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Spatiotemporal application of small molecules in fracture healing

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ABSTRACT

Skeletal injuries and disorders are major causes of physical disability worldwide, posing an intractable clinical challenge. Within the field of regenerative medicine, researchers are continuously developing new therapeutic strategies to promote bone regeneration. Small molecules, defined as bioactive compounds with a molecular weight of <1,000 Da, have emerged as promising agents capable of precisely regulating intracellular signaling pathways to enhance bone regeneration. Their cost-effectiveness, superior membrane permeability, and minimal immunogenicity have positioned them at the forefront of both fundamental research and clinical applications. In recent years, advancements in artificial intelligence have accelerated the development and screening of small-molecule drugs, broadening their potential therapeutic applications. Furthermore, innovations in dynamic drug delivery systems have advanced the concept of spatial precision, enabling the controlled release of drug doses over time and achieving the spatiotemporal application of small molecules. These systems release specific small molecules in a sequence, synchronizing therapeutic interventions with the dynamic process of bone healing. Spatiotemporal delivery strategies, which effectively replicate the complex and highly ordered processes of bone healing, have the potential to reduce drug side effects and enhance healing efficacy. However, clinical translation remains hindered by insufficient spatiotemporal control and limited pharmacokinetic precision, challenges that this review explores in depth. We systematically examine stage-specific molecular targets of signaling pathways and their corresponding small molecule modulators. In addition, we discuss current approaches to spatiotemporal delivery strategies, such as stimuli-responsive delivery systems. Finally, we explore the status of clinical applications, the challenges encountered, and potential solutions regarding the spatiotemporal release strategy. We hope this review will contribute to the development of future spatiotemporal delivery strategies, ultimately improving outcomes for patients with impaired fracture healing.

Keywords:
Bone regeneration; Fracture healing; Sequential application; Signaling pathways; Small molecules; Spatiotemporal strategy

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1. Introduction

With the global population aging rapidly, the incidence of fracture has surged, adversely impacting quality of life and burdening healthcare systems. Although bone possesses a unique regenerative capacity, studies indicate that non-union or delayed union fractures occur in approximately 5% – 10% of cases.[1] In the realm of regeneration medicine, growth factors have been employed to activate osteogenic pathways, but their efficacy is inconsistent, and safety concerns persist.[2] For example, food and drug administration (FDA)-approved recombinant bone morphogenetic proteins (BMPs) have adverse effects, including inflammation, immunogenicity, excessive bone growth, and severe cervical swelling.[3,4]

Small molecule drugs are alternatives to growth factors and promising candidates for bone regeneration. These compounds, characterized by molecular weights below 1 kDa, can penetrate cell

membranes and activate osteogenic signaling pathways.[5] Compared with growth factors, small molecules offer several advantages, including lower immunogenicity, cost-effective production, and a reduced risk of cross-species contamination. Furthermore, advancements in artificial intelligence (AI) have revolutionized the drug discovery process, reducing costs, expanding therapeutic options, and improving drug efficacy, thereby demonstrating the broad prospects of small molecule drugs.

Recent advances in drug delivery systems have revolutionized fracture treatment paradigms. The current delivery technologies enable spatially precise drug release, allowing targeted delivery of small-molecule drugs to specific organs. However, spatial precision alone is insufficient, as it does not adapt to the dynamic changes in the healing environment. Therefore, efforts are now focused on achieving spatiotemporal precision, combining spatial control with stage-specific drug release. For instance, the rapid release of simvastatin (SIM) during the inflammatory phase enhances stem cell recruitment, followed by sustained pargyline (PGL) release to drive osteogenic differentiation, significantly improving bone formation compared to simultaneous administration.[6] Moreover, traditional long-term, high-dose regimens often result in adverse effects. In contrast, precisely timed small-molecule release at specific healing stages enables short-term treatments that minimize side effects while maximizing therapeutic efficacy.

Despite these promising advances, critical gaps persist. The current delivery systems predominantly enable concurrent rather than sequential drug release, with inadequate precision in spatiotemporal control and pharmacokinetic modulation. A thorough understanding of stage-specific small molecules and the development of systems enabling sequential drug delivery are essential for optimizing outcomes – an issue that this review explores in depth. We begin by introducing phase-specific therapeutic molecules involved in key signaling pathways during the healing process. We then critically evaluate current spatiotemporal delivery platforms, discussing their clinical translation, associated challenges, and potential solutions. Finally, we explore strategies for screening small molecule drugs and the role of AI in facilitating this process. We hope this review will serve as a foundation for the development of future spatiotemporal strategies and ultimately promote the treatment of fracture healing in clinical practice.

2. Retrieval strategy

To identify small molecules promoting fracture healing, we conducted a PubMed search using the keywords: ((“fracture healing”) OR (“bone regeneration”) OR (“bone formation”)) AND (“small molecule”). For temporal strategies in promoting fracture healing, we used the keywords: ((“fracture healing”) OR (“bone regeneration”) OR (“bone formation”)) AND ((“temporal”) OR (“sequential”)) AND (“drug”). We excluded studies from Q3 and Q4 journals, selecting publications from the past 5 years, and manually reviewed the articles to ensure their relevance to the field.

3. Stages of fracture healing and corresponding signaling pathways

Fracture healing is an intricately coordinated process, comprising three partially overlapping phases: inflammation, renewal, and remodeling.[7,8] We introduced each stage of fracture healing in chronological order, along with the corresponding signaling pathways that regulate each stage. Synergistic and antagonistic effects between signaling pathways were also discussed (**Figure 1**).

3.1. Inflammatory phase and corresponding signaling pathways

The inflammatory phase involves hematoma formation and the release of inflammatory mediators.[9,10] Upon bone fracture, blood vessels rupture, leading to the activation of platelets and the formation of a hematoma. Neutrophils, acting as “first responders,” migrate to the injury site and recruit monocytes through the secretion of chemotactic mediators, the most important of which is tumor necrosis factor-alpha (TNFα). These cells also release inflammatory cytokines, triggering the mobilization of fibroblasts and progenitor cells from their respective secluded niches. The recruited cells contribute to new collagen production and hematoma cross-linking, facilitating the initial stages of bone healing.[7]

The inflammatory phase is driven by the TNFα pathway, which plays a dual role in fracture healing.[11] On one hand, TNFα can stimulate the migration of bone marrow mesenchymal stem cells (BMSCs) to the fracture site, promoting osteogenic differentiation and facilitating fracture repair.[12] On the other hand, activation of the TNFα pathway also triggers both nuclear factor kappa-B (NF-κB) and mitogen-activated protein kinase (MAPK) signaling pathways, which inhibit Sma- and Mad-related protein (Smad) 1/5/8 signaling and suppress osteoblast differentiation.[13] Furthermore, the TNFα pathway disrupts wingless-related integration site (Wnt)/β-catenin signaling. A study showed that 24 h of TNFα treatment in BMSCs reduced β-catenin expression in both the cytoplasm and nucleus, inhibiting its recruitment to the promoters of key osteogenic transcription factors, Runt-related transcription factor 2 (*Runx2*) and Osterix.[14]

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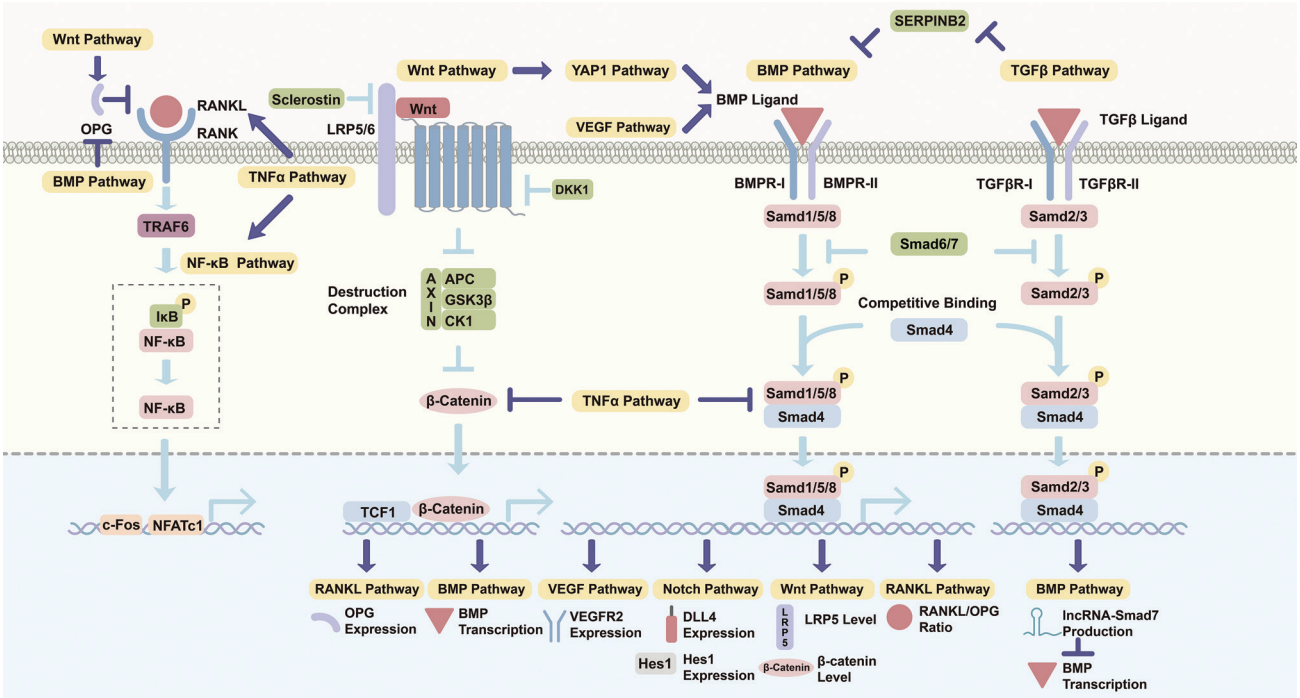

Figure 1. A simplified overview of the synergistic and antagonistic interactions between signaling pathways. BMP signaling promotes the expression of VEGFR2, regulates the expression of the Notch pathway ligands DLL4 and Hes1, increases the ratio of RANKL/OPG, and elevates the levels of Wnt receptors LRP5 and β-catenin. In contrast, BMP signaling antagonizes the TGFβ pathway by competitively binding to Smad4, while TGFβ signaling, in turn, can suppress BMP expression. Wnt signaling promotes BMP expression and inhibits the RANKL pathway by increasing OPG levels. In contrast, the inflammatory TNFα pathway suppresses Smad complex formation and reduces β-catenin levels, thereby inhibiting osteogenesis, while simultaneously enhancing RANKL expression and activating the NF-κB pathway to promote osteoclastogenesis. Abbreviations: APC: Adenomatous polyposis coli; BMP: Bone morphogenetic protein; BMPR: BMP receptor; CK1: Casein kinase 1; DLL4: Delta-like ligand 4; GSK3β: Glycogen synthase kinase 3 beta; Hes1: Hairy and enhancer of split-1; IκB: Inhibitor of kappa kinase; lncRNA: Long non-coding RNA; LRP: Low-density lipoprotein receptor-related protein; NFATc1: Nuclear factor of activated T-cells, cytoplasmic 1; NF-κB: Nuclear factor kappa-B; OPG: Osteoprotegerin; P: Phosphate; RANK: Receptor activator of nuclear factor kappa-B; RANKL: Receptor activator of the nuclear factor kappa-B ligand; SERPINB2: Serpin family B member 2; Smad: Sma- and Mad-related protein; TCF-1: T cell-specific DNA-binding protein; TGFβ: Transforming growth factor beta; TGFβR: Transforming growth factor beta receptor; TNFα: Tumor necrosis factor alpha; TRAF6: TNF receptor-associated factor 6; VEGF: Vascular endothelial growth factor; VEGFR2: Vascular endothelial growth factor receptor 2; Wnt: Wingless-related integration site; YAP1: Yes-associated protein 1.

3.2. Repair phase and corresponding signaling pathways

The repair phase begins before the inflammation stage subsides, characterized by angiogenesis, soft callus formation, and hard callus formation. This stage occurs through both intramembranous and endochondral ossification, with the latter process being more significant and intricate.[9,15] Intramembranous ossification occurs at the end of the fractured bony, where mesenchymal stem cells (MSCs) differentiate into osteoblasts and directly form woven bone. In contrast, endochondral ossification occurs within the central hypoxic core,[7,9] where endogenous MSCs develop into chondroblasts and form cartilage-specific matrix.[9] Simultaneously, hypertrophic chondrocytes secrete angiogenic factors (such as vascular endothelial growth factor (VEGF)), stimulating vascular ingrowth and transforming the non-vascular cartilaginous matrix into a vascularized osseous tissue.[16] Gradually, osteoprogenitor cells (OPCs) are recruited and differentiate into osteoblasts, which secrete mineral vesicles containing calcium and phosphate, facilitating the transition to a hard bony callus.[17]

VEGF signal is indispensable for endothelial proliferation and maturation, driving the formation of new blood vessels, and is a key factor in osteo-angiogenic coupling.[18] Notably, only optimal levels of VEGF could stimulate vascular invasion and progenitor cell osteogenic differentiation, thereby promoting bone formation. This is because while increasing VEGF levels enhances vascular density, it also slows the rate of vascular ingrowth, which is critical for progenitor cell survival and proliferation.[19] VEGF signaling also aids in bone regeneration by increasing vascular permeability, which induces MSCs and OPCs,[20,21] and by inducing the expression of BMP to activate the osteogenesis pathway.[22]

The Notch pathway achieves a balanced regulation of angiogenesis through two competing ligands, Jagged1 and delta-like ligand 4 (DLL4). DLL4 limits epithelial cell mitosis and blood vessels sprouting by downregulating VEGF receptor expression, while Jagged1 competes with DLL4 to interact with the Notch receptor, activating the canonical Notch pathway and promoting angiogenesis.[23] The role of Notch signaling in bone regeneration is context-dependent, leading to contradictory findings in the literature. Certain studies reported that Notch signaling enhanced osteoblast metabolic activity,[24] promoted bone mineralization,[25] and maintained the pool of bone progenitor cells.[26] In contrast, other research indicated that

Notch signaling reduced the number of osteoblasts,[27] inhibited osteoblast differentiation,[28] and decreased bone mass.[29]

BMP signaling is one of the most well-established pathways involved in promoting fracture healing, initiating a series of organized processes leading to chondro-osteogenesis. Most BMP pathways promote the final maturation of committed osteoblastic precursors and osteoblasts, with BMP2, BMP6, and BMP9 being particularly effective in driving the differentiation of mesenchymal progenitor cells.[30] However, some studies suggest that changes in BMP signaling in adult osteoblasts have a limited effect on bone mass, indicating that reducing BMP dosage in clinical settings might minimize side effects.[31] The BMP pathway exhibits extensive crosstalk with multiple signaling pathways. BMP2 promotes VEGF-mediated endothelial sprouting by regulating DLL4, while BMP6 modulates VEGF signaling by regulating VEGF receptor 2 expression.[22] BMP9 induces the expression of the key Notch signaling effector molecule hairy and enhancer of split-1 through the Smad pathway.[32] Moreover, BMP2 upregulates low-density lipoprotein receptor-related protein (LRP) 5 levels and prevents β-catenin degradation, thereby activating the canonical Wnt pathway.[33] BMP signaling also influences the receptor activator of the nuclear factor kappa-B ligand (RANKL) pathway, increasing the RANKL/osteoprotegerin (OPG) ratio to promote osteoclast formation.[33] BMP receptor (BMPR) type II interacts with receptor activator of nuclear factor kappa-B (RANK), activating both phosphorylated Smad1/5/8 and NF-κB signaling.[33]

The transforming growth factor beta (TGFβ) pathway plays a dual role in fracture healing,[34,35] promoting the chemotaxis, expansion, and early differentiation of osteoprogenitors while inhibiting the maturation, mineralization, and osteogenic differentiation of osteoblasts during the later stages of bone formation.[36] For instance, in MC3T3-E1 (mouse embryo osteoblast precursor) cells, TGFβ1 treatment resulted in increased alkaline phosphatase (ALP) expression in the early stages while reducing mineralization and decreasing ALP levels in later stages. This suggests that while TGFβ1 might initially promote osteogenic differentiation, it subsequently exerts an inhibitory effect.[37] The TGFβ pathway can also facilitate BMP signaling. Elsafadi *et al.*[38] demonstrated that exogenous SERPINB2 suppresses BMP signaling, whereas TGFβ counteracts this suppression, facilitating the differentiation and commitment of human BMSCs. In addition, TGFβ1 synergizes with BMP9 to enhance BMSC osteogenic differentiation, potentially through non-canonical pathways.[39] However, TGFβ signaling has also been reported to inhibit BMP signaling by competing for Smad4.[40] Furthermore, the TGFβ pathway induces the production of long non-coding RNA-Smad7, which inhibits BMP expression, thereby regulating cell fate determination between osteocytes and myocytes in mouse myoblasts.[41] Notably, low doses of TGFβ activate Smad3 and upregulate BMP2 expression in BMSCs, whereas high TGFβ levels inhibit Smad3 and attenuate BMP2 transcription.[42]

The Wnt/β-catenin pathway is critical for osteoblast development, promoting osteogenesis while simultaneously inhibiting chondrogenesis.[43-45] Canonical Wnt signaling mediated the differentiation of BMSCs into osteoblast precursor cells.[46] Notably, local delivery of β-catenin messenger RNA has been demonstrated to promote callus formation in a mouse tibial fracture model.[47] Targeting and silencing Wnt antagonists, such as schnurri-3 and sclerostin, with microRNA enhances Wnt/β-catenin signaling, improves osteoblast function, and facilitates the repair of impaired fracture healing.[48] Activation of the canonical Wnt pathway also enhances the transcription of BMP2[49] and BMP7.[50] Wnt1 also promotes fracture healing and bone formation by activating the yes-associated protein (YAP) 1/BMP signaling pathway.[51]

The Hedgehog pathway plays an essential role in stimulating osteoblast proliferation and differentiation.[52] For instance, activation of the Hedgehog signaling pathway through agonists has been shown to increase the production of mineralized fibrocartilage and improve tendon-to-bone healing.[53] In addition to its role in osteoblast activity, the Hedgehog pathway also significantly influences both osteogenesis and angiogenesis. This may occur through mechanisms such as TNF receptor-associated factor (TRAF) 6 protein stabilization,[54] smoothened (Smo)-glioma-associated oncogene family zinc finger (Gli) 1/2 axis activation,[55] and upregulation of RANKL expression.[56]

3.3. Remodeling phase and corresponding signaling pathways

The remodeling phase involves the gradual replacement of the initial fracture callus by mature mineralized tissue, restoring the normal bone tissue structure. Osteoclasts, recruited by macrophage colony-stimulating factor and RANKL, create absorptive lacunae on the bone surface, removing immature woven bone and the underlying cartilage matrix.[9,57] Following this, osteoblasts move to the resorption lacuna, re-establish a superior lamellar bone structure, and eventually transform into a quiescent state.[58]

The RANKL pathway serves as a pivotal trigger for the activation of osteoclastogenic signaling,[59,60] regulating the transformation of pre-osteoclast to multi-nucleated osteoclasts and the activation of mature osteoclasts.[61] The inflammatory TNFα pathway upregulates RANKL expression in osteocytes, contributing to this crosstalk.[62] The RANKL pathway also exacerbates inflammation and promotes the activation of thymic stromal lymphopoietin, which, in turn, activates the phosphoinositide 3-kinase (PI3K), MAPK, caspase-1, and NF-κB pathways.[63] Furthermore, several signaling crosstalks are involved in balancing the RANKL pathway. RANKL pathway activation induces the production of interferon-β in osteoclast precursors, which inhibits osteoclast differentiation by antagonizing RANKL-induced c-Fos expression. In mouse models, the absence of interferon signaling led to increased osteoclast numbers and decreased bone density.[64] Furthermore, the Wnt/β-catenin signaling pathway indirectly suppressed the RANKL pathway and osteoclast differentiation by promoting the secretion of OPG.[65]

3.4. Emerging insights into osteogenic signaling mechanisms

In addition to classical osteogenic pathways, several novel mechanisms have been identified. For instance, nanostructured

topographies can activate Piezo1, a mechanosensitive ion channel located in the cell membrane, thereby enhancing mitochondrial oxidative phosphorylation and increasing intracellular acetyl-coenzyme A levels. This cascade ultimately activates β-catenin signaling, promoting bone formation.[66] Furthermore, vestigial-like family member 4 has been reported to function as a transcriptional repressor of YAP-transcriptional enhancer factor TEF-1 (TEAD) in the Hippo pathway. TEAD interacts with *Runx2* and inhibits its transcriptional activity; however, vestigial-like family member 4 disrupts TEAD-mediated suppression of *Runx2*, thereby facilitating osteoblast differentiation and skeletal development.[67] Certain signaling pathways regulate the neurogenic promotion of bone regeneration. For example, the delivery of nerve growth factor to fracture sites has been shown to activate its receptor, tropomyosin receptor kinase A, thereby inducing nerve-mediated bone regeneration in rat cranial defect models.[68]

Advancements in sequencing technologies have significantly deepened our understanding of osteogenic molecular mechanisms. One study employed RNA interference (RNAi) screening of kinases in primary murine osteoblasts and identified cyclin-dependent kinase 5 (Cdk5) as a negative regulator of osteoblast differentiation in both mice and humans. RNA sequencing analysis of Cdk5 knockdown osteoblast revealed that Cdk5 knockdown promotes osteogenic differentiation by activating the MAPK/extracellular regulated kinases (ERK) signaling pathway.[69] Another study used RNA sequencing to analyze gene expression changes in BMSCs during biomaterial-induced bone regeneration, identifying a significant increase of semaphorin 7A, which promotes osteogenesis through the integrin subunit beta 1/focal adhesion kinase/ERK signaling cascade.[70] In addition, Liu *et al.*[71] demonstrated that tripartite motif 21 depletion enhances bone mass. Proteomic screening further revealed that tripartite motif 21 regulates osteoblast differentiation by modulating YAP1/β-catenin signaling.

4. Small molecules targeting pathways to promote fracture healing

Despite the critical role of cytokines in regulating osteogenic pathways, their clinical application faces numerous challenges, including short half-life, high-dose requirements, expensive costs, undesired side effects, and the risk of immune reactions.[72] Therefore, the discovery and application of small molecules, which serve as validated stable alternatives to cytokines, represents a key milestone for further progress in bone regeneration. In this section, we discuss several osteogenic small molecules, including their effects and the underlying mechanisms involved (**Table 1**).

4.1. Small molecules in inflammatory phase for fracture healing

4.1.1. Regulation of the TNFα pathway by small molecules

The TNFα pathway is initiated by the binding of TNFα to TNFα receptor 1, which subsequently activates NF-κB signaling,[112] exacerbating inflammation and inhibiting BMP2-induced osteogenesis (**Figure 2**).[113]

High concentrations of inflammatory factors are known to inhibit osteogenesis,[114] suggesting that anti-inflammatory drugs may promote bone regeneration. Notoginsenoside R1 alleviates TNFα expression in human mesenchymal stem cells (hMSCs). Incorporating notoginsenoside R1 into a hyaluronic acid/nanosized hydroxyapatite scaffold activates the MAPK/ERK signaling pathway and downregulates TNFα expression, ultimately controlling inflammation and promoting superior osteogenic effect in the rat skull defect model.[73] TNFα activates downstream NF-κB signaling through TRAF2/6, which inhibits the Wnt pathway and impairs osteogenesis. The TRAF6 inhibitor 6877002 has been shown to successfully block TNFα-stimulated NF-κB signaling, reducing TNFα-mediated osteoclastogenesis.[74] Quercetin has been demonstrated to safely act as an NF-κB signaling repressor, reversing TNFα-induced osteogenic damage in BMSCs and significantly increasing bone mass.[76] In addition, a scaffold loaded with nano-formulated quercetin promotes the healing of femoral bone defects in rats.[75]

4.2. Small molecules in bone renewal phase for fracture healing

4.2.1. Regulation of the VEGF pathway by small molecules

The VEGF pathway, a critical regulator of osteo-angiogenic coupling,[115] is activated in the hypoxic environment of the fracture site through hypoxia-inducible factor 1-alpha (HIF-1α) upregulation. Its downstream signaling pathway includes MAPK-ERK1/2 and PI3K- protein kinase B (Akt), among others (**Figure 3**).[116]

Small molecules that upregulate VEGF secretion can promote fracture healing. Statins, including SIM, atorvastatin, and pravastatin, have been reported to enhance VEGF mRNA expression in osteoblasts, thereby stimulating angiogenesis and facilitating bone repair.[77] Forskolin, an activator of adenylyl cyclase that raises intracellular cyclic adenosine monophosphate (cAMP) levels, induced cAMP activation in mouse OPCs and rabbit MSCs after 24 h of treatment. This upregulated VEGF through a cascade reaction, exerting long-term osteogenic effects.[78,79] Treatment with cilostazol, an approved drug to inhibit phosphodiesterase-3 and increase cAMP levels in clinical practice, elevated VEGF levels, increased microvessels formation, and enhanced bone formation in both the atrophic non-union mouse model and the aged mouse fracture model.[80,81]

Hypoxia-inducible factor 1-alpha, an oxygen-sensitive transcription factor that mediates VEGF production, is hydroxylated by prolyl hydroxylases and subsequently degraded under oxygen conditions. Stabilizing HIF-1α is a critical target for activating the VEGF pathway. The prolyl hydroxylase inhibitor, iron chelator 2 (IOX2), was shown to activate the HIF-1α pathway, promote VEGF production, enhance BMSC proliferation and migration, and increase bone density in a rat femoral fracture model.[82] Deferoxamine, an FDA-approved iron chelator that mimics a hypoxic environment, upregulates HIF-1α signaling. Loading deferoxamine onto hydrogel scaffolds improved the viability and adhesion of human umbilical vein endothelial cells and exerted angiogenic effects.[83] Salidroside also upregulated HIF-1α to activate the VEGF

Table 1. Summary of small molecule drugs targeting signal pathways for bone healing

Pathways	Small molecules	Mechanisms	Models	References
TNFα pathway	NGR1	Activate the MAPK/ERK pathway to downregulate TNFα expression	hMSC model; rat skull defect model	73
	6877002	Inhibit TNFα/TRAF6/NFκB axis	Osteoclast precursor model	74
	Quercetin	Repress NF-κB signaling and activate Wnt/β-catenin	BMSC model; Rat femoral bone defect model	75,76
VEGF pathway	Statins	Increase VEGF mRNA expression	Osteoblast model	77
	Forskolin	Activate cAMP pathway to increase VEGF expression	Mouse osteogenic progenitor cell MC3T3-E1 model; rabbit MSC model	78,79
	Cilostazol	Increase VEGF secretion	Mouse atrophic fracture non-healing model; Elderly mice fracture model	80,81
	IOX2	Stabilize HIF-1α to activate VEGF pathway	SD rat femoral fracture model	82
	DFO	Stabilize HIF-1α to activate VEGF pathway	HUVEC model	83
	SAL	Stabilize HIF-1α to activate VEGF pathway	Fetal mouse long bone model	84
Notch pathway	VPA	Activate Notch signaling	MC3T3-E1 osteoblasts; OVX rats with femoral epiphyseal defect	85
	Imiquimod	Activate Notch signal	iMAD cell model	86
	DAPT	Inhibit γ-secretase	Embryonic and induced pluripotent stem cell model; aged BMSC model	87,88
BMP pathway	SY-LB-35	Act as BMP receptor agonists	C2C12 cell model	89
	SY-LB-57	Act as BMP receptor agonists	C2C12 cell model	89
	Trapidil	Increase reactivity of BMPR	Rat skull defect model	90
	FK506	Activate BMPR	Rat ectopic subcutaneous implantation model; rat femoral defect model; rabbit spinal fusion model	91,92
	NVP	Promote nuclear translocation of phosphorylated Smad1/5/9	C2C12 cell model; rabbit skull defect model	93
	ONO-1301	Enhance the effect of BMP	MSCs; rat skull defect model	94
TGFβ Pathway	Amygdalin	Promote Smad2/3 phosphorylation	Gli1+MSCs; mouse tibial fracture model	10
	Zg	Inhibit Smad7	MSC model	95
	Csn-B	Stimulate Nr4a1 and reverse the side effects of TGFβ pathway	BMSCs; mouse skull defect model; mouse tibia defect model	96
	SB431542	Downregulate TGFβ-induced inhibitory Smads expression	Mouse skull defect model; miniature pig maxillofacial bone severe defect model	97,98
Hh pathway	PUR	Activate Smo to activate Hedgehog pathway	MSC; mouse skull defect model	99,100
	SAG	Activate Smo to activate Hedgehog pathway	Primary neonatal mouse skull cell model; mouse skull defect model	101
	SAG21k	Activate Smo to activate Hedgehog pathway	Mouse segmental femoral model defect	102
Wnt/β-catenin pathway	1-Azakenpaullone	Inhibit GSK-3β to upregulate β-catenin	hMSC model	103
	C91	Inhibit GSK-3β to upregulate β-catenin	BMSC model	104
	VA1	Inhibit sclerostin to activate Wnt pathway	Rat ectopic mineralization model; rabbit posterior spinal arthrodesis surgery model	105
	C07	Inhibit sclerostin to activate Wnt pathway	Rat ectopic mineralization model; rabbit posterior spinal arthrodesis surgery model	105
	WAY-262611	Inhibit DKK1 to activate Wnt pathway	Lumbar fusion model in osteoporotic rats	106
RANKL pathway	ZA	Downregulate RANK expression	Rabbit femoral defect model	107
	Aspirin	Inhibit NF-κB and NFATc1 activation	Dendritic cell model; Rat mandibular defect model	108
	XAV-939	Increase OPG expression and decrease RANKL expression	hMSCs model	109
	W9	Bind competitively with RANKL	Rat femur delayed-union model	110,111

Abbreviations: BMP: Bone morphogenetic protein; BMPR: BMP receptor; BMSC: Bone marrow mesenchymal stem cell; cAMP: Cyclic adenosine monophosphate; C91: CHIR99021; DFO: Deferoxamine; DKK1: Dickkopf-related protein 1; ERK: Extracellular regulated kinase; Gli1: Glioma-associated oncogene family zinc finger; GSK3β: Glycogen synthase kinase 3 beta; HIF-1α: Hypoxia-inducible factor 1-alpha; hMSC: Human mesenchymal stem cell; HUVEC: Human umbilical vein endothelial cells; iMAD: Multipotent adipose-derived cells; IOX2: Iron chelator 2; mRNA: Messenger RNA; MAPK: Mitogen-activated protein kinase; MSC: Mesenchymal stem cell; MC3T3-E1: Mouse embryo osteoblast precursor' NFATc1: Nuclear factor of activated T-cells, cytoplasmic 1; NF-κB: Nuclear factor kappa-B; NGR1: Notoginsenoside R1; Nr4a1: Nuclear receptor 4 A1; NVP: N-Vinyl-2-pyrrolidone; OPG: Osteoprotegerin; OVX: Ovariectomized; PUR: Purmorphamine; RANK: Receptor activator of nuclear factor kappa-B; RANKL: RANK ligand; SAG: Smoothened agonist; SAL: Salidroside; SD: Sprague Dawley; Smad: Sma- and Mad-related protein; Smo: Smoothened; TGFβ: Transforming growth factor beta; TGFβR: Transforming growth factor beta receptor; TNFα: Tumor necrosis factor alpha; TRAF6: TNF receptor-associated factor 6; VA1: VCP activator 1; VEGF: Vascular endothelial growth factor; VPA: Valproic acid; Wnt: Wingless-related integration site; W9: WP9QY; ZA: Zoledronic acid; Zg: Zingerone.

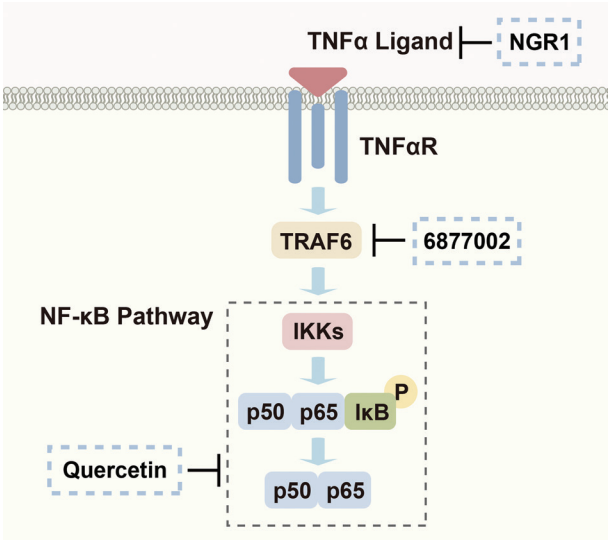

Figure 2. Small molecules targeting the inflammatory phase to promote fracture healing. The TNFα ligand binds to TNFαR, initiating intracellular signaling cascades through TRAF6, including the NF-κB pathway. This pathway subsequently regulates osteogenesis through NF-κB subunits. At present, small molecules can modulate the TNFα ligand, TRAF6, or the NF-κB pathway to influence TNFα signaling.
Abbreviations: IKKs: IκB kinase complexes; IκB: Inhibitor of kappa B; NF-κB: Nuclear factor kappa-B; NGR1: Notoginsenoside R1; NF-κB: Nuclear factor kappa-B; P: Phosphate; p50: Nuclear factor NF-κB p50 subunit; p65: Nuclear factor NF-κB p65 subunit; TNFα: Tumor necrosis factor alpha; TNFαR: Tumor necrosis factor-alpha receptor; TRAF6: TNF receptor-associated factor 6.

pathway, promoting endothelial cell proliferation, migration, and capillary-like structure formation, demonstrating angiogenic activity in mouse fetal long bone.[84]

4.2.2. Regulation of Notch pathway by small molecules

Notch signaling consists of five ligands (Jagged-1, Jagged-2, DLL1, 3, and 4) and four transmembrane receptors (Notch 1 – 4). Ligand-receptor interactions trigger the proteolytic cleavage of the Notch receptor by the γ-secretase complex, releasing the intracellular domain, which subsequently induces the transcription of classical Notch target genes, including those from the Hes and Hey families **(Figure 3)**.

Co-culturing MC3T3-E1 osteoblasts with valproic acid (VPA) was found to activate the Notch pathway, significantly promoting cell mineralization and the expression of osteogenic proteins.[85] The delivery of VPA through hydrogel to femoral metaphyseal defects in ovariectomized (OVX) rats accelerated both vascular and bone formation.[85] Imiquimod treatment of mouse immortalized multipotent adipose-derived cells (iMAD) activated the Notch pathway, increasing ALP activity and matrix mineralization, leading to a 2.8-fold enhancement in the cells' osteogenic differentiation capacity and a 1.6-fold increase in BMP-9-induced osteogenic differentiation.[86]

Conversely, Notch pathway inhibitors have also demonstrated therapeutic potential by counteracting the inhibitory effects of Notch signaling on bone formation, such as γ-secretase inhibitors like DAPT.[87,88] Notably, DAPT application enhanced the osteoblastic commitment of embryonic and induced pluripotent stem cells[87] and improved the diminished osteogenic differentiation potential of aged BMSCs.[88]

4.2.3. Regulation of TGFβ pathway by small molecules

Transforming growth factor-beta and BMPs are both members of the TGFβ superfamily, each with both canonical and non-canonical signaling pathways,[34] mediated by Smad 2/3 and MAPKs, PI3K-Akt, Ras homolog family member A, protein phosphatase 2, etc. (**Figure 4**).[117]

Transforming growth factor-beta activators have shown promise in enhancing fracture healing. Amygdalin upregulated phosphorylated Smad2/3, enhanced cartilage formation in $Gli1^+$ MSCs, and promoted tibial fracture healing in C57BL/6 mice through the TGFβ/Smad signaling pathway.[10] Similarly, zingerone exhibited a potent osteoinductive effect by inhibiting Smad7, a negative regulator of TGFβ1 signaling, which in turn promoted osteoblast differentiation and calcium deposition in MSCs.[95]

On the other hand, TGFβ inhibitors have also been reported to enhance bone healing. TGFβ signaling activation suppressed transcription of the Wnt4 agonist, nuclear receptor 4 A1. Treatment with nuclear receptor 4 A1 agonist, cytosporone B, promoted BMSC differentiation *in vitro* and enhanced skull and tibial defect repair, accelerating fracture healing *in vivo*.[96] TGFβ1 receptor inhibitor, SB431542, downregulated TGFβ-induced inhibitory Smad expression, and potentially accelerated BMP-stimulated osteogenesis, functioning as a skeletal repair promoter in mouse skull defect models and swine jawbone defect models.[97,98]

4.2.4. Regulation of BMP pathway by small molecules

BMP pathway begins with the interaction between BMPs and specific type I or type II receptors,[34,35] subsequently regulating target gene expression through the phosphorylated Smad1/5/8 complex (canonical pathway)[118] or by activating various protein kinases, such as c Jun N-terminal kinase/P38, PI3K/Akt, Ras homolog family member-GTPase, and MAPK (non-canonical pathways) (**Figure 4**).[34]

BMPR agonists have been shown to promote fracture healing. SY-LB-35 and SY-LB-57, full BMPR agonists, elevated the expression of phosphorylated Smad and activated the PI3K/Akt signaling pathway, significantly increasing cell proliferation and viability in the C2C12 myoblast cell line.[89] Trapidil increased BMPR reactivity, induced Smad1/5/9 phosphorylation, and enhanced *Runx2* expression, thus promoting osteoblastic differentiation and bone regeneration in a rat cranial defect model.[90] FK506-binding protein 12 is an intracellular BMP inhibitor targeting BMPR-I. FK506, an FDA-approved immunosuppressant, can bind to FK506-binding protein 12 and inhibit its function, thereby activating BMPR and elevating pSmad1/5 levels.[91] Sangadala *et al.*[92] found that FK506 could induce ALP activity in C2C12 cells, and local delivery of FK506 on collagen sponges in a rat subcutaneous implantation model resulted in mineralization comparable to BMP2, suggesting FK506's potential as a future

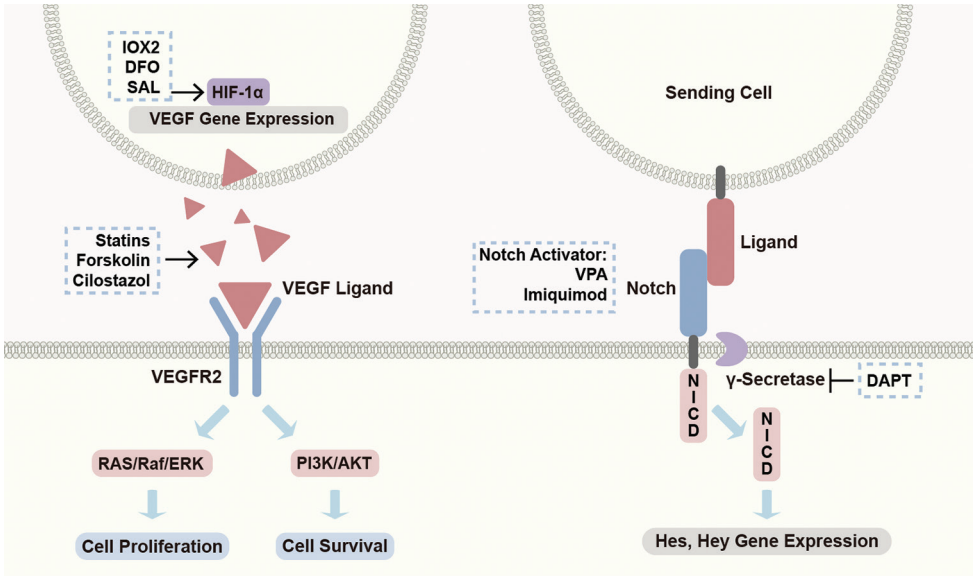

Figure 3. Small molecules targeting angiogenesis. VEGF pathway: VEGF ligands bind to VEGFR2, activating the downstream ERK pathway to promote cell proliferation and the PI3K pathway to support cell survival. Small molecules can regulate VEGF ligand levels and modulate HIF-1α expression to influence this pathway. Notch pathway: Ligands expressed on neighboring cells bind to Notch receptors, leading to the release and nuclear translocation of NICD, which activates the transcription of osteogenic genes such as Hes and Hey. Small molecules can regulate Notch signaling by modulating γ-secretase activity.
Abbreviations: AKT: Protein kinase B; DFO: Deferoxamine; ERK: Extracellular regulated kinase; Hey: Hes-related repressor Herp, Hesr, Hrt, CHF, gridlock; Hes: Hairy and enhancer of split; HIF-1α: Hypoxia-inducible factor 1 alpha; IOX2: Iron chelator 2; NICD: Notch intracellular domain; PI3K: Phosphoinositide 3-kinase; Raf: Raf protein kinase; RAS: Rat sarcoma; SAL: Salidroside; VEGF: Vascular endothelial growth factor; VEGFR2: Vascular endothelial growth factor receptor 2; VPA: Valproic acid.

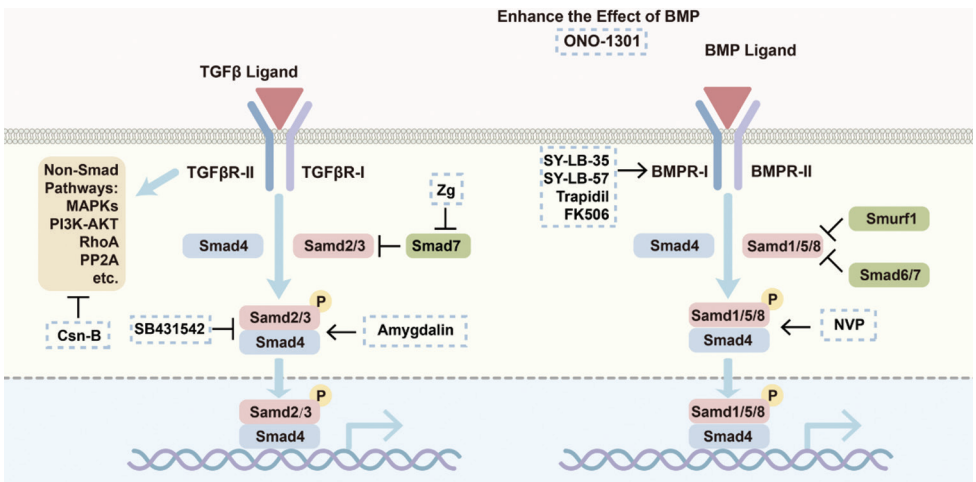

Figure 4. Small molecules for callus formation. TGFβ pathway: Binding of TGFβ to its receptor activates Smad2/3, which then forms a complex with Smad4. This complex translocates to the nucleus, influencing gene expression involved in both osteoclast and osteoblast differentiation. Small molecules can regulate the expression of Smads, Smad complexes, and non-canonical signaling pathways to modulate TGFβ signaling. BMP pathway: BMP ligands bind to type I and II receptors, leading to the phosphorylation of Smad1/5/8. These phosphorylated Smads form complexes with Smad4 and translocate to the nucleus to regulate osteogenic genes such as *Runx2*. Small molecules can modulate BMPR-I activity and Smad complexes to influence BMP signaling. Non-canonical pathways, such as MAPK and PI3K, are activated downstream, influencing the transcription of genes involved in bone formation and resorption.
Abbreviations: AKT: Protein kinase B; BMP: Bone morphogenetic protein; BMPR: BMP receptor; Csn-B: Cytosporone B; MAPK: Mitogen-activated protein kinase; NVP: N-Vinyl-2-pyrrolidone; PI3K: Phosphoinositide 3-kinase; PP2A: Protein phosphatase 2; Samd: Sterile alpha motif domain containing; Smad: Sma- and Mad-related protein; Smurf1: Smad ubiquitination regulatory factor 1; RhoA: Ras homolog family member A; TGFβ: Transforming growth factor-beta; TGFβR: TGFβ receptor; Zg: Zingerone.

osteogenic agent. Another study demonstrated that FK506 promoted osteogenic differentiation of hMSCs and induced bone formation in rat femoral defect and rabbit spinal fusion models, showcasing its efficacy across different models.[91]

Assisting in enhancing the osteogenic effect of the BMP pathway is another approach to promoting fracture healing. N-Vinyl-2-pyrrolidone (NVP), an FDA-approved drug, enhanced the nuclear translocation of phosphorylated Smad1/5/9 in a

dose-dependent manner, thereby increasing ALP activity in BMP2-induced C2C12 cells.[93] Local delivery of NVP through a biodegradable membrane promoted healing in a rabbit cranial defect model.[93] ONO-1301, a prostacyclin analog known for its pro-angiogenic properties, when combined with BMP2 on a scaffold, improved MSC migration and osteogenic differentiation *in vitro* and promoted bone regeneration in a rat cranial defect model, surpassing the effects of scaffolds with BMP2 alone.[94]

4.2.5. Regulation of Wnt pathway by small molecules

Upon Wnt binding to its receptor, Frizzled, along with the co-receptor, LRP5/6, β-catenin accumulates in the cytoplasm and subsequently translocates to the nucleus, where it drives the transcription of osteogenesis-related genes (**Figure 5**).[45]

Glycogen synthase kinase 3β (GSK-3β) phosphorylates and reduces β-catenin levels, positioning GSK-3β inhibitors as promising candidates for promoting bone formation, including 1-Azakenpaullone[103] and CHIR99021(C91).[104] 1-Azakenpaullone enhanced Wnt signaling and nuclear β-catenin accumulation in hMSCs, leading to elevated *Runx2* expression and enhanced osteoblastic differentiation and mineralization.[103] Similarly, C91 boosted ALP activity and secretion in BMSCs. When incorporated into an artificial bone scaffold mimicking the *in vivo* environment, the slow release of C91 promoted osteogenic differentiation and mineralization of BMSCs.[104]

Sclerostin, a protein that inhibits Wnt signaling by competitively binding to LRP5/6, is another therapeutic target for enhancing fracture healing. VA1 (an FDA-approved drug) and C07, small molecules that block the extracellular sclerostin interaction with LRP5/6, activated Wnt pathways, produced dose-dependent ectopic mineralization in a non-bony environment in rats, and significantly increased bone healing in a posterior spinal arthrodesis model in rabbits.[105] BMP pathway activation was also involved in the process, mediated by GSK-3β-enhanced Smad1 phosphorylation.[105] Dickkopf-1 (DKK1) inactivates the Wnt pathway by blocking Wnt binding to the Lrp5/6 receptor. The small molecule WAY-262611 inhibited DKK1, thereby activating Wnt/β-catenin signaling and significantly improving lumbar fusion in osteoporotic rats.[106]

4.2.6. Regulation of Hedgehog pathway by small molecules

The Hedgehog pathway is initiated by three ligands – Sonic Hedgehog, Indian Hedgehog, and Desert Hedgehog – binding to its receptor, Patched.[119] This binding relieves the suppression of Smo by Patched, enabling Smo to trigger intracellular signaling cascades that activate the Gli family of transcription factors, which modulate various cellular activities (**Figure 5**).[120]

Activation of Smo is a key target for regulating the Hedgehog pathway. Purmorphamine (PUR) can activate Smo to trigger the Hedgehog pathway, thereby promoting osteogenic differentiation of endogenous stem cells and MSCs.[100] Another study utilized 20S-hydroxycholesterol, a naturally occurring small molecule that targets Smo, to form sterosomes with PUR, which were then immobilized onto scaffolds. This scaffold significantly improved osteogenic differentiation of MSCs through Hedgehog pathway activation and enhanced bone repair in a mouse cranial defect model.[100]

Smoothened agonist (SAG), another Smo activator, induced osteogenesis in primary neonatal mouse calvarial cells and dose-dependently promoted bone healing and angiogenesis in a mouse cranial defect model.[101] SAG21k, a derivative of SAG,

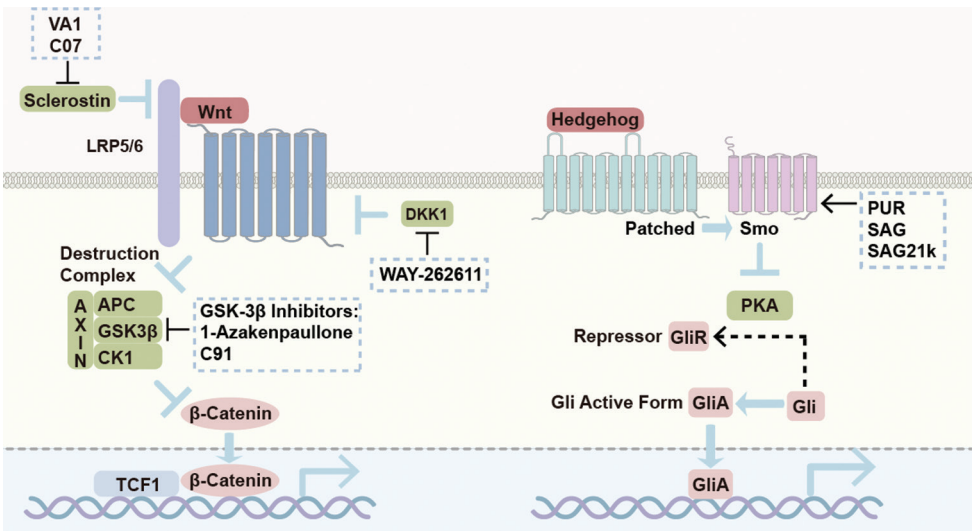

Figure 5. Small molecules for callus formation. Wnt pathway: In the Wnt signaling pathway, Wnt ligands bind to Frizzled and LRP5/6 receptors, leading to the degradation of GSK-3β and the accumulation of β-catenin, which then translocates to the nucleus to drive gene transcription. Small molecules can regulate GSK-3β levels and modulate Wnt inhibitors like DKK1 and sclerostin to control the Wnt pathway. Hedgehog pathway: Hedgehog proteins bind to Patched receptors on target cells, activating Smo and subsequently activating Gli transcription factors to regulate downstream target genes. Small molecules can modulate Smo activation to regulate Hedgehog signaling.
Abbreviations: APC: Adenomatous polyposis coli; CK1: Casein kinase 1; C91: CHIR99021; DKK1: Dickkopf-related protein 1; GSK3β: Glycogen synthase kinase 3 beta; Gli: Glioma-associated oncogene family zinc finger; LRP: Low-density lipoprotein receptor-related protein; PKA: Protein kinase A; PUR: Purmorphamine; SAG: Smoothened agonist; Smo: Smoothened; TCF1: T-cell factor 1; Wnt: Wingless-related integration site.

activated the Sonic Hedgehog pathway and enhanced cartilage formation.[102] Sequential application of SAG21k and IOX2 yielded significant effects in a mouse femoral segmental defect model, with SAG21k promoting cartilage development in the callus and IOX2 facilitating its conversion to bone, showing potential in the healing of challenging bone injuries.[102]

4.3. Small molecules in bone remodeling phase for fracture healing

4.3.1. Regulation of RANKL pathway by small molecules

Receptor activator of nuclear factor kappa-B ligand is a ligand of the transmembrane protein RANK, which recruits TRAFs (particularly TRAF6). This interaction activates downstream MAPKs, NF-κB, PI3K/Akt, ultimately leading to the activation of key transcription factors such as c-Fos and nuclear factor of activated T cells, cytoplasmic 1 (NFATc1) (**Figure 6**).[121]

Overactivation of the RANKL pathway can lead to osteoclast dysregulation and bone loss. Therefore, biomolecules that finely tune RANKL signaling are key for managing this process. Zoledronic acid (ZA), a member of the bisphosphonate family, downregulates RANKL-related gene expression and inhibits osteoclast-mediated bone resorption. Local delivery of ZA through hydroxyapatite exhibited dual effects, promoting osteogenesis while suppressing osteolysis, and facilitating bone defect repair in rabbit femur models.[107] Aspirin reduced RANKL-induced dendritic cell differentiation into osteoclasts by inhibiting NF-κB and NFATc1 activation, which reduced the number of osteoclasts and promoted bone regeneration in a rat mandibular defect model.[108]

OPG, a soluble decoy receptor produced by osteoblasts, inhibits RANKL/RANK binding.[61] Treatment of hMSCs with XAV-939 increased OPG expression while decreasing RANKL expression, thereby inhibiting osteoclast differentiation.[109] WP9QY (W9) competes with RANK for binding with RANKL, thus inhibiting RANK-RANKL interactions and blocking osteoclast differentiation.[110,111] Local administration of W9 promoted callus formation and aided fracture healing in a rat femur delayed-union model.[111]

5. Spatiotemporal precision in drug delivery strategies

Bone tissue engineering has made significant progress in achieving spatially precise delivery of small molecules. However, spatial delivery alone may present challenges, as the therapeutic effects of these drugs can vary depending on the phase of the treatment. For instance, the early-stage release of magnesium ions promotes the reconstruction of the neurovascular network, whereas its later-stage release inhibits mineralization.[122] Controlled, spatiotemporal drug release over time can help address this issue. Spatiotemporal precision delivery, which allows for sequential drug release and dose modulation in accordance with the temporal progression of healing, can significantly enhance overall repair outcomes. Numerous approaches have been explored to regulate the rate and sequence of release for multiple drugs.

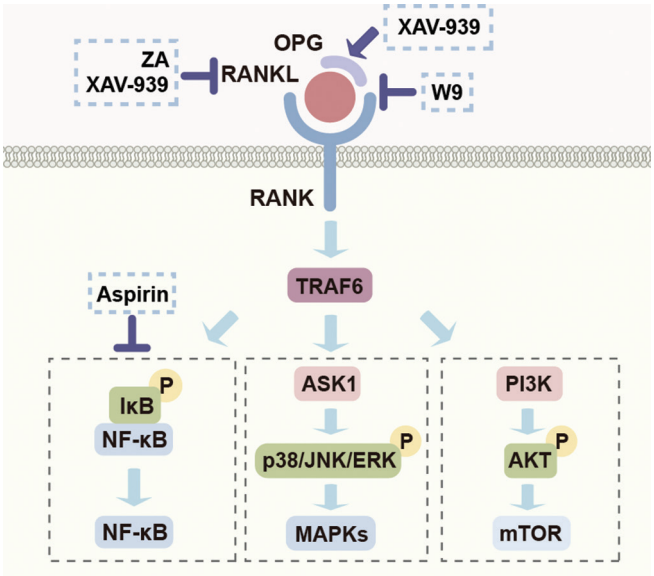

Figure 6. Small molecules for bone remodeling. RANKL, produced by osteoblasts, binds to RANK receptors on osteoclast precursors, initiating signaling cascades through TRAF6, including MAPK, NF-κB, and PI3K/AKT pathways, ultimately enhancing NFATc1 expression. OPG serves as a decoy receptor for RANKL, blocking RANK-RANKL interactions. Small molecules can modulate RANKL expression, inhibit RANK-RANKL binding, increase OPG levels, and block NF-κB signaling to suppress RANKL-mediated osteoclastogenesis.

Abbreviations: AKT: Protein kinase B; ASK1: Apoptosis signal-regulating kinase 1; ERK: Extracellular signal-regulated kinase; IκB: Inhibitor of kappa B; JNK: c-Jun N-terminal kinase; MAPK: Mitogen-activated protein kinase; mTOR: Mechanistic target of rapamycin; NFATc1: Nuclear factor of activated T-cells, cytoplasmic 1; NF-κB: Nuclear factor kappa-B; OPG: Osteoprotegerin; P: Phosphate; PI3K: Phosphoinositide 3-kinase; RANK: Receptor activator of nuclear factor kappa-B; RANKL: RANK ligand; TRAF6: TNF receptor-associated factor 6; W9: WP9QY; ZA: Zoledronic acid.

5.1. Drug affinity for targeted microenvironments

In the development of spatiotemporal delivery systems, researchers have capitalized on the differential affinity of drugs to their environments for controlled release. Zhang *et al.*[123] engineered a spatiotemporal scaffold system that leveraged the varying solubility profiles of drugs. The highly hydrophilic strontium ranelate demonstrated a strong affinity for aqueous environments, exhibiting substantial burst release (70% of the payload within 24 h). Conversely, the hydrophobic resveratrol showed preferential retention in polycaprolactone/tricalcium phosphate (PCL/TCP) matrices, achieving sustained release over 21 days. This dual-release system significantly enhanced angiogenesis, suppressed osteoclastic activity, and promoted MSC osteogenesis in rat mandibular defect models. Lee *et al.*[124] developed a sequential delivery platform through differential drug-scaffold interactions. The anti-inflammatory substance P, loaded through a simple hydration method, displayed burst release, discharging approximately 90% of the payload within 14 days. Concurrently, 4-hexylresorcinol was tightly bound to the scaffold matrix, maintaining therapeutic concentrations for 70 days. This system promoted angiogenesis and osteogenesis, offering a novel therapeutic approach to osteoporosis (OP)

healing. Furthermore, Sun *et al.*[125] achieved sustained delivery through affinity-based conjugation by coupling BMP2 with PlGF-2123-144 peptide, which had a strong affinity for extracellular matrix-based materials, thereby achieving a slow and sustained release of BMP2. Apt19s, which effectively recruited MSCs, exhibited a binding affinity for BMSCs and released rapidly in the early phase. The sequential release of Apt19s to recruit BMSCs and BMP2 to promote osteogenic differentiation demonstrated effective osteogenic potential both *in vitro* and *in vivo*.

5.2. Release kinetics differences across delivery platforms

Poly (lactic-co-glycolic acid) (PLGA)-based systems are widely utilized in drug delivery due to their ability to release drugs continuously over extended periods as PLGA degrades. Given the degradation rate of PLGA is adjustable, the drug release profile from PLGA microspheres can, in theory, be controlled. Lee *et al.*[126] developed a spatiotemporal scaffold loaded with BMP2 and biodegradable microspheres encapsulating alendronate (ALN). Initially, BMP2 was released rapidly, followed by the slow release of ALN from the PLGA microspheres. The sequential release of BMP2 and ALN effectively promoted MSC osteogenic differentiation while inhibiting osteoclast activity, significantly enhancing osteogenic activity. Hao *et al.*[127] incorporated platelet-derived growth factor with two B subunits (PDGF-BB) directly into hydrogel scaffolds, while kartogenin (KGN) was encapsulated in PLGA microspheres and integrated into the scaffold. This system achieved the early release of PDGF-BB to recruit MSCs and the sustained release of KGN to promote MSC chondrogenic differentiation, facilitating enhanced bone regeneration *in situ* for tissue engineering applications. By adjusting the initial concentrations of silk fibroin (SF) and polyvinyl alcohol, the drug release rate of the nanosphere-incorporated matrix could be modulated. A 5% nanosphere-incorporated matrix achieved a rapid initial release of drugs that promoted BMSC migration, while a 0.2% nanosphere-incorporated matrix provided a slow and sustained release of molecules that promoted chondrogenesis, effectively facilitating osteochondral regeneration.[128]

5.3. Stimuli-responsive delivery systems for controlled release

Stimuli-responsive delivery systems enable controlled biomolecular release through exogenous stimuli (e.g., temperature, light, and electric/magnetic fields) or endogenous stimuli (e.g., enzymes, pH changes, and redox gradients). Dong *et al.*[6] designed a near-infrared light-responsive hydroxyapatite/collagen-based three-dimensional (3D)-printed scaffold. This system initially released SIM to recruit MSCs, and upon exposure to near-infrared light, precisely modulated the release of PGL, a compound that promoted osteogenic differentiation, thereby optimizing bone regeneration outcomes. Xue *et al.*[129] synthesized an enzyme-responsive hydrogel system based on matrix metalloproteinase-7, an enzyme that naturally appears 3 days post-fracture. Initially, most physically encapsulated nanoparticles were released through diffusion. In the presence of matrix metalloproteinase-7, chemically bonded nanoparticles were rapidly released as the hydrogel degraded, enabling drug release in response to the fracture microenvironment. Zha *et al.*[130] developed pH-responsive nanocomposite hydrogel using zinc-gallium-humic acids (soluble at pH ≥ 7). In the inflammation stage (pH < 7), the nanoparticles released zinc ions and gibberellic acid to combat bacteria. As the pH increased in the later stage (pH ≥ 7), the humic acids dissolved and promoted tissue regeneration, facilitating rapid healing of infectious fractures in mice.

At present, substitutes for artificial bone grafts primarily include bone cement, bioglass, alloy materials, naturally derived materials, and synthetic polymer materials.[131,132] Among these, natural materials are favored for their superior degradation properties, while synthetic polymers are widely used in bone repair-related research due to their mechanical properties and stable and adjustable structures.[133] When designing scaffolds with spatiotemporal drug release capabilities, it is essential to consider additional characteristics such as mechanical properties, biocompatibility, degradability, appropriate porosity, and the potential for convenient surgical application.[134] These characteristics are crucial for the scaffold's future clinical applications.

6. Clinical applications of small molecules in fracture healing: From conventional to spatiotemporal strategies

6.1. Translation of conventional small molecules

To date, numerous small-molecule drugs have demonstrated osteogenic effects in clinical applications. For instance, a randomized controlled trial in healthy elderly women showed that intermittent administration of dasatinib and quercetin over a short period (2 – 4 weeks) promoted bone formation (NCT04313634). Several prospective randomized controlled trials have confirmed that SIM enhances osteoblastic activity and facilitates autologous bone formation following tooth extraction.[135-137] ZA, a bisphosphonate used to treat OP, has improved bone health in various conditions, including in elderly women with cognitive impairment.[138] In addition, the combination of ZA and calcitriol in diabetic OP patients post-fracture has been reported to enhance bone density and metabolism, alleviate pain, improve knee joint function, and reduce the risk of re-fracture.[139] These studies highlight the clinical translational potential of small-molecule drugs. However, their clinical application faces several challenges, including low drug efficacy, local adverse effects, and systemic off-target effects.

6.1.1. Low drug efficacy

Small molecule drugs may exhibit suboptimal clinical efficacy due to limitations such as poor water solubility, instability in weakly alkaline conditions, and low oral bioavailability. For example, the natural osteogenic small molecule quercetin has poor solubility and low oral absorption, limiting its practical application.[140] The conventional oral administration of bisphosphonates results in poor gastrointestinal absorption, typically <1%, with further reductions when taken alongside food or calcium.[141]

Encapsulating small molecules in biodegradable and biocompatible delivery systems can address these issues.[75] A Phase III clinical trial demonstrated that an oral

phospholipid-based quercetin formulation significantly improved oral bioavailability, achieving plasma levels 20 times higher than unformulated quercetin, with no reported adverse effects in human volunteers.[140] Similarly, calcium phosphate cement/PLGA composite materials have been used for controlled local delivery of ALN, facilitating bone defect repair in osteoporotic rat femoral condyles.[141]

6.1.2. Local adverse effects

Some small molecule drugs, even when administered at therapeutic doses, can cause unintended local adverse effects. For instance, dexamethasone has been shown to induce osteogenic differentiation by inhibiting *SOX9* expression and simultaneously promote adipocytic differentiation in a dose-dependent manner through upregulation of peroxisome proliferator-activated receptor gamma, impairing bone marrow osteogenesis.[142] Similarly, multiple cAMP pathway activators, such as 8-bromo-cAMP and forskolin, enhance osteogenesis while inhibiting MSC proliferation.[143]

To mitigate these local adverse effects, strategies such as short-term treatment, dose reduction, and reduced administration frequency have been proposed.[144] For example, short-term, low-dose administration of dexamethasone during early differentiation stages has been reported to minimize adipogenic side effects.[142] A phenotypic mini-screening study identified forskolin (100 μM) as the most potent osteogenic agent with minimal toxicity when administered as a single 24-h dose to rabbit BMSCs.[145] Furthermore, forskolin-loaded scaffolds implanted in a rabbit radial defect model significantly promoted *in vivo* bone regeneration, comparable to recombinant human BMP-2, while reducing cAMP-related adverse effects.[79]

6.1.3. Systemic off-target effects

One of the major limitations of small molecules in clinical applications is their systemic off-target effects. Due to their high diffusivity, these compounds may induce non-specific systemic adverse reactions.[145] For instance, natural drug psoralen has been demonstrated to accelerate femoral defect repair in rats through the ERK and BMP pathways,[146] but its accumulation in the liver depletes glutathione, leading to hepatotoxicity.[147] Intravenous ZA administration may alter endogenous cytokine release, triggering infusion-related inflammatory responses,[107] which can result in flu-like symptoms, hypocalcemia, gastrointestinal disturbances, and renal impairment.[148]

Targeted drug delivery using scaffolds can minimize off-target effects by localizing drug action to the damaged bone tissue. For example, psoralen-loaded polyelectrolyte multilayer-coated titanium mesh scaffolds enhanced spinal fusion in rats while preventing post-operative complications.[149] Similarly, the localized release of ZA from hydroxyapatite scaffolds minimized systemic exposure, reducing adverse effects while promoting osteoblast activity and bone defect repair in rabbits.[107] These findings underscore the importance of precise spatiotemporal drug targeting to minimize systemic adverse reactions and maximize therapeutic efficacy.

Systemic side effects also contribute to discrepancies between fundamental research and clinical trials. For example, VPA has been shown to enhance fracture repair in OVX rats through local hydrogel-based release.[85] However, clinical studies have reported that systemic VPA use as an antiepileptic drug reduces bone mass.[150-153] This discrepancy may be attributed to VPA-induced osteoclast activation,[154] which, in localized delivery, facilitates bone remodeling by clearing necrotic bone tissue but, in systemic administration, leads to overall bone loss. Similarly, aspirin promoted osteogenesis in cellular and rat models by inhibiting NF-κB and NFATc1 activation.[108] However, a clinical trial in elderly individuals found that aspirin did not reduce fracture risk and instead increased the risk of serious falls (ACTRN12615000347561). This divergence may stem from aspirin's differential effects on osteoblasts and osteoclasts in fracture sites versus normal bone tissue.

6.2. Translation of spatiotemporal delivery systems

Several small molecule-releasing materials have advanced to clinical and pre-clinical evaluation. Injectable PLGA polymer microsphere formulations for anticancer drug delivery have been approved for clinical use.[155] SF, derived from silkworms, has gained widespread application in tissue engineering due to its excellent biocompatibility, controllable degradation rates, strong mechanical properties, and non-inflammatory byproducts. SF has been approved by the FDA for clinical use in settings such as sutures and support structures in reconstructive surgery.[128] A keratin-based hydrogel has been developed for use in a porcine burn model, enabling the controlled release of fluoroquinolone, an inhibitor of fibrosis that works by inhibiting collagen synthesis. This system fostered a favorable microenvironment for burn wound healing *in vivo*.[156] Kim *et al.*[157] developed Inventage Lab Inc. Precision Particle Fabrication Microsphere® technology to fabricate PLGA microspheres encapsulating finasteride. The inclusion of PLA02A prolonged drug release in beagle dogs for up to 3 months.

Pre-clinical studies have demonstrated the efficacy of spatiotemporally controlled small molecule release in large animal models. Zhang *et al.* incorporated nanospheres into a sericin porous matrix, achieving controlled release by adjusting the initial SF/polyvinyl alcohol concentration. This system facilitated early-stage recruitment of BMSCs through E7 release, followed by sustained KGN release to induce chondrogenesis, enhancing cartilage defect repair in rabbits (**Figure 7**).[128] A sequentially releasing scaffold delivered SIM for early-stage chemotaxis, followed by late-stage PGL release to promote MSC osteogenic differentiation. This scaffold enhanced ALP activity, upregulated osteogenic gene expression, and improved bone regeneration in a rabbit cranial defect model.[6]

6.2.1. Pre-clinical insights into spatiotemporal delivery in disease models

Despite significant advancements in medical treatments, fractures that cannot spontaneously heal remain a major challenge in clinical practice, particularly osteoporotic fractures and large bone defects.[131,158] Osteoporotic fractures, which are severe consequences of OP, are difficult to treat and are associated with high mortality rates. Due to decreased bone strength and

microstructural degradation in OP, the bone's repair capacity is impaired, delaying the healing of bone defects.[158] In this context, spatiotemporal drug release offers a potential therapeutic strategy for osteoporotic fractures. Lee *et al.*[124] designed a biomimetic scaffold that rapidly releases anti-inflammatory SP during the early stages of fracture healing. The sustained release of 4-hexylresorcinol promoted endothelial cell proliferation through MAPK signaling and inhibited osteoclastogenesis by disrupting the IκB/NF-κB signaling pathway. The sequential release of SP and 4-hexylresorcinol endowed the scaffold with multiple osteogenic abilities in the osteoporotic microenvironment, significantly promoting femoral defect healing in OVX rats. Another study grafted ALN, a drug that inhibits osteoclast activity, onto the surface of microspheres, while parathyroid hormone, which promotes osteogenesis, was loaded into the internal cavity of the microspheres. The spatiotemporal, sustained release of these drugs effectively suppressed osteoclast activity while stimulating osteogenesis, providing an effective treatment for osteoporotic bone defects in OVX rats.[159]

Treating large segmental bone defects also remains a formidable clinical challenge.[131] Autologous bone grafts are considered the "gold standard" for treatment; however, they are limited by donor site morbidity and a scarcity of donor

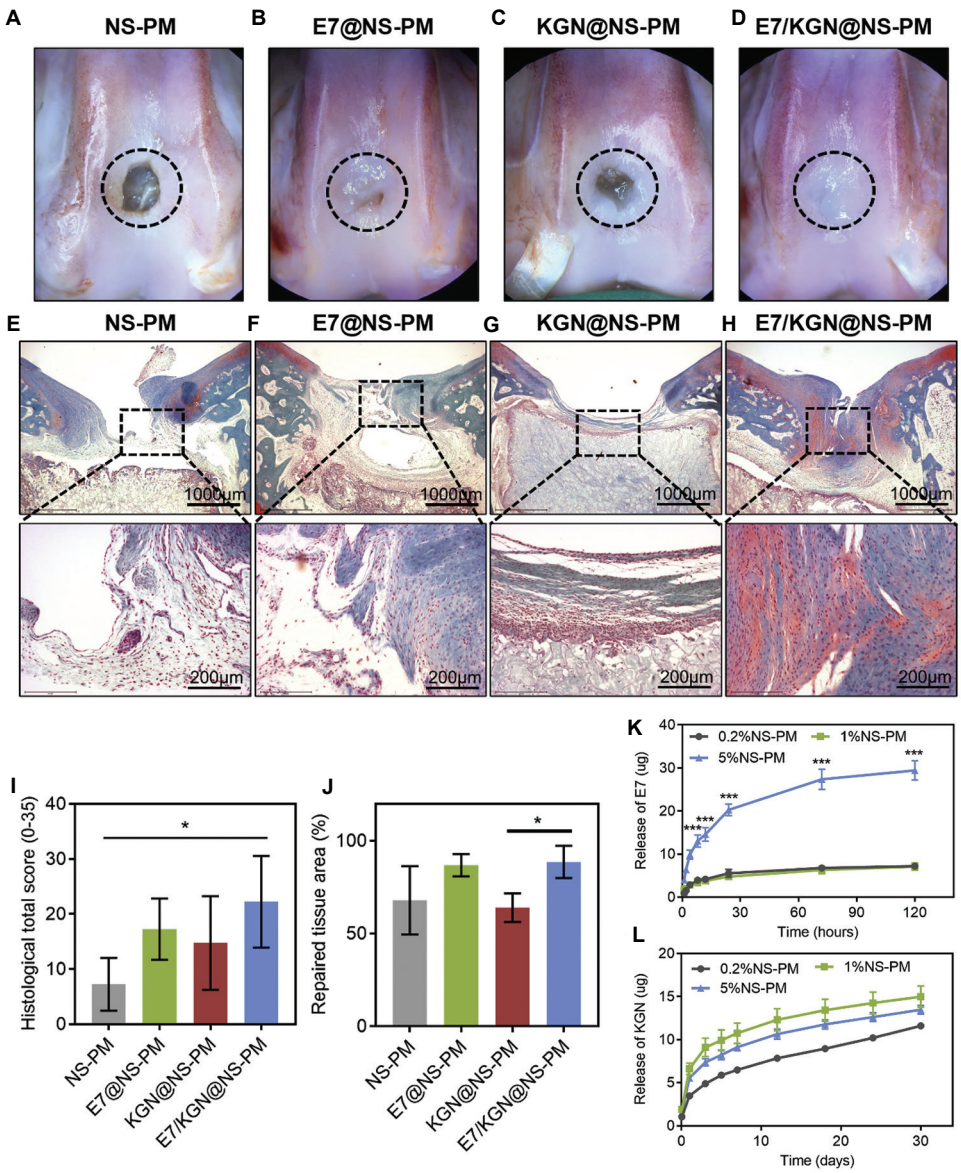

Figure 7. Spatiotemporal delivery strategy enhancing cartilage defect repair in rabbit models. (A-D) Macroscopic morphology of joint specimens collected 12 weeks post-surgery. Black circles indicate the boundaries of the original defect. (E-H) Safranine-O staining images. The bottom panels show higher magnification images of the corresponding black boxes in the top panels. Scale bars = 1,000 μm (top panels), 200 μm (bottom panels). (I) Histological evaluation according to the established histological scoring system. (J) Quantification of the repair tissue area as a percentage of the total defect area. (K and L) Cumulative E7 and kartogenin (KGN) release from SF nanosphere matrices at 37 C. *p<0.05, **p<0.01. Reprinted from Zhang *et al.*[128] Copyright 2020 Authors.
Notes: E7@NS-PM: E7-loaded nanosphere-porous matrix; E7/KGN@NS-PM: E7/KGN-loaded nanosphere-porous matrix; KGN@NS-PM: KGN-loaded nanosphere-porous matrix; NS-PM: Unloaded nanosphere-incorporated matrix.

sites, severely restricting their use. Although allografts are less limited by supply, they present challenges related to the risk of disease transmission and immune rejection, which limit their clinical success.[131] In this context, spatiotemporal drug release represents a potential strategy for treating large bone defects. A study demonstrated that a composite scaffold, which released the chemokine PDGF-BB to recruit and proliferate endogenous stem cells in the early stages, followed by the long-term release of KGN to promote MSC chondrogenesis, effectively promoted the healing of large bone defects in critical-sized rabbit knee joint defects.[127]

6.2.2. Clinical translation challenges and mitigation strategies

Despite these advancements, clinical studies remain limited. Several challenges hinder clinical translation, including the complexity of design and fabrication processes, as well as the low bioactivity of certain materials. Current spatiotemporally controlled release systems have made progress in fundamental research, achieving effective release of multiple small molecules. However, challenges persist in achieving precise temporal control over drug release and reducing the *in vivo* immunogenicity of carrier materials. Consequently, the clinical translation of spatiotemporal strategy remains in its early stages.

The functional requirements for spatiotemporally controlled release of small molecule drug materials are as follows: (i) the ability to effectively load multiple small molecules; (ii) precise recognition of target tissues *in vivo*; (iii) the ability to release small molecules in an ordered manner within target tissues to achieve therapeutic concentrations during the treatment window; (iv) minimal drug leakage during non-treatment periods; and (v) good bioactivity. These complex requirements lead to intricate design and fabrication processes, contributing to the complexity of production and high manufacturing costs. These factors hinder large-scale production and clinical translation. At present, nanodrug delivery systems and prodrug strategies show promise in meeting these criteria while minimizing challenges in clinical translation and application.[160]

6.2.3. Nanodrug delivery systems

In recent years, human serum albumin (HSA) has been widely explored as a multifunctional nanodrug delivery system in biomedical applications due to its superior biodegradability, biocompatibility, non-toxicity, and non-immunogenicity. The advantages of using HSA as a carrier for small molecules include: (i) its inherent biocompatibility as an endogenous substance; (ii) its unique spatial structure and abundant surface functional groups that provide strong drug-loading capacity; (iii) the ability to significantly extend the half-life of drugs in circulation; and (iv) its ability to protect exogenous drugs from enzymatic degradation, thereby reducing drug leakage. Although this approach offers unique advantages, there are still unresolved issues and mechanisms to explore. First, while HSA-based nanodrug delivery systems have proven effective in cancer treatment, their efficacy in other biomedical disease models remains to be validated. Second, how HSA can covalently conjugate drugs for controlled release at the target site and subsequent transport pathways requires further investigation.[161]

6.2.4. Prodrug strategies

Nanodrugs typically consist of a small amount of active drug components and carrier materials, which have long been used to improve pharmacokinetics and biodistribution, enhance therapeutic efficacy, and reduce side effects. However, the low drug-loading capacity inevitably requires the use of excess carrier materials to deliver the desired active pharmaceutical dose, posing an additional burden on patients, particularly when repeated dosing is required. Prodrug nanodrugs can increase bioavailability through chemical modification of small molecule drugs, enabling spatiotemporal control of drug release and activation. Amphiphilic prodrugs, which involve modifying hydrophobic/hydrophilic drugs into amphiphilic prodrugs capable of self-assembling into nanostructures, have emerged as a convenient method for manufacturing nanodrugs. This amphiphilic prodrug strategy offers several advantages, including minimal use of inert carrier materials, well-characterized prodrug structures, high and stable drug-loading rates, and controlled drug release without explosive release. These advantages facilitate the faster clinical translation of self-assembled small molecule nanodrugs.[162,163]

The therapeutic mechanisms of small molecule drugs in fracture healing primarily involve the regulation of key signaling pathways related to bone metabolism. We expect that the research and application of these strategies will enable the spatiotemporal release of small molecule drugs in fracture healing, allowing for precise and effective modulation of signaling pathways while minimizing systemic side effects, thereby maximizing medical benefits for patients.

7. Small molecule drug screening: Strategies and AI integration

Drug screening refers to the process of evaluating compounds for their bioactivity, pharmacological effects, and therapeutic potential. This process can be categorized into experimental screening and virtual screening. Experimental screening involves standardized laboratory procedures to identify bioactive compounds from a large pool of small molecules, whereas virtual screening employs computational methods to preselect molecules most likely to bind target proteins, significantly reducing the number of compounds requiring experimental validation. Advancements in screening technologies have expanded beyond traditional methods – such as activity-based screening and high-throughput screening (HTS) – to more advanced approaches, including structure-based virtual screening (SBVS), ligand-based virtual screening (LBVS), and DNA-encoded compound libraries (DEL). These innovations have significantly broadened the market potential of small-molecule drugs.

AI, defined as a technological framework for simulating human intelligence through computational systems, has emerged as a valuable tool in the design and development of small-molecule drugs. AI-driven approaches enable the rapid screening of vast numbers of small molecules, significantly reducing the cost and time compared with traditional drug discovery.[164] The number of small molecules discovered through AI methods has risen exponentially, paralleling the output of conventional methods by 2022, with AI-discovered drugs demonstrating higher

success rates in Phase I clinical trials compared to the historical industry average.[165] This section focuses on the screening strategies of small molecule drugs, with particular emphasis on the integration and applications of AI in this field.

7.1. HTS and AI integration

HTS employs automated platforms to conduct large-scale experimental assays, enabling rapid data collection and computational analysis to identify lead compounds. Its advantages include high standardization, automation, sensitivity, and minimal sample consumption. However, HTS is constrained by high screening costs and extended processing times.

A recent HTS study utilized OPCs as *in vivo* probes, employing dual-parameter screening based on phosphorylated Akt signaling (indicative of cell survival and growth) and OPC-binding affinity. This approach successfully identified two osteogenic small molecules, YLL3 and YLL8, which enhanced osteogenic differentiation in OPCs and accelerated femoral fracture healing.[166] Another study leveraged a human extended pluripotent stem cell-derived cartilage organoid model equipped with dual fluorescent reporters, COL2A1-mCherry and COL10A1-eGFP, to monitor chondrogenesis and hypertrophy in real time. This platform identified the α2-adrenergic receptor antagonist phentolamine as a potent enhancer of chondrocyte differentiation. *In vivo* studies in murine and mini-pig cartilage defect models further demonstrated phentolamine's ability to promote hyaline cartilage regeneration.[167]

The integration of AI with HTS addresses several inherent challenges, including high false-positive rates, data imbalance, and low screening efficiency. Machine learning algorithms improve the predictive accuracy and reliability of HTS by assigning weighted scores to data points based on their importance, thereby reducing unnecessary validation experiments and enhancing screening efficiency.[168] Furthermore, AI-driven Bayesian active learning has revolutionized combinatorial drug screening by dynamically adjusting experimental designs based on iterative feedback, maximizing information gain while overcoming the combinatorial explosion of experimental conditions – a fundamental limitation of HTS in multi-drug synergy studies.[169]

7.2. SBVS and AI integration

SBVS utilizes computational tools to identify small molecules that optimally interact with 3D structures of biological targets. Compared to traditional HTS, SBVS offers significantly improved hit rates and reduced costs while enabling precise predictions of receptor-ligand binding interactions. However, its reliance on complete structural data of target proteins limits its applicability, and it does not inherently predict the pharmacodynamic properties of identified compounds.

SBVS has facilitated the discovery of novel therapeutic agents in bone metabolism research. For example, a series of 3-acetylindole derivatives were identified as selective inhibitors of bromodomain and PHD finger–containing protein1 bromodomains, which are effective targets for inhibiting RANKL activity. Further experiments revealed that compound 18 displayed the highest *in vitro* efficacy without cytotoxicity in osteoclast precursor cells.[170] Molecular docking and molecular dynamics (MD) simulations are important components of SBVS. Molecular docking simulates and predicts intermolecular interactions, while MD simulations calculate binding energies to verify the effective binding of proteins to small molecule ligands.[171] For instance, molecular docking successfully identified seven compounds as fibroblast growth factor receptor 3 binders, a target positively associated with osteogenesis, and MD simulations confirmed that compound 14977614 exhibited the most stable binding. Treating organ-cultured mouse skulls with 14977614 enhanced mineralization and upregulated bone formation markers.[172] Similarly, five anti-TNFα lead-like complexes were identified through molecular docking, and further MD simulations revealed the stability of these small molecules in a dynamic state, confirming their ability to bind to and antagonize TNFα.[173]

Recent AI-driven advancements in SBVS have further enhanced predictive accuracy and efficiency. Zhou *et al.*[174] developed Rosetta virtual screening, which integrated flexible sidechain modeling and limited backbone adjustments to improve docking accuracy. In addition, Gentile *et al.*[175] introduced the Deep Docking platform, which iteratively refined docking predictions for a subset of a chemical library while using ligand-based predictions to estimate docking scores for the remaining dataset. This approach enables efficient screening of billion-scale molecular libraries without excessive computational resource demands.

7.3. LBVS and AI integration

LBVS is employed when structural data of a biological target are unavailable. Instead, it analyzes known ligands with specific binding affinities to infer the structural features of potential lead compounds. Its advantages include shorter development timelines and the ability to discover novel small-molecule drugs; however, it is generally less accurate than SBVS.[176]

Zhao *et al.*[177] employed LBVS to screen anti-OP drugs and summarized chemical assembly rules, based on which they identified a series of potential compounds. Among them, compound 10a exhibited the most significant inhibition of RANKL-induced osteoclast differentiation and effectively prevented bone loss in OVX rats. Three-dimensional quantitative structure–activity relationship (3D-QSAR) is an important tool in LBVS, as it elucidates the structural characteristics and physicochemical properties of molecules related to their biological activity. Through 3D-QSAR, the characteristic structure of TGFβI receptor inhibitors was identified, leading to the identification of seven lead molecules, which exhibited superior docking scores compared to standard TGFβI receptor inhibitors (SB431542 and galunisertib).[178]

The integration of AI with LBVS has further advanced drug discovery. One study introduced the Molecular Prediction Model Fine-Tuning approach, which was pre-trained on a dataset of one million unlabeled molecules to learn molecular representations before being fine-tuned on various QSAR tasks using smaller chemical datasets with specific endpoints.[179] AI integration also facilitates the identification of novel drug candidates. For instance, chemical language

models utilize Simplified Molecular Input Line Entry System strings to explicitly describe ligand molecular structures. These models learn the intrinsic features of these strings and generate customized sequences that meet pre-defined design requirements, enhancing the efficiency of LBVS.[180]

7.4. DEL and AI integration

DEL technology enables HTS of small molecules by tagging compounds with unique DNA barcodes. By analyzing the DNA tags, researchers can efficiently identify high-affinity small molecules from libraries containing billions of compounds, offering substantial time and cost advantages. However, DEL screening presents technical challenges, including the complexity of DNA-conjugated molecules and uncertainties regarding selection specificity.

To date, DEL technology has not been applied to small molecule screening for fracture healing. The first DEL-identified clinical candidate was GSK2256294, an inhibitor of soluble epoxide hydrolase, a target implicated in cardiovascular and anti-inflammatory processes. Previous attempts using HTS and fragment-based drug discovery failed to yield viable lead compounds, but screening an 800-million-compound DEL library identified a triazine compound with promising activity, which was ultimately optimized into GSK2256294.[181] Phase I trials confirmed its safety and tolerability (NCT01762774, NCT02262689), and it is currently undergoing a Phase IIa clinical trial for diabetic complications (NCT03486223).

AI integration has further refined DEL screening precision. Suo *et al.*[182] demonstrated that Graph Convolutional Network, Message Passing Neural Network, and Attentive FP outperform traditional machine learning models, such as random forests, in DEL-based screening. A hybrid joint model combining these AI approaches improved predictive accuracy. Furthermore, a co-model integrating DEL affinity and photocross-linking screening data dynamically adjusted weights based on dataset characteristics, yielding optimized small molecule candidates with lower molecular weights and enhanced modification potential.

8. Limitations

Despite the comprehensive scope of this review, several limitations must be acknowledged. Due to space constraints, we were unable to provide an in-depth discussion of small-molecule drug delivery systems. The review mainly focuses on the most extensively studied signaling pathways involved in fracture healing, and other potentially relevant pathways were not covered. Furthermore, while temporal strategies for small molecules in fracture healing show promise, they have yet to be clinically translated, and the number of studies on this topic remains limited.

9. Conclusions

In summary, this review highlights the spatiotemporal delivery of small-molecule drugs as a novel strategy for enhancing fracture healing. The spatiotemporal strategy offers a promising approach to achieving precise control over the healing process, improving therapeutic efficacy while minimizing adverse effects. Future research should focus on refining these strategies to address current clinical challenges, ultimately providing cost-effective and efficient therapeutic solutions for bone defects and fractures.

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The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.
Author contributions
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