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REVIEW

Signaling network regulating osteogenesis in mesenchymal stem cells

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Abstract

Osteogenesis is an important developmental event that results in bone formation. Bone forming cells or osteoblasts develop from mesenchymal stem cells (MSCs) through a highly controlled process regulated by several signaling pathways. The osteogenic lineage commitment of MSCs is controlled by cell–cell interactions, paracrine factors, mechanical signals, hormones, and cytokines present in their niche, which activate a plethora of signaling molecules belonging to bone morphogenetic proteins, Wnt, Hedgehog, and Notch signaling. These signaling pathways individually as well as in coordination with other signaling molecules, regulate the osteogenic lineage commitment of MSCs by activating several osteo-lineage specific transcription factors. Here, we discuss the key signaling pathways that regulate osteogenic differentiation of MSCs and the cross-talk between them during osteogenic differentiation. We also discuss how these signaling pathways can be modified for therapy for bone repair and regeneration.

Keywords Osteogenesis · Stem cells · Bone regeneration · BMP signaling · Wnt signaling · Hedgehog signaling · Nell signaling · Mechanotransduction

Abbreviations

AD-MSCs	Adipose tissue-derived mesenchymal stem cells
ALP	Alkaline phosphatase
ATF4	Activating transcription factor 4
BM-MSCs	Bone marrow-derived MSCs
BMP	Bone morphogenetic protein
BMPR	BMP receptor
BSP	Bone sialoprotein
Cbfa1	Core binding factor 1
Cntnap4	Contactin associated protein family member 4
Col1	Collagen type 1
Dkk1	Dickkopf 1
Dlk1	Delta homolog-like 1
Dll1/2/3	Delta-like 1/2/3
Dlk5	Distal-less homeobox 5
DNER	Delta/Notch-like EGF-related receptor
ERK1/2	Extracellular-signal regulated kinase1/2
FAK	Focal adhesion kinase
FGF	Fibroblast growth factor
FOXO	Forkhead box type O
Fzd	Frizzled
GSK3β	Glycogen synthase kinase-3 β
Hey	HES-related with a YRPF motif
Ihh	Indian hedgehog
Jag1	Jagged 1
JNK	Jun amino-terminal kinase
LRP	Low-density lipoprotein receptor-related protein
MAPK	Mitogen activated protein kinase
MSCs	Mesenchymal stem cells
NELL-1	Neural epidermal growth factor-like 1 protein
NICD	Notch intracellular domain
Opn	Osteopontin
Ocn	Osteocalcin
Osx	Osterix
PCP	Planar cell polarity
PPARγ	Peroxisome proliferator-activated receptor gamma
PTH	Parathyroid hormone
PTHrP	Parathyroid hormone-related protein
Rbpjκ	Recombination signal binding protein for immunoglobulin kappa J region
rhBMP	Recombinant human BMP

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ROCK	Rho associated protein kinase
Runx2	Runt-related transcription factor 2
sFRP	Secreted frizzled-related protein
Shh	Sonic hedgehog
SMO	Smoothened
SOST	Sclerostin
Sox9	SRY box transcription factor 9
TGFβ	Transforming growth factor β
WIF1	Wnt-inhibitory factor 1
YAP/TAZ	Yes-associated protein/transcriptional coactivator with PDZ-biding motif

Introduction

Osteoblasts develop from the MSCs through a process called osteogenesis. MSCs, give rise to osteo-chondro progenitors, which develop into osteo-precursors upon Runx2 (Runt-related transcription factor 2) activation or chondrocytes if Sox9 (SRY box transcription factor 9) is activated (Martinez et al. 2016). Pre-osteoblasts, the osteogenic precursors, proliferate further and mature to form osteoblasts and, finally, osteocytes (Maes et al. 2010). MSCs, isolated from the bone marrow and other tissue sources, can differentiate into osteoblasts, adipocytes, and chondrocytes (Pittenger et al. 1999). Osteogenic differentiation ability was observed in MSCs isolated from several tissue sources such as bone marrow (Shima et al. 2015), adipose tissue (Si et al. 2019), the dental pulp (Kawashima et al. 2017), placenta (Ulrich et al. 2015), umbilical cord blood (Rebelatto et al. 2008), extraocular muscle (Mawrie et al. 2016) and ocular adipose tissue (Mawrie et al. 2019). However, bone marrow MSCs (BM-MSCs) possess epigenetic memory for osteo-lineage and have high osteogenic differentiation ability compared to MSCs from other tissue sources (Xu et al. 2017), whereas adipose tissue-derived MSCs (AD-MSCs) do not show the donor-dependent change in characteristics (Beane et al. 2014).

Osteogenesis is controlled by several signaling pathways such as bone morphogenetic proteins (BMPs), Notch, Hedgehog, neural epidermal growth factor-like 1 protein (NELL-1), and Wnt/β-catenin signaling (Fig. 1). Activation of transcription factors Runx2, Osterix (Osx), distal-less homeobox 5 (Dlx5), TWIST, Msh homeobox 2 (Msx-2), activating transcription factor 4 (ATF4) and forkhead box type O (FOXO) members through interaction with various signaling pathways induce osteogenic lineage commitment and differentiation of MSCs. Runx2, the master regulator of osteogenesis, has an important role in early osteogenic lineage commitment, and induce Hedgehog and Wnt signaling molecules for further maturation (Qin et al. 2018). When core binding factor 1 (Cbfa1), the mouse homolog of Runx2 was knocked out, it abrogated ossification due to maturational arrest in osteoblasts (Komori et al. 1997). Cbfa1 null mice has delayed osteogenesis while heterozygotes show skeletal abnormalities and hypoplasia similar to that seen during cleidocranial dysplasia (CCD) (Otto et al. 1997). When Osf2/Cbfa1 was expressed in stem cells, it induces pre-osteoblast development even in non-osteoblastic cells (Ducy et al. 1997). However, when Cbfa1 was forcibly expressed in late stage of osteogenesis, it resulted in failed osteoblast maturation and osteopenia (Liu et al.

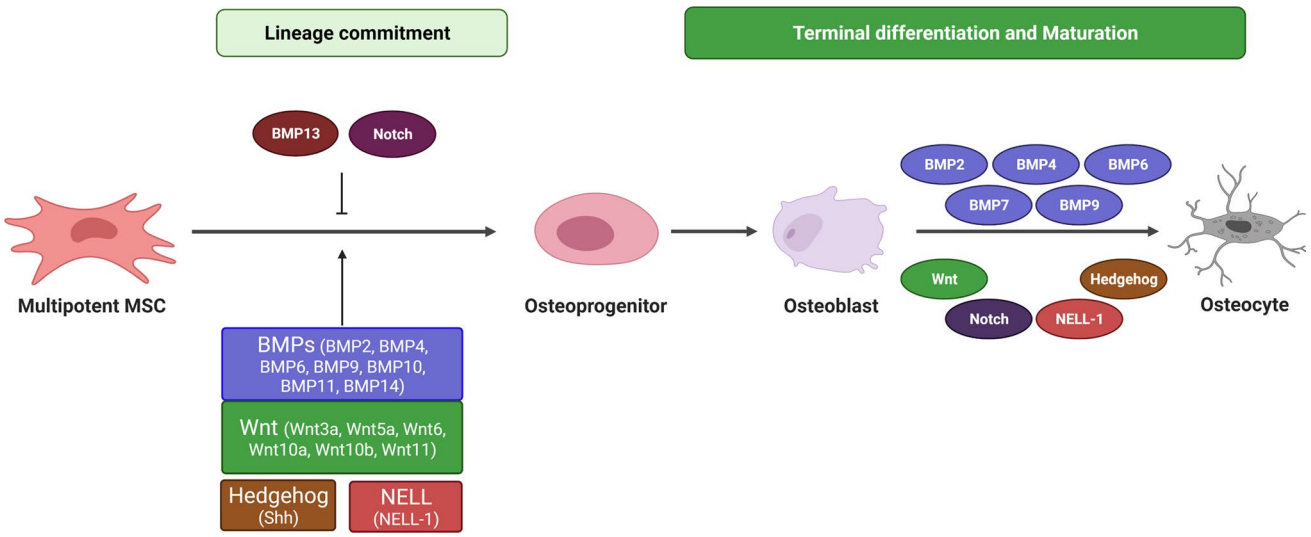

Fig. 1 Osteogenic lineage commitment of MSCs. The initial commitment of pluripotent MSC into osteogenic fate is induced by BMP, Wnt, Hedgehog and Nell-1 signaling pathways. While many BMP ligands promote osteogenesis of MSCs, BMP13 inhibits osteogenic differentiation of BM-MSCs by inhibiting calcium mineralization and alkaline phosphatase (ALP) activity. Notch plays an inhibitory role during initial commitment into osteogenic lineage, whereas it promotes the terminal differentiation of osteoblasts

2001). Another transcription factor Osterix (Osx), also inhibits osteoblast maturation at later stages of differentiation, although it is necessary for maintaining bone homeostasis, craniofacial bone development, and spine formation (Chen et al. 2014; Li Wang and Fei 2015). Further, MSCs from Osx null mice does not mineralize the cartilage matrix or form mature bone, and Osx acts downstream of Runx2 (Nakashima et al. 2002). In this review, we discuss the various signaling pathways that modulate osteogenesis and osteogenic differentiation of MSCs, secondly, how the signaling pathways cross-talk to induce osteogenesis and finally, the modulation of signaling pathways to enhance osteogenesis for therapeutic purposes.

BMP signaling

BMPs are members of the transforming growth factor- β (TGF-β) superfamily, secreted by osteoblasts and other cells (Wang et al. 2014). BMP signaling is initiated by ligand binding to the heterodimeric BMP receptors (BMPRs) (Nickel and Mueller 2019) formed of BMPR1 and BMPR2 (Nickel and Mueller 2019). BMP receptors can signal through either canonical Smad-dependent or non-canonical Smad independent pathways (Derynck & Zhang, 2003). BMP molecules function in autocrine, paracrine, and endocrine manner, and to date, more than 15 different types of BMP molecules have been discovered in mammals with distinct and overlapping functions. Mutation in BMP-2 causes Brachydactyly type A2 characterized by bone shortening, also seen in BMPR1B mutants (Lehmann et al. 2003; Liu et al. 2014). Smad4 mutation leads to Myhre syndrome, which causes short stature and facial dysmorphism (Le Goff et al. 2011). Mutations in BMPR1 receptor results in osseous deformation and fibro dysplasia ossificans progressiva (FOP) (Shore et al. 2006).

Several studies have investigated the effect of exogenous addition to BMPs in regulating osteogenesis of MSCs. Among the BMP ligands, BMP-2, BMP-6, and BMP-9 significantly induce osteogenic lineage commitment of MSCs whereas BMP-2, BMP-4, BMP-7, and BMP-9 promote terminal differentiation of osteoblast progenitors (Cheng et al. 2003) (Fig. 2). BMP-4 expression is regulated in an autocrine manner and by Noggin and inhibited by BMP-2 and BMP-6 (Pereira et al. 2000). Exogenous BMP-2 and BMP-7 enhance osteogenic differentiation and induce endogenous BMP-2, 3, and 8a expression but inhibit endogenous BMP-3b, 4, and 6 expression in BM-MSCs (Edgar et al. 2007). In MSCs, the continuous presence of BMP-7 was required to maintain osteogenesis and its withdrawal led to adipogenic differentiation (Shea et al. 2003). The addition of BMPs such as BMP-4, BMP-9, BMP-10, BMP-11, and BMP-14 induces the expression of osteogenic transcription factors Runx2 and Osx in mouse myoblasts. Maximum osteogenic activity is exhibited by BMP-4, BMP-9 and BMP-14, they induce Smad1/5 activity and expression of ALP, Bone sialoprotein (BSP) and Osteopontin (Opn) (Bessa et al. 2009). Furthermore, BMP-2 overexpression in human MSCs significantly increased its osteogenic potential and N-cadherin (CDH2) expression (Cai et al. 2021). Co-expression of BMP-2 and TGF-β3 significantly increased Runx2 and Osx protein levels and improved the osteogenic activity of BMP-2 in rabbit BM-MSCs (Wang et al. 2016b). Porcine MSCs transfected with rhBMP-6 had significantly high ALP expression, calcium deposition, and ectopic bone formation than rhBMP-2 (Mizrahi et al. 2013), and BMP-1 overexpression in rabbit BM-MSCs promoted ALP and type 1 collagen expression (Su et al. 2020). On the contrary, exogenous addition of BMP-13 inhibited osteogenic differentiation of BM-MSCs in a dose-dependent manner by inhibiting calcium mineralization and alkaline phosphatase (ALP) activity (Shen et al. 2009).

BMPs also induce the expression of BMP antagonists in vivo that limit the exposure of BMPs to regulate osteogenesis. BMP antagonists are spatiotemporally regulated molecules that negatively regulate the BMP pathway by interfering directly with BMP ligands, BMP receptors, or Smad proteins. Some known BMP antagonists are Gremlin, Noggin, Sclerostin, Chordin, CTGF, and Follistatin (Rosen, 2006). Gremlins bind to BMP ligands and inhibit their interaction with the BMP receptors (David R. Hsu 1998). Gremlin2 inhibits BMP-2 induced osteogenesis in human BM-MSCs by competing with BMP receptors, and its suppression leads to an enhanced healing of defective femur in animal models (Wang et al. 2017). Noggins are secreted molecules that inhibit BMP signaling by blocking the molecular interface of the binding epitopes of type I and type II BMP receptors (Groppe et al. 2002). Noggin expression induces BMP-7 expression in the niche during skeletal development in mice, which in turn suppresses the BMP-7 activity (Nifuji and Noda 1999). Noggin inhibits osteoinduction by BMP-2, BMP-4, and BMP-6, however, BMP-7 and BMP-9 induced osteogenesis is resistant to Noggin mediated inhibition. Noggin does not inhibit BMP-9 induced nuclear translocation of Smad1/5/8 (Wang et al. 2013). However, a balance between Noggin and BMP expression is essential for normal development, and imbalance leads to pathological conditions (McMahon et al. 1998; Warren et al. 2003). For instance, BM-MSCs from patients with ankylosing spondylitis (AS) show decreased Noggin expression, leading to increased action of BMP-2 and high Smad1/5/8, extracellular-signal regulated kinase1/2 (ERK1/2) phosphorylation resulting in pathological osteogenesis (Xie et al. 2016). Chordin, an antagonist of BMP signaling, inhibits osteogenesis of human AD-MSCs by suppressing BMP-2 signaling and its deletion leads to osteoinduction (Schneider et al.

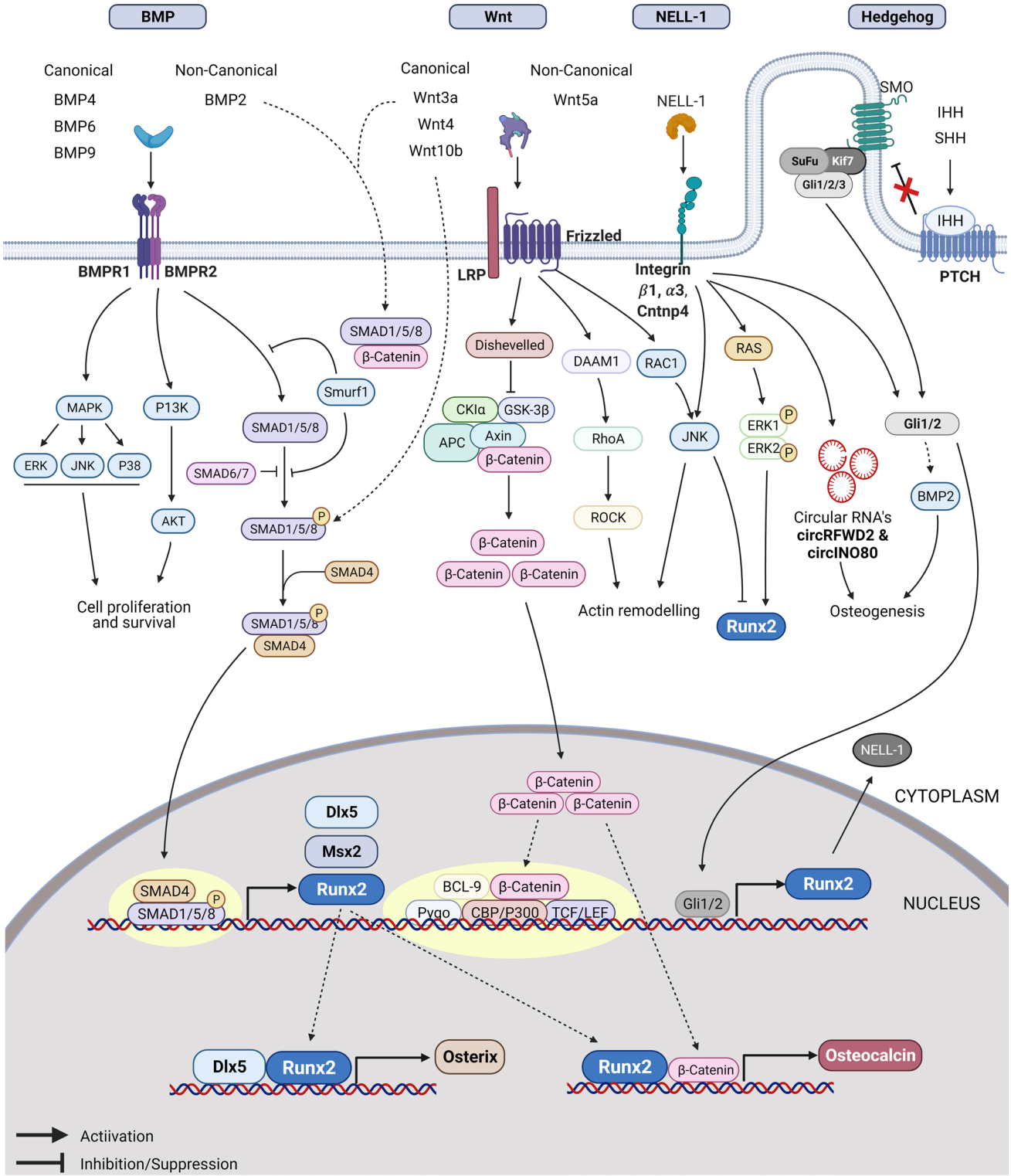

Fig. 2 Signaling networks that regulate osteogenesis in MSCs. Figure representing various signaling pathways and their cross-talk in enhancing osteogenesis in MSCs. Several signaling pathways activate Runx2 expression, and in association with other genes, Runx2 activates the expression of Osterix and Osteocalcin

2014). Chordin-like 1, a BMP inhibitor, increases the proliferation of human MSCs in a dose-dependent manner without affecting the early osteogenic marker ALP (Fernandes et al. 2010). Thus, BMPs are the major regulators and inducers of osteogenesis in MSCs, which is confirmed by the fact that Food and Drug Administration (FDA) had approved the use of recombinant BMP-2 and BMP-7 for the treatment of long bone non-unions.

WNT signaling

Wnt, a highly conserved signaling pathway, regulates cell polarity, proliferation, fate determination, migration, inflammation, primary axis formation, and energy homeostasis in several cell types (Sethi and Vidal-puig 2015). Wnt ligands are cysteine-rich, highly hydrophobic proteins composed of 300 to 400 amino acids that bind to transmembrane receptor Frizzled (Fzd) and low-density lipoprotein receptor-related proteins 5 or 6 (LRP5/6) and receptor tyrosine kinase-like orphan receptor-1/2 (ROR-1/2) act as co-receptors. 19 different Wnt ligands are known in vertebrates, and the type of co-receptors engaged determines the downstream effect of the ligand binding. Low levels of Wnt signaling increase the proliferation of uncommitted human MSCs, whereas high levels lead to inhibition of adipogenic differentiation but promote osteo-lineage commitment (Jan De Boer and Clemens 2004). Wnt ligand binding can activate distinct signaling pathways such as canonical Wnt/β-catenin dependent pathway, non-canonical Wnt/planar cell polarity (PCP) pathway, and Wnt/Ca^{2+} pathway.

Mutations in the Wnt receptor LRP5 is associated with low bone mineral density in humans, and loss of function mutation in LRP5 or LRP6 in mature osteoblasts results in trabecular bone loss (Riddle et al. 2013). The absence of both LRP5 and LRP6 receptors leads to osteopenia and failure in osteogenic differentiation, whereas heterozygous mutations cause limb defects in mice (Holmen et al. 2004). Conversely, a gain of function mutation in the LRP5 coding sequence increases Wnt signaling resulting in high bone density in the mutated individuals and is resistant to inhibition by dickkopf 1 (Dkk1) (Boyden et al. 2002). Wntless (Wls)/Gpr177, a chaperone protein directing Wnt ligand secretion, regulates bone mass in mature osteoblasts through Wnt/β-Catenin signaling and conditional deletion of Wls inhibits Wnt expression and induces a defect in osteoblast differentiation and mineralization (Zhong et al. 2012), hence pointing to a positive role for Wnt signaling in osteogenesis.

Several studies have reported autocrine Wnt signaling in the fate determination of MSCs. Several Wnt signaling components such as LRP5, secreted Frizzled related peptides sFRP2, sFRP3, sFRP4, Disheveled (Dvl), Glycogen synthase kinase-3 β (GSK3β), APC, β-catenin, Kremen 1, Dkk1, and T-cell factor (TCF1 and TCF4) were identified in BM-MSCs obtained from different donors (Etheridge et al. 2004). However, Wharton's jelly derived MSCs (WJ-MSCs) express low levels of Wnt components, Wnt receptors, targets, and have upregulated Wnt inhibitor DKK1 (Batsali et al. 2017). Canonical Wnt signaling modulates osteogenic differentiation mainly through its interaction with Runx2 promoter and putative TCF-DNA binding sites, and a Wnt responsive element was identified in the Runx2 promoter (Gaur et al. 2005) (Fig. 2). Upregulated Wnt signaling prevents osteoblasts from entering chondrocyte lineage, whereas inactivation of β-catenin in progenitors induces chondrocyte differentiation at the expense of osteoblast differentiation (Day et al. 2005; Liu et al. 2009). Further, Wnt ligand Wnt10b inhibits adipogenic transcription factor peroxisome proliferator-activated receptor gamma (PPARγ) and induces trabecular bone formation in mice by enhancing Runx2, Dlx, and Osx expression (Bennett et al. 2007). Wnt10b also mediates BMP-9 induced osteogenesis (Liao et al. 2019) and conditional Wnt10b expression induces osteoblastogenesis in transgenic mice, whereas bone formation rate is reduced in Wnt10b knock out mouse (Bennett et al. 2007). When human MSCs were treated with Wnt5a, it induced osteogenesis by activating non-canonical Wnt signaling and subsequent osteopontin expression (Bilkovski et al. 2010). Treatment with Wnt11 upregulates Wnt5a and subsequent commitment to osteogenic lineage in human BM-MSCs (Boyan et al. 2018). Further, Wnt6 and Wnt10a, when overexpressed, stimulates osteoblastogenesis and upregulation of osteolineage specific genes in murine MSCs, whereas its knock down enhances adipogenesis (Cawthorn et al. 2012). In addition, β-catenin overexpression inhibits adipogenesis by increasing Runx2 and ALP levels. On the other hand, inactivation of β-catenin promotes adipogenesis and inhibits osteogenic differentiation of MSCs (Cai et al. 2014; Cawthorn et al. 2012) and lineage committed pre-osteoblasts (Song et al. 2012).

Wnt antagonists, mainly secreted molecules that inhibit Wnt signaling belong to two families, sFRP (secreted Frizzled-related protein) and Dkk class of molecules (Kawano and Kypta 2003). While sFRP molecules inhibit by binding with Wnt ligands, inhibiting canonical and non-canonical signaling, Dkk molecules inhibit canonical pathway by binding to Wnt receptor components. The sFRP family includes the sFRP, WIF1 (Wnt-inhibitory factor 1), and Cerberus, and the Dkk family is comprised of four members (Dkk1 to Dkk4). The expression pattern of endogenous Wnt antagonists in mice osteoblasts suggests a Wnt feedback loop during osteoblast maturation (Vaes et al. 2005). Hsieh et al. reported that WIF1 treatment enhances adipogenesis by downregulating the transcriptional activity of β-catenin/T-cell factor (TCF) (Hsieh et al. 1999), whereas WIF1 level gets downregulated when murine MSCs differentiate into osteogenic cells (Cho et al. 2009). Dkk downregulates Wnt

signaling in maturing osteoblasts so that the mineralized matrix can be formed (van der Horst et al. 2005) and mice heterozygous for Dkk1 display increased bone formation and bone mass (Morvan et al. 2006). Dkk1 blocks canonical Wnt/β-catenin signaling by antagonizing LRP5/6, and inhibition of Wnt signaling by Dkk-1 is responsible for reduced bone mass (Li et al. 2006) and osteolytic bone lesions during multiple myeloma (Qiang et al. 2008). Sclerostin (SOST), an inhibitor of canonical Wnt signaling is expressed by osteocytes, whose absence leads to a pathological condition termed Sclerosteosis characterized by high bone mass (Balemans et al. 2001). SOST competes with BMPs-2,4,5,6 for binding sites on type I and type II BMP receptors thus interrupting BMP signaling (Winkler et al. 2003). SOST is negatively regulated in osteocytes by parathyroid hormone (PTH) (Bellido et al. 2005) and expression of SOST was found restricted to osteocyte canaliculi, lacunae, cell processes in cortical and trabecular bones and primary osteoblasts but absent in active bone forming osteoblasts and osteoclasts (Van Bezooijen et al. 2004; Winkler et al. 2003). SOST expression is controlled by the mechanosensory property of osteocytes as mechanical loading of rodent limbs leads to a reduction in sclerostin positive osteocyte cell bodies through the action of Wnt/Lrp5 (low-density lipoprotein receptor-related protein5) signaling (Robling et al. 2008). Taken together, Wnt signaling exerts a positive influence on osteogenesis and osteogenic differentiation of MSCs.

Notch signaling

Notch, initially discovered in *Drosophila melanogaster* is named so since its inactivation resulted in notches in the wing blade. Four different Notch receptors (Notch 1, 2, 3 and 4) are identified in mammals, and its ligands are membrane-bound proteins mediating canonical or non-canonical signaling. Canonical Notch ligands are Jagged like—Jag1 and 2, Delta-like—Dll1, 2 and 3 that are distinguished by multiple tandem EGF repeats in the extracellular domain, while non-canonical ligands are Delta homolog-like—Dlk1, Delta/Notch-like EGF-related receptor (DNER), and Contactins (Zanotti and Canalis 2010). Following ligand binding, the receptors undergo sequential proteolytic cleavage; consequently, the Notch intracellular domain (NICD) is released from the attachment site in the plasma membrane and activates canonical and non-canonical signaling pathways (Zanotti and Canalis 2010). The importance of Notch signaling during skeletogenesis and various diseases associated with Notch mutations was reviewed extensively elsewhere (Zieba et al. 2020).

Notch signaling exhibits dimorphic effects during bone homeostasis, and a pathological gain of function of Notch1 in osteoblasts leads to osteosclerosis due to upregulated cyclin D, cyclin E, and Osx (Engin et al. 2008). Conditional deletion of Jag1 inhibits mineralization, whereas conditional activation of Notch through NICD overexpression in osteocytes increases mineralization and bone formation (Liu et al. 2016). Conditional deletion of Jag1 in osteochondral progenitors and osteoblasts has a negative effect on the differentiation of MSCs into the osteoblast phase (Youngstrom et al. 2016) and mice lacking Jag1 shows enhanced osteoblast function and differentiation leading to more cells leaving the osteoprogenitor cell pool (Lawal et al. 2017). Further, osteosclerotic phenotype characterized by early proliferation and arrested maturation of osteoblasts occurs in transgenic mice expressing human Notch ligands Dll1 or Jag1 under the control of ColA1 promoter (Muguruma et al. 2017). However, Jag1 is necessary for bone homeostasis and the maintenance of osteoprogenitor pool in mice (Lawal et al. 2017). Canalis et al. reported that Notch1/2 conditional null mice showed increase in osteoblasts, leading to increased trabecular bone volume. Nevertheless, conditional Notch1 deletion under osteocalcin (Ocn) promoter did not affect the osteogenesis and subsequent Notch2 downregulation enhanced osteogenesis in vitro (Zanotti et al. 2008). Conditional expression of NICD resulted in high trabecular bone volume due to increased bone formation through activation of Wnt signaling (Canalis et al. 2013a). Further, conditional expression of NICD in committed osteoblasts under collagen α1 promoter induced osteosclerosis, which could be rescued by selective deletion of Rbpj (Tao et al. 2010) (Fig. 3). Conversely, Zanotti et al. reported inhibition of osteogenesis, decreased bone volume, and osteopenia when NICD was conditionally overexpressed under collagen I promoter. (Zanotti et al. 2008). Several studies found a repressive action of Runx2 on Notch through direct physical interaction (Ann et al. 2011; Engin et al. 2008; Hilton et al. 2008), and Runx2 expression during early stages of osteogenesis might likely contribute to Notch inhibition (Canalis et al. 2013a).

Several studies demonstrated the role of Notch signaling in the osteogenic differentiation of MSCs. Human MSCs, dental pulp and periodontal ligament cells cultured on Jag1 immobilized surfaces had increased osteogenic differentiation and mineralization (Liang et al. 2019; Sukarawan et al. 2016). NICD and Jag1 facilitated osteogenic differentiation of MSCs in a dose-dependent manner, but at high doses, it was found to have an inhibitory effect (Semenova et al. 2020). Similarly, expression of NICD in mouse MSCs enhances ALP expressing osteoblasts (Tezuka et al. 2002), however, He et al. found that silencing of Notch1 promoted osteogenic differentiation but reduced the viability of murine BM-MSCs (He & Zou, 2019). Conditional expression of Dlk1 under type I collagen promoter decreases bone progenitors and inhibits the osteogenic capacity of MSCs (Figeac et al. 2018). Using a multiple fracture model, Wang et al. found that disruption of Notch signaling in skeletal

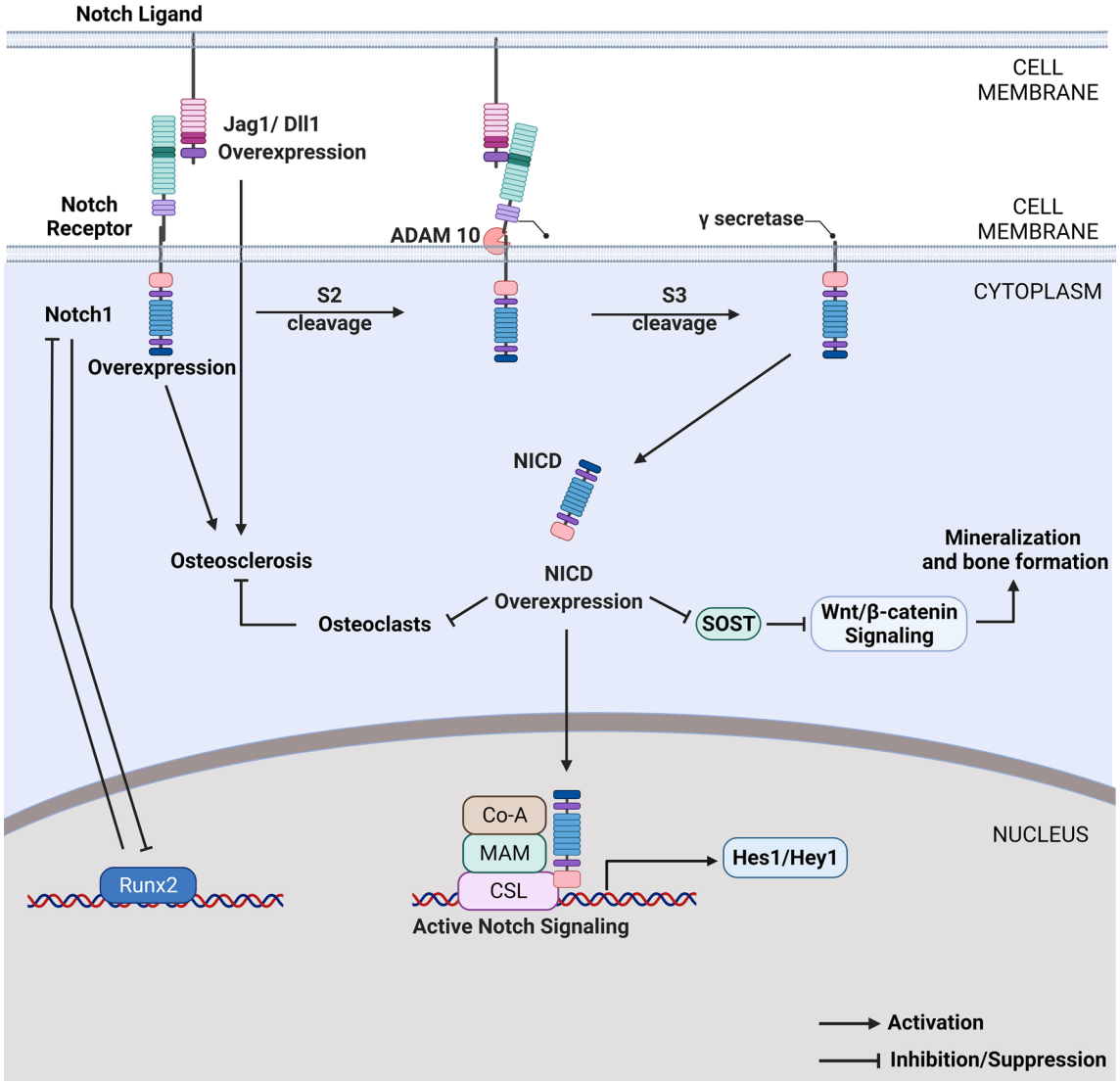

Fig. 3 Notch signaling in osteogenesis. Notch signaling inhibits the initial commitment of MSCs to osteolineage, whereas it is required for the terminal differentiation of osteoblasts. Notch induces osteocyte mineralization through Wnt signaling activation

progenitors leads to a reduction in BM-MSCs and fracture repair, whereas no negative effect was observed when disrupted in differentiated osteoblasts (Wang et al. 2016a). Under hypoxic conditions, activation of Notch signaling downregulated osteogenic differentiation of MSCs, and Notch1 inhibited the transcriptional activity of Runx2 by direct binding (Xu et al. 2013) (Fig. 3). Further, the age-related decrease in osteogenic ability was due to upregulated Notch signaling, and osteogenic differentiation was restored when mouse MSCs were treated with γ-secretase (Tang et al. 2016). Since Notch is downregulated during early differentiation but activated when osteoblasts differentiate into osteocytes, Notch activation inhibits the commitment of MSCs into osteoblasts resulting in the accumulation of immature pre-osteoblasts. Activation of Notch signaling at the later stage of differentiation enhances osteogenesis and mineralization (Ji et al. 2017). Given the contradictory roles reported for Notch in osteogenesis, Canalis et al. found that the role of Notch in osteogenesis was context-dependent, where activation in the early osteoblasts inhibited further differentiation but was essential for terminal differentiation (Canalis et al. 2013b) and mineralization in osteocytes (Shao et al. 2018).

Hedgehog signaling

The hedgehog signaling pathway is one of the fundamental pathways with diverse roles in embryo development, skeletogenesis, and bone formation (Yang et al. 2015) and three structural homologs, Sonic Hedgehog (Shh), Indian

Hedgehog (Ihh), and Desert Hedgehog (Dhh) are present in mammals. Shh and Ihh are essential during embryological development, and Shh plays a crucial role in skeletogenesis (Alman 2015). Mutations in Gli3, a downstream effector of hedgehog signaling, leads to Pallister syndrome that affects limb, head, face and causes abnormalities such as extra toes and fingers (Kang et al. 1997). Gli3 is a known repressor of osteogenic activity, and experimental deletion leads to a defective condition called Grieg cephalon-polysyndactyly syndrome characterized by abnormal skeletal growth (Hui and Joyner 1993).

Ihh has an important role in skeletal development and regulates endochondral bone formation, and intramembranous ossification (Bitgood and McMahon 1995), and Ihh null mice lack mature osteoblasts, exhibit severe dwarfism, failed digit segmentation and incomplete joints and die postnatally (St-Jacques et al. 1999). Gli2 overexpression and Ihh treatment induce Runx2 expression and osteogenic activity through direct physical interaction (Shimoyama et al. 2007) (Fig. 2). Shh induces Osx expression and enhances osteogenic differentiation of BM-MSCs (Cai et al. 2012) and murine ADSCs in both Runx2-dependent and independent manner (Tian et al. 2012). Transplantation of Shh treated ADSCs significantly increased bone regeneration in a tibial injury model (James et al. 2010) and combined activation of Hh, NELL-1 signaling by Shh-N (Shh, N-terminus protein) and NELL-1 addition had an additive pro-osteogenic effect on human AD-MSCs (James et al. 2012). Shh overexpression significantly increases the osteogenic ability in rat BM-MSCs in vitro and bone formation in vivo and reverses diabetes induced osteogenesis defects (Jiang et al. 2019b). Further, Gli1 overexpression reverses oxidative stress-mediated reduction in osteogenic differentiation of murine MSCs (Kim et al. 2010). Thus, hedgehog signaling positively regulates osteogenicity of MSCs and might produce a contradictory effect on MSCs from certain tissue sources.

NELL signaling

Neural EGF like proteins are secreted glycoproteins that belong to the NELL family with two members NELL-1 and NELL-2 (Pakvasa et al. 2017). NELL-1, initially identified in neural tissue, has roles in normal growth and development of various tissues, especially the bone tissues (Matsuhashi et al. 1995), whereas NELL-2 supports neuronal cells in hippocampus and cerebral cortex (Aihara et al. 2003). Unlike other signaling pathways whose mechanism of action is well documented, the NELL-1 mediated signaling activation and its downstream effectors still largely remain to be identified. However, receptors such as Integrin β1, Integrin α3 and contactin associated protein family member 4 (Cntnap4) were found to be binding targets of NELL-1 (Li et al. 2019; Zhang et al. 2010).

A loss of function mutation in Nell-1 induces neonatal lethality in mice with several skeletal abnormalities whereas Nell-1 heterozygotes are normal at birth (Desai et al. 2006) but later develop osteoporosis (James et al. 2015). However, in humans, NELL-1 overexpression is associated with craniosynostosis (CS) (Ting et al. 1999). The osteogenesis promoting effects of Nell-1 was observed in Runx2 haplo-insufficient mice, where Nell-1 overexpression partially rescued the calvarial defects through activation of ERK1/2 and JNK1 mitogen activated protein kinase (MAPK) pathways and Runx2 phosphorylation (Zhang et al. 2011) (Fig. 2). Several transcripts of NELL-1 are identified and the full length isoform, NELL-1_{810} is expressed during embryonic development and controls skeletal development. A truncated isoform, NELL-1_{570} expressed postnatally, induces osteogenic differentiation and proliferation in MSCs (Pang et al. 2015). The mitogenic effect of NELL-1_{570} is age-dependent where BM-MSCs from aged mouse does not respond to NELL-1_{570} induction (Meyers et al. 2019) and the number of Nell-1 positive bone lining cells decreases significantly with increasing age (James et al. 2015).

NELL-1 binds with apoptosis related protein 3 (APR3) (Zou et al. 2011) and colocalizes with Cntnap4 on the cell surface to promote osteogenic differentiation (Li et al. 2018). NELL-1 is also a transcriptional target of Runx2 and Osx, where Runx2 increases Nell-1 transcription in a dose-dependent manner (Truong et al. 2007) and Osx represses Nell-1 expression (Chen et al. 2011). Moreover, exogenous NELL-1 induces osteogenic differentiation through upregulation of circular RNAs such as circRFWD2 and circINO80 (Huang et al. 2019). Long noncoding RNAs (lncRNAs) induced during NELL-1 signaling inhibits the hedgehog pathway and activates the Wnt pathway to induce osteogenesis (Xia et al. 2020). Exogenous addition of Nell-1 to pre-osteoblasts significantly induces osteogenesis on modified titanium surfaces through ERK and JNK activation (Shen et al. 2018) and mineralization through activation of Pit-1 and Pit-2 channels (Cowan et al. 2012). NELL-1 application promoted bone formation in several animal models (James et al. 2016) and induced ectopic bone formation (Askarinam et al. 2013). PEGylated NELL-1 formed by adding polyethylene glycol (PEG) to NELL-1, improved proliferation of BM-MSCs, accelerated bone regeneration, and fracture union (Tanjaya et al. 2018). Altogether, NELL-1 positively influences osteogenic differentiation.

Cross-talk between pathways

Although several pathways individually influence the bone formation, osteogenesis is achieved by the coordinated action of multiple signaling pathways (Fig. 2). For instance,

in rat BM-MSCs, Osx is transcriptionally activated by the combined effects of BMP-2 and canonical Wnt3a through direct interaction of Smads and β-catenin (Rodríguez-Carballo et al. 2011). Further, during osteogenic differentiation, BMP-9 induces Wnt10b expression, which in turn enhances BMP-9 induced phosphorylation of Smad1/5/8 (Liao et al. 2019). Similarly, Wnt3a enhances BMP-9 induced osteogenic gene expression and BMP-9 promotes recruitment of Runx2 and β-catenin to the osteocalcin promoter. However, when β-catenin is downregulated, it inhibits BMP-9 induced ectopic bone formation in vivo (Tang et al. 2009). An intact Notch signaling is essential for osteogenesis promoting effect of BMP-9, and activation of Notch signaling augments BMP-9 induced Runx2, Col1a1 expression in MSCs (Cao et al. 2017). Interestingly, Hey1, a Notch signaling target gene, was significantly upregulated during BMP-9 induced osteogenesis, and Hey1 expression augmented BMP-9 induced osteogenesis and found to act upstream of RUNX2 (Sharff et al. 2009). Furthermore, Wnt and Hh signaling pathways regulate each other (Ding and Wang 2017), and Ihh signaling is activated during the early stages of osteogenesis, whereas Wnt signaling is required for osteoblast maturation (Day and Yang 2008). Further, Shh has an additive effect on BMP-2 during osteogenic differentiation and Shh signaling induced BMP-2 expression is mediated by Gli2 (Zhao et al. 2006), indicating a cross-talk between BMP and hedgehog pathways. Cowan et al. found a synergistic effect of Nell-1 on BMP-2 in inducing osteogenic differentiation in murine myoblasts (Cowan et al. 2007) and co-treatment of NELL-1 and BMP-2 enhanced fracture union, bone strength in mouse bone defect model, and augmented osteogenesis of human BM-MSCs. This co-treatment also enhanced the nuclear accumulation of β-catenin, thus activating canonical Wnt signaling to facilitate osteogenesis (Shen et al. 2016). In human AD-MSCs, recombinant NELL-1 enhanced osteogenesis and inhibited adipogenesis by activating the hedgehog signaling pathway (James et al. 2011); thus a cross-talk between these two pathways is required for osteogenesis. Further, fibroblast growth factor-2 (FGF-2) induces osteogenesis through ERK mediated TAZ expression (Byun et al. 2014), and FGF-2 promotes TAZ and RUNX2 interaction to induce Ocn expression in murine and human MSCs (Zhu et al. 2018).

Hormone signaling in osteogenesis

Hormones such as PTH, melatonin, and triiodothyronine play integral roles in determining the osteogenic fate of MSCs. PTH enhances osteogenesis by inducing BMP signaling (Yu et al. 2012); PTH treatment increases Smad phosphorylation, antagonizes the inhibitory effect of Noggin, and increases the endocytosis of PTH1R/LRP6, which further increases the access of BMPs to its receptors. Transgenic mice with PTH/-Parathyroid hormone-related protein (PTHrP) receptor (PTH1R) deletions in MSCs have low bone formation, high resorption, and increased levels of adipose tissue in the bone marrow (Fan et al. 2017). Intermittent administration of PTH activates Wnt and BMP signaling (Ogura et al. 2016), enhances osteogenesis (Kuo et al. 2017) by inhibiting adipogenesis (Yang et al. 2019), and increases osteoblast numbers in vivo (Balani et al. 2017). The anabolic action of PTH is exerted mainly through the activation of Wnt signaling by downregulation of SOST expression (Silva & Bilezikian, 2015). Continuous PTH treatment, however, causes proteasomal degradation of Runx2 (Bellido et al. 2003). Several studies have reported osteoinductive effects of estrogen, and Zhao et al. found that estrogen treatment induces osteogenic differentiation over chondrogenic differentiation and activates ERK, JNK signaling in rat BM-MSCs (Zhao et al. 2016). Activation of estrogen receptor signaling in mouse MSCs through estradiol treatment or ERα expression increases the Wnt3a induced osteogenic differentiation and matrix mineralization (Gao et al. 2013). A synergy between BMP-9 and melatonin was observed in inducing osteogenic differentiation of mice MSCs where it enhances late osteogenic markers expression, matrix mineralization, and ectopic bone formation (Jiang et al. 2019a). In addition, BMP-9 and triiodothyronine, a thyroid hormone, synergistically induces AMPK/p38 signaling and enhances osteogenesis of MSCs (Chen et al. 2020).

Physical factors inducing osteogenesis

Mechanical signals such as matrix elasticity and cyclic strain induce osteo-lineage commitment and related gene expression in MSCs. When rat MSCs were subjected to oscillatory fluid flow, Runx2 and Sox9 expression were upregulated, leading to increased osteogenesis (Arnsdorf et al. 2009). Further, stiffer matrices enhance α2-integrin expression, which in turn activates ERK1/2 through ROCK and FAK, leading to increased osteogenesis in human MSCs (Shih et al. 2011). Yes-associated protein/Transcriptional coactivator with PDZ binding motif (YAP/TAZ) mediated Hippo signaling induces osteogenesis in MSCs during physical stimuli such as matrix stiffness and surface topography modifications. Rough surfaces and increased extracellular matrix (ECM) stiffness activate YAP/TAZ leading to higher substrate adhesion, cell spreading through increased actin polymerization, cytoskeletal tension and enhanced osteogenic differentiation (Dupont et al. 2011; Heng et al. 2020). Barreto et al. found that mechano-sensitivity of MSCs was linked to the skeletal maturity and age, where MSCs from children had higher mechanosensitivity and showed better osteogenesis

compared to adult MSCs (Barreto et al. 2017). Simmons et al. found that cyclic strain induced the osteogenic lineage commitment of hMSCs by enhancing MAPK signaling (Simmons et al. 2003). In contrast, Shi et al. reported that under continuous cyclic mechanical tension, human as well as rat MSCs had significantly low expression of osteogenic markers such as ALP, Opn, Col1, and Runx2 (Shi et al. 2011). Sugimoto et al. recently demonstrated that hydrostatic pressure enhances osteogenesis of MSCs by activating the calcium channel protein Piezo1 (Sugimoto et al. 2017). Actin modification, cell density, cell shape, and composition of ECM have all been implicated in regulating the osteogenic differentiation potential of MSCs (McBeath et al. 2004; Somaiah et al. 2015; Sonowal et al. 2013).

Conclusions

Multiple signaling pathways interact in intricate ways to regulate osteogenic differentiation and mineralization in MSCs. BMP signaling pathway plays a major role in regulating osteogenic differentiation of MSCs (Bessa et al. 2009; Edgar et al. 2007) and shows synergy with NELL-1 (Cowan et al. 2012; Shen et al. 2016), Wnt (Liao et al. 2019; Rodríguez-Carballo et al. 2011; Tang et al. 2009), and Hedgehog (Yuasa et al. 2002; Zhao et al. 2006) signaling to enhance osteogenesis. Wnt (Bennett et al. 2007; Bilkovski et al. 2010; Gaur et al. 2005) and hedgehog (James et al. 2012; Jiang et al. 2019b) pathways have positive effects on osteogenic differentiation of MSCs, whereas Notch has a context-dependent role in modulating osteogenesis. Activation of the Notch pathway inhibits the initial commitment of MSCs into osteoblasts, however, it is necessary for osteocyte maturation (Canalis et al. 2013b). In addition, several hormones (Chen et al. 2020; Fan et al. 2017; Jiang et al. 2019a) and physical factors such as matrix elasticity and mechanical stress (Shih et al. 2011; Sugimoto et al. 2017) promote osteogenesis, and a combinatorial application of these factors might enhance osteoblastogenesis and thus bone formation under therapeutic conditions. Given the positive influence of several signaling pathways, MSCs can be pre-conditioned or genetically modified to activate osteo-promoting signaling before transplantation to enhance the therapeutic benefits.

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Declarations

Conflict of interest The authors declare that they have no conflicts of interest.

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