretation of the literature can be difficult due to variance in protocols and in the relative differentiation state cells, particularly in vitro, as well as the reliance on Cre drivers that may lack specificity. Notch signaling has distinct phenotypes even across different compartments of the same bone [42], making this area of inquiry especially sensitive to confounding variables.

Osteoblasts

Switching off Notch signaling appears to be necessary, but not sufficient, for entry into the earliest pre-osteoblast lineages. Forty-eight hours of treatment with the γ-secretase inhibitor DAPT abrogates expression of neural crest stemness markers (c-Myc, Sox2, Oct4a, Nanog, etc.) in dental pulp MPCs, yet these cells perform poorly during in vitro osteogenesis assays, in part due to parallel downregulation of the Wnt pathway [43]. Wnt likely participates as a contextual regulator in the transition from pre-osteoblast to mature osteoblast [44] and is downregulated in later stages [45•]. Liver-derived MPCs downregulate Notch receptors after the first few days of osteogenic conditioning [46]. After passing a presently undefined checkpoint, Notch switches to perform a stimulatory function in osteoblast maturation.

Cultured bone marrow MPCs from transgenic Notch reporter mice suggest that Notch signaling is not active during early osteoblast cell commitment, but reactivates during late stages of mineralization, and that pulsatile activation of Notch can enhance its osteoanabolic function [47]. It has been repeatedly shown that Jag1 stimulates maturation and mineralization in pre-osteoblast cultures in vitro [48, 49]. Encapsulated in PEG (polyethylene glycol maleimide) hydrogels, human embryonic palatal MPCs exposed to bound Jag1 undergo a 50-fold upregulation of the Notch downstream target gene Hes1 expression preceding the induction of a dose-dependent late-stage osteoblastic gene expression profile (defined as low expression of the early osteoblastic marker gene Alp (alkaline phosphatase) and high expression of the late osteoblastic marker gene Bsp (bone sialoprotein)) [50]. Notch signaling is activated by hypoxia-induced osteogenesis in mouse embryonic stem cells [51], and transplanted rat MPCs overexpressing Hif-α (hypoxia-inducible factor 2-alpha) promote Notch signaling and angiogenesis in adjacent tissue [52]. Notch1/Hey2 downregulation by nicotine reduces proliferation and mineralization of primary rat parietal osteoblasts, arresting lineage progression in immature (defined as high Alp, low Bsp) stages [53]. Conversely, shRNA silencing of Notch3 promotes osteogenesis in primary rat marrow MPCs [54].

Notch signaling is necessary but not sufficient for osteogenesis through the Bmp (bone morphogenetic protein) pathway [55]. Bmp9-induced osteogenesis fails when Notch signaling is abrogated by γ-secretase inhibitor (GSI) Compound E, dominant-negative Notch1 or Presenilin1/2-DKO in mouse embryonic fibroblasts, while ectopic Jag1 enhances osteogenesis [56•]. This phenotype is, at least in part, believed to be due to the role of Notch in mediating the proliferative MPC phenotype of Bmp treatment. Downstream of Bmp/Smad signaling, Notch also functions in a positive feedback loop to amplify osteoinductive stimuli: including by upregulation of the Bmp receptor Alk2 (activin receptor-like kinase-2) [57]. Furthermore, there is evidence of γ-secretase-independent Notch activation by Bmps in certain contexts, which may play a role in epithelial-mesenchymal transition of tumors outside of bone [58]. As a reoccurring trend, the influence of Notch downstream of Bmp is dependent on the differentiation state of the cell, with disparate evidence in the literature [59].

Paradoxically, in other contexts, different ligands [60] or receptors [61] have been shown to have opposite effects. Osteoblast-targeted Col1a1(2.3 kb)-Cre (targeting the 2.3 kb upstream promoter of collagen, type I, alpha 1) Dll1 (delta-like 1)-overexpressing mice develop profound sclerosis of trabecular bone that was not reported in matched Jag1-overexpressing mice [62]. Significant work remains before we understand the ligand- and receptor-specific dynamics of Notch signaling in all bone lineages. It is unclear if differences in receptor expression in intramembranous versus endochondral ossification represent distinct Notch activation pathways in common progenitor cells, or differences in basal receptor expression across different cell types [63]. Therefore, while some aspect of ligand/receptor-specific biology likely plays a role in osteoblast differentiation, this review focuses on Jagged-class-mediated receptor activity as "Notch signaling."

Osteoblast-lineage phenotypes result from certain congenital Notch defects [42, 64] as well as from drug treatment. There is a case report of Notch1 gene duplication inducing precocious puberty including a stark acceleration of bone growth [65]. Suppression of Notch target gene transcription may be a mechanism by which glucocorticoids induce bone resorption in addition to their pro-osteoclastic role in upregulation of Rankl [66], though interestingly, glucocorticoids

increase Notch signaling in muscle, leading to atrophy [67]. In a model of osteomyelitis, mice infected with *Staphylococcus aureus* exhibited decreased levels of bone Jag1/Notch1/Hes1 concurrent with bone loss: a phenotype which could be largely rescued with shRNA knockdown of the inflammatory glycoprotein Chi3l1 (chitinase-3-like protein 1) [68].

Osteocytes

Notch is also believed to promote osteocyte differentiation. The early osteocyte marker E11 (podoplanin) is a transcriptional target of Hes1 [45•], though the receptor profile and transcriptional landscape changes upon transition to osteocyte [69]. Hes1-dependent deactivation of Wnt signaling is necessary for maturation of IDG-SW3 (osteocytic) cells but not MC3T3 (osteoblast) cells in vitro [45•].

Mouse calvarial osteocytes show consistent Notch activity in adult transgenic Notch reporter mice [47]. Conditional N1ICD activation in either (Osx (osterix/SP7)-Cre) osteoblasts or (Dmp1 (dentin matrix acidic phosphoprotein 1)-Cre) osteocytes (and their progenies) results in a greater than twofold increase in femoral and vertebral trabecular bone volume fraction in 3-month-old mice [70]. This does not occur due to regulation of osteocyte number nor is there an osteocyte phenotype with conditional deletion of Notch1/2 using these Cres [71]. The downstream anabolic activity of N1ICD in the trabecular compartment, as well as the accompanying trabeculation of cortical bone, is believed to occur largely via abnormal osteocyte-mediated signaling to differentiating osteoclasts by Opg (osteoprotegerin) and Wnt [70]. As one mechanism, N1ICD overactivation in the Dmp1+ lineage induces Rbpj-dependent canonical Notch signaling resulting in downregulation of Sost (sclerostin) and Dkk1 (dickkopf Wnt signaling pathway inhibitor 1) and upregulation of Wnt signaling [72]. However, activity of Dmp1-Cre earlier in the osteoblast lineage than intended may confound these phenotypes [73], and the lack of inducible Cres precludes separation of developmental/formation/remodeling features. Nevertheless, this has been corroborated in tissue culture: intermittent compressive stress promotes Jag1/Notch signaling in differentiating MC3T3-E1 cells [74], with downstream suppression of Sost expression that only occurs under cyclic loading. Thus, Notch signaling in osteocytes serves as a mechanoresponsive mechanism for altering bone metabolism by modulation of osteoblast and osteoclast activity.

Osteoclasts

Premature truncation of the C-terminal PEST domain of Notch2 causes the Notch-activating Hajdu-Cheney syndrome (HCS) [75]. Notch2 receptor turnover is normally regulated by Fbw7 (F-box and WD repeat domain-containing 7)/Gsk3 (glycogen synthase kinase 3) degradation; conditional deletion of Fbw7 in osteoclasts (Ctsk-Cre) phenocopies HCS osteoporosis by pathological osteoclastogenesis [76]. The widespread osteopenia in HCS mice is caused by upregulation of osteoclast activity by osteoblasts, despite the increase in osteoblast number [77•]. This has been confirmed in HCS conditional mice [78]. αNotch2 inhibitory antibody rescues the hyperosteoclastic phenotype of HCS mice [79].

Similar to the mesenchymal/osteoblast lineage, Notch signaling can be either stimulatory or inhibitory in the osteoclast lineage [80]. Exposure to Rankl may mark a switch, after which Notch signaling stimulates osteoclast maturation [81]. Both direct Jag1 stimulation and conditioned medium from Jag1-treated periodontal ligament cells promotes osteoclast maturation in RAW 267.4 transformed monocytes [82]. Dll1 misexpression by osteoblasts impairs physiological Rankl expression, arresting early osteoclast specification and exacerbating the anabolic phenotype [62].

Hematopoiesis and Immunity

Notch signaling is one method by which MPCs signal to the hematopoietic lineage to orchestrate innate and adaptive immunity. Specifically, Jag1 is expressed by a Pdgfrα (platelet-derived growth factor receptor alpha)$^{+}$/Lepr (leptin receptor)$^{+}$ subpopulation of MPCs supporting hematopoiesis in bone marrow [83]. This Jag1 activates Notch2 in CD34^{+}/CD38 (cyclic ADP ribose hydrolase)$^{-}$ hematopoietic stem cells and serves as a protective niche factor against radiation damage by anti-apoptotic Bcl2 (B cell lymphoma 2) and inhibition of p63 (transformation-related protein 63) [84]. Osteoblast-derived Notch signaling to hematopoetic stem cells is distinct from a cell-autonomous function, as demonstrated in Gipr (gastric inhibitory polypeptide receptor)-deficient mice [85]. Notch signaling does not directly regulate self-renewal of hematopoietic stem cells [86], but primary MPC-derived osteoblasts facilitate the continuous expansion of these cells in tissue culture via Notch [87]. Conversely, diseases of hematopoiesis can produce phenotypes in the osteoblast lineage: including increased Jag1 and decreased osteoprogenitor cell number and differentiation capacity in cells from patients with myelodysplastic syndrome [88].

Disorders of hematopoietic stem cells and their niche overlap with disorders of MPCs. For example, Notch signaling is upregulated in bone marrow MPCs derived from multiple myeloma patients. MPCs and multiple myeloma cells activate Notch via paracrine Jag2/Notch2 signaling [89], with additional crosstalk with endothelial cells [90]. These MPCs undergo impaired in vitro osteogenesis, which can be rescued by DAPT, Notch1-siRNA, or the multiple myeloma drug lenalidomide, which inhibits Notch [91]. MPC-derived Notch also likely serves an immunomodulatory function by suppressing maturation of dendritic cells. In the myeloid lineage, the transcription factor Irf8 (interferon regulatory factor

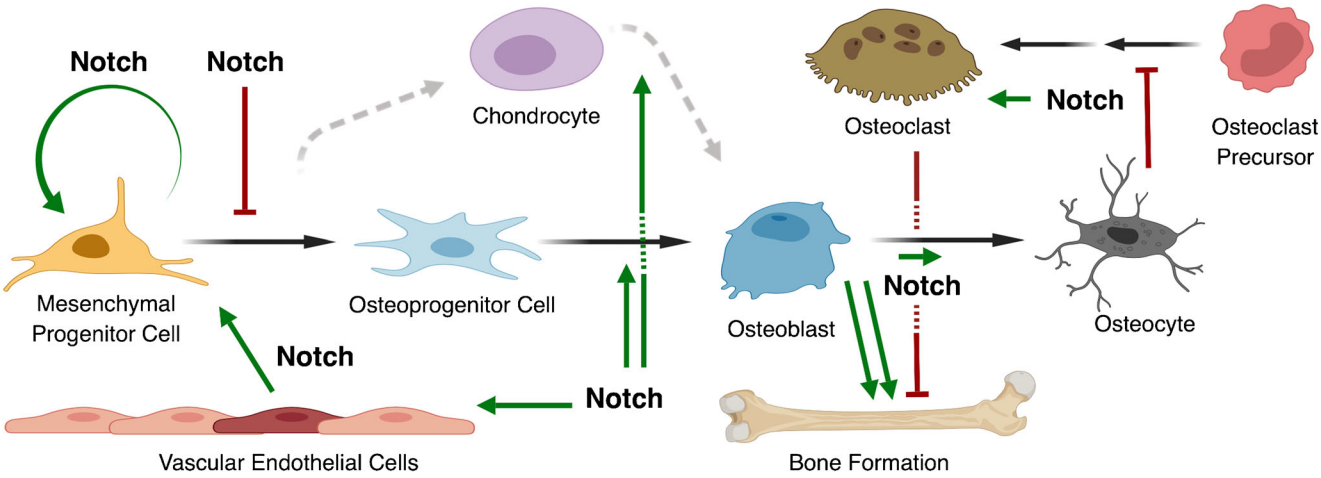

Fig. 1 Notch signaling orchestrates discrete phases of osteoblast lineage progression during bone development and regeneration. A putative molecular switch early in the differentiation of committed bone progenitor cells demarcates the proliferative versus differentiative phenotypes of Notch activity. There is crosstalk between osteoblast-lineage cells and cells of endothelial and hematopoetic origin. The basic biology underlying this highly contextual regulation, as well as specific ligand–receptor interactions, may prove fruitful targets of ongoing investigation

8), which is essential to differentiation into monocytes, is suppressed by Rbpj-dependent Notch signaling [92]. Reduced Jag1 in MPCs derived from patients with autoimmune thrombocytopenia may lead to impaired regulatory dendritic cell differentiation, contributing to the disease phenotype [93].

Endothelial Cells

CD31 (platelet endothelial cell adhesion molecule)$^+$/Emcn (endomucin)$^+$ (type-H capillary) vascular endothelial cells serve as a niche for Osx$^+$ osteoprogenitor cells [94]. While these cells decrease in number with aging, Cdh5 (VE-cadherin)-Cre^{ERT2} conditional N1ICD overexpression promotes neovascularization and bone formation in aged mice [95]. Thus, the osteoanabolic function of Jagged-mediated Notch signaling is partially orchestrated by interactions with endothelial niche cells. Notch1 knockdown in MPCs impairs angiogenesis in co-cultured human umbilical vein endothelial cells [96], and human transgenic Jag1 (but not Dll1) mouse HESS-5 MPCs stimulate angiogenesis of human cord blood cells [97]. Dietary phosphate-restricted mice exhibited downregulated Notch signaling and decreased tibial marrow vascular volume fraction [98]. Notch signaling functions downstream of Pdgf in co-cultured human MPCs and endothelial cells to enhance measures of angiogenesis [99].

Fracture Healing

Notch signaling is active during the proliferative phase of early fracture healing [63, 100]. Lineage tracing and microarray analysis of αSMA (alpha smooth muscle actin)$^+$ periosteal progenitor cells following mouse tibial fracture revealed downregulation of Notch signaling at 2–6 days post-fracture, during which time MPC markers Sca1 and Pdgfrα/β are upregulated alongside induction of an osteochondral gene program [101]. A protein–protein interaction network constructed from this microarray revealed Notch1 interactions with Bmp4 and Vegf (vascular endothelial growth factor) receptor Flt1 (VEGFR1) [102]. Inducible systemic blockade of Notch by dnMaml (dominant negative Maml1) using the interferon-inducible Mx1-Cre supports the notion that canonical Notch is dispensable for early callus formation but is required for sustained callus proliferation [100], as well as normal remodeling by both osteoblasts and osteoclasts [103].

In intramembranous healing, Jag1 deletion in αSMA$^+$ cells impairs bone formation, and in delivery of recombinant Jag1 protein improves bone healing in several rodent injury models [104•]: whether this occurs primarily through regulation of the osteoblast lineage or via alterations of neovascularization is unknown. In endochondral healing following long bone fracture, Notch signaling reactivates during hard callus formation, marking a subpopulation of late-stage chondrocytes at the chondro-osseous junction [63] that may participate in endochondral transdifferentiation [105] adjacent to the invading vasculature [106]. Interestingly, Notch1/Hes1 signaling is active in osteoarthritic chondrocytes in mice, suppressing Hedgehog signaling and promoting hypertrophy and osteophyte formation [107].

Notch activation and Notch inhibition are both being investigated as fracture therapies. Transient DAPT treatment promotes both osteoblast and osteoclast differentiation during the first few days after fracture, with the greatest benefit to bone strength from pulsed treatment at day 2 [108]. Sustained transgenic N1ICD decreases mineralization potential of

transplanted αSMA^{+} cells in an ectopic bone model [101]. Interestingly, Prx1-Cre;Rbpjκ$^{f/f}$ mice but not their Col1a1(2.3 kb)-Cre or Acan (aggrecan)-Cre counterparts experience sustained fracture non-union [109]. Blocking Dll4/Notch1-dominant Jag-low signaling by DAPT promotes angiogenesis and bone formation in a rat Masquelet technique model [110]. Significant work remains to increase the spatiotemporal specificity of genetic mouse models and to isolate variables, particularly in complex fracture models that combine all of the described cell types in proximity.

Conclusions

Jagged/Notch signaling serves as a critical regulator of bone metabolism. Our current understanding of this versatile pathway suggests that (a) Notch positively regulates mesenchymal progenitor cell expansion and inhibits passage into the earliest osteochondral fates and (b) activation of Notch signaling in committed osteoblasts is anabolic: promoting maturation and mineralization. These conclusions are summarized in Fig. 1. Notch signaling within the osteochondral lineage has far-reaching consequences beyond skeletal development and regeneration, with paracrine Notch signaling regulating crosstalk between bone cells and endothelial, hematopoetic, and immune cells. Considerable work remains before we thoroughly understand the role of Notch signaling in the osteochondral lineage, as well as its interrelationships with other tissues in health and disease.

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Compliance with Ethical Standards

Conflict of Interest Kurt Hankenson reports he was co-founder of Skelegen LLC, and has received a research grant from Orthofix. Both are outside the submitted work.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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