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REVIEW ARTICLE

Nerve–bone crosstalk manipulates bone organoid development and bone regeneration: A review and perspectives

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Abstract

As an innovative regenerative medicine technology, bone organoids represent a promising therapy for refractory bone injury repair, whereas the key to fabricating bone organoids is grounded in the utilization of biomaterials with osteogenesis cues. Considering the intricate crosstalk between neurons and osteocytes would support bone organoid development and bone wound healing, it is extremely essential to predicate biomaterial design and osteo-organoid construction on understanding the roles of neural growth in ossification center formation and bone-like tissue development. Therefore, this review presents the recent advances of bone organoids with innervated ossification centers after the detailed introduction in the nerve–bone crosstalk. Beginning with the exploration of underlying interaction mechanisms within the osteogenic microenvironment, the importance of the nerve–bone crosstalk on skeleton development and bone regeneration is emphasized at first. The following discussions mainly include diverse biomaterial strategies designed to enhance osteogenesis through early innervation, such as the incorporation of bioactive minerals, controlled release of neurotrophic factors, and exosome-mediated nerve regeneration. Last but not least, the review highlights the advanced technologies in fabricating tissue-engineered bone organoids, with a focus on the applications of cell-laden and multicellular 3D-bioprinted bone microtissues for constructing bone organoids with neurovascularization. These insights are critical to the development of novel biomaterials to construct innervated osteo-organoids, with significant implications for future clinical applications, while also exploring the potential to advance bone regenerative therapies through enhanced nerve–bone crosstalk and laying the foundation for innovative translational research in this field.

Keywords: Biomaterials; Bone organoids; Bone regeneration; Innervated osteogenesis microenvironment; Nerve–bone crosstalk; 3D-bioprinted osteo-organoids

1. Introduction

Bone defect repair and healing is a meticulously orchestrated physiological process that features a complex interplay of diverse cells and signaling molecules facilitating bone formation and wound healing. Such an intricate process involves many cell types and signaling molecules orchestrated in specific transduction pathways, a complexity that, without proper understanding, leads to failed attempts to develop optimal therapies and unsatisfactory repair outcomes in clinical practices. Nowadays, autologous bone transplantation, as a gold-standard clinical treatment, has been widely employed for bone regeneration but is limited by the low bone donor availability and high infection risk, thereby resulting in a lower success rate of transplantation than 90% in the majority of reported cases.[1-3] Especially when the size of a lesion surpasses the innate regenerative capacity of bone tissues, the process of bone repair is often impeded, leading to delayed healing and demand for the exploration of novel methods, with osteoinductive biomaterials and bone organoids emerging as novelty interventions at this juncture.[4]

Emerging as a promising avenue for refractory bone defect repair, bone organoids have been proposed and constructed from a three-dimensional (3D) culture of stem cells and their spheroids, exhibiting differentiation capability toward diverse osteochondral lineages. Several studies have developed different hydrogel matrixes for culturing stem cell-derived spheroids, such as induced pluripotent stem cells and bone marrow-derived mesenchymal stem cells (BMSCs), which form bone-like organoids that present robust osteogenesis capacity through intramembranous ossification or endochondral ossification processes.[5,6] To better fulfill their osteogenic functions, the stem cell-derived bone organoids should experience homing, migration, and orchestrating bone integration in the host, regulated by intracellular or extracellular biochemical factors and biophysical cues similar to those of natural bone microenvironment.[7] Recent years have witnessed a surge in studies surrounding biomaterials that deliver osteogenesis cues into stem cells to activate or inhibit intracellular signal pathways.[4,8,9] Therefore, it is of vital importance to find out the biomaterial design principles and biological mechanisms for bone organoid culture and osteogenesis microenvironment remodeling, but it has been still not fully understood up to now.

The bone microstructure is a well-assembled architecture, whereas the osteogenic microenvironment is a certainly complex system characterized not only by various cell lineages and signaling factors but also by dense vascular and neural networks that collectively maintain bone integrity and homeostasis.[9,10] Previous studies implied that vascularization is a key regulator for the integration between the tissue-engineered bone implants and the host bone tissues by improving the osteogenic microenvironment for osteoblast differentiation, infiltration, and metabolism.[1,11,12] Similarly, neural development and nerve invasion also happen in skeleton formation processes, providing an ossification cue for activating osteogenic signaling pathways and guiding osteoblast lineage progression.[13-17] Therefore, the interplay between bone and neural as well as vascular tissues attaches paramount importance to bone development and regeneration. While extensive researches have been conducted on vascularized bone regeneration, studies about the interaction between neural development and osteogenesis processes, particularly innervated bone regeneration, remain relatively nascent and underdeveloped.

This review article illustrates the necessity and challenges associated with constructing innervated bone organoids from the view of nerve–bone crosstalk. With a focus on innervated bone regeneration, this article first details interaction mechanisms between neurogenesis and skeleton development and summarizes several major strategies for developing bioactive materials and tissue-engineered bone organoids that osteogenesis could be enhanced by early neurogenesis through those approaches. As displayed in Figure 1, the article is structured around three primary aspects: (1) the interaction mechanisms of the nerve–bone crosstalk within the osteogenic microenvironment for bone regeneration; (2) diverse strategies for biomaterials to improve the osteogenic microenvironment with early innervation,

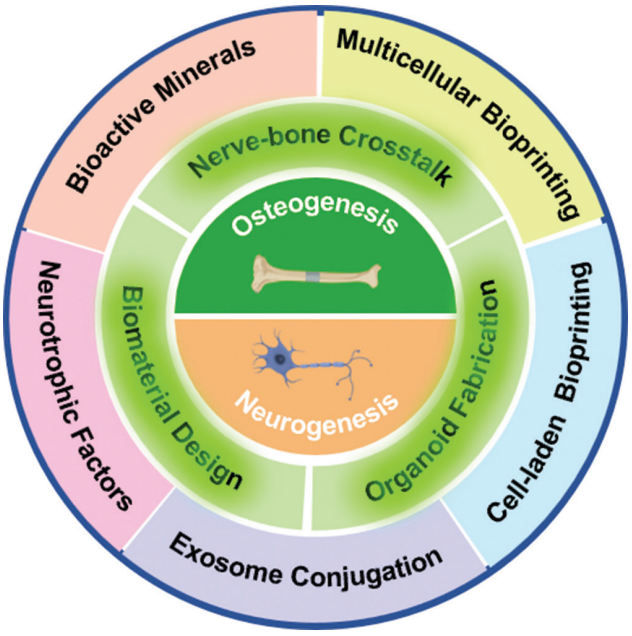

Figure 1. Schematic illustration of nerve–bone crosstalk mechanisms, biomaterial design strategies, and fabrication approaches for bone organoid development and innervated bone regeneration.

including the introduction of bioactive minerals, the release of neurotrophic factors, and exosome-mediated innervation; and (3) major approaches to fabricating tissue-engineered bone organoids with enhanced nerve–bone crosstalk, highlighting the applications of cell-laden and multicellular 3D-bioprinted constructs in endogenous organoid activation and *in vitro* organoid construction, respectively. We hope this review will be instructive in designing the novelty biomaterials for building innervated osteo-organoids and accelerating bone regeneration, which may be of important value for further clinical translation.

The literature search was conducted using the keyword Boolean operation, which includes "osteo-organoids," "bone organoids," "neurogenesis," "nerve," "bone regeneration," and "tissue engineering," on PubMed and Web of Science databases. The accessible literatures were selected according to the criteria that focus on the applications of neurogenesis and nerve–bone crosstalk in skeleton development, biomaterial synthesis, bone tissue engineering, and regeneration medicine.

2. Nerve–bone crosstalk within osteogenesis microenvironment

2.1. Distributions and functions of skeletal nerves

As presented in Figure 2, the skeletal system is innervated by a dense nerve network originating from both the central nervous system (CNS) and the peripheral nervous system (PNS). These nerves play a crucial role in the regulation of bone metabolism and the response to pathological changes after bone injury.[18-20] As a signal transduction hub of mammal bodies, the CNS mainly contains the brain and spinal cord and influences bone metabolism through the production of neurotransmitters, such as serotonin (5-HT), which can modulate bone formation, absorption, and energy consumption.[21] In addition, the CNS can express high levels of semaphorin 3A (Sema3A) in the hypothalamus, which may help direct the migration and innervation of neurons within the skeletal system.[22]

The PNS, distributed in the periosteum, bone marrow, cortex, and trabecular bone, is an information transmitter associating the CNS with the skeleton system and is divided into sensory and motor nerves, with the latter consisting of both sympathetic and parasympathetic components.[18,23] Similar to the CNS, these peripheral nerves also secrete neuroactive components surrounding osteocytes, such as neurotransmitters, neuropeptides, axonal growth, and neurotrophic factors, which regulate bone metabolism through the communications between the nerve resident cells and skeletal cells.[24] As an important motor nerve of the PNS in the skeleton system, the sympathetic nervous system innervates the periosteum with high expression of norepinephrine (NE), neuropeptide Y (NPY), acetylcholine (ACh), and choline acetyltransferase, which are widely distributed in the Haversian system and the Volkmann's canals.[25] Similarly, sensory nerves also play a significant role in bone metabolism and regeneration with abundant neuropeptide secretion (calcitonin gene-related peptide [CGRP] and substance P [SP]) in the periosteum, bone marrow, epiphyseal, and metaphyseal regions. Notably, these sensory nerves extend along the growth plates of the epiphysis, reaching into the bone tissue and establishing direct contact with osteoclasts and secreting nerve growth factors (NGFs) essential for the survival and recruitment of peptide fibers.[25-27]

2.2. Phenotypic changes of skeletal nerves during bone injury

When the skeletal system is subjected to mechanical injury or pathological changes, the nerves within bone tissues are often interfered with and rapidly respond to traumas. Following a bone injury, the signals from the lesion travel backward along the proximal axons to the cell bodies, followed by transmission into the brain through dorsal root ganglion (DRG), whereas various neuropeptides and neurotransmitters, such as CGRP and SP expressed around the injury site, initiate a series of feedback processes of neuromodulation for nerve restoration and bone healing.[28,29]

Recent research has shown an increase in NGF at the early stage of fracture, directly activating sensory neurons with high expression of the NGF receptor tyrosine kinase (TrkA+) to transmit the injury signals.[28] Simultaneously, the distal axons undergo Wallerian degeneration, leading to the deterioration of axons, myelin, and blood-nerve barrier. Cells residing in the nervous system, such as mesenchymal cells and Schwann cells (SCs), undergo transformation and proliferation following nerve degeneration.[30] Subsequently, the SCs relocate to the lesion sites to clear debris for restoration of axonal connection and simultaneously recruit macrophages to aid in the clearance process.[31] The temporary channels formed by the SC basal tubes guide them to the target organ for bone reactivation.[32] On the other hand, the inflammation at the fracture site sensitizes the sensory nerves, and the inflammatory factors released from the activated immune cells also elevate the extremely high nerve sensitivity with neurotransmitter accumulation, as evidenced by the high expression of brain-derived neurotrophic factor (BDNF) and its receptor TrkB in endothelial cells and osteoblasts,[33] as well as the high local expression of Sema3A.[34] Furthermore, the DRG, a main connection pathway between the CNS and the skeleton system, also plays a pivotal role in bone remodeling and homeostasis after bone fracture healing. It has been presumed by the DRG transcriptome analysis that several

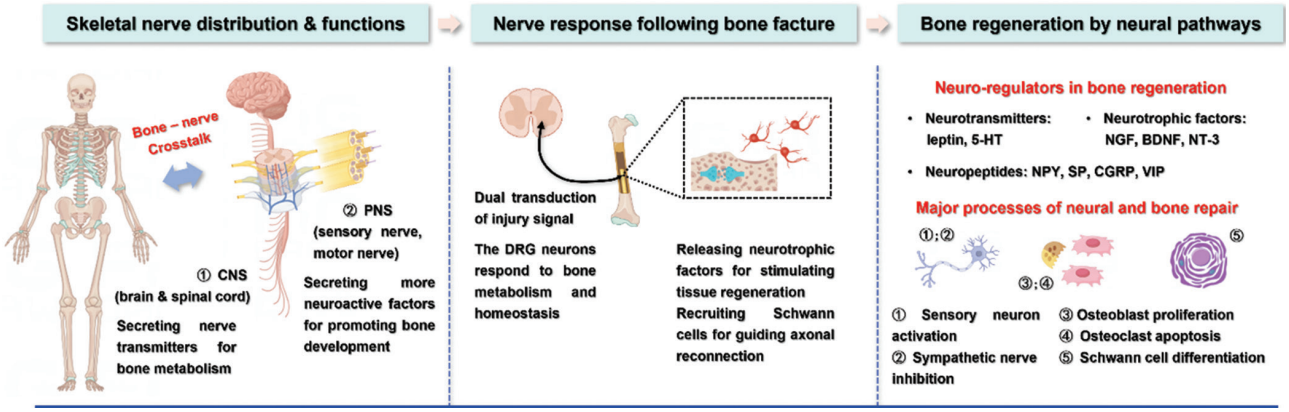

Figure 2. Schematic illustration of the skeletal nerve functions in bone tissue development and regeneration. Part of the graphic materials used in this schematic illustration were obtained from Figdraw.

Abbreviations: 5-HT: Serotonin; BDNF: Brain-derived neurotrophic factor; CGRP: Calcitonin gene-related peptide; CNS: Central nervous system; DRG: Dorsal root ganglion; NGF: Nerve growth factor; NPY: Neuropeptide Y; NT-3: Neurotrophin-3; PNS: Peripheral nervous system; SP: Substance P; VIP: Vasoactive intestinal peptide.

osteogenic signaling pathways are involved for the DRG neurons to respond to bone injury with the secretion of various neuroskeletal regulatory factors.[35] The DRG neurons can be sensitized by the macrophage-neuron-osteoblast axis to participate in regulating bone metabolism and homeostasis through CGRP secretion for stimulating vascularized bone neogenesis.[36]

2.3. The main neuro-modulatory pathways of bone regeneration

The CNS and PNS play a fundamental role in regulating skeletal metabolism through different neuromodulation pathways.[37] CNS-related pathways and neurotransmitters can maintain a delicate balance between bone formation and absorption. For example, leptin, a hormone secreted by adipocytes, interacts with the hypothalamus to trigger the release of NE, which activates β2 receptors on osteoblasts, interfering with bone mass regulation and suppressing bone formation (Table 1).[38,39] In addition, 5-HT can modulate sympathetic nerve activity, indirectly impacting bone resorption and promoting bone formation (Table 1).[40]

Neurotrophic factors, such as NGF, BDNF, and neurotrophin-3 (NT-3), play critical roles in the development of diverse nerve cells in both the CNS and PNS (Table 1).[41] Among these factors, the NGF has been demonstrated to facilitate osteoblast proliferation and differentiation and inhibit their apoptosis, contributing to osteoblast generation (Table 1).[33,42,43] Furthermore, the NGF binds to the TrkA receptor, enhancing the survival rate and regenerative capacity of BMSCs,[44] and upregulating the expression of transforming growth factor beta (TGF-β) and bone morphogenetic protein 9 (BMP-9) to promote osteogenic differentiation.[45] Studies have indicated that the NGF can induce load-induced nerve sprouting in a rabbit mandibular distraction osteogenesis model,[33] facilitating the sensory nerve and inferior alveolar nerve recovery and indirectly promoting bone regeneration.[46,47] Similarly, the BDNF, primarily found in the brain,[48] has also been implicated in influencing bone healing processes (Table 1).[49] Activation of the ERK1/2 and AKT signaling pathways through the TrkB receptor by BDNF has been shown to increase integrin β1 expression, thereby promoting the proliferation and differentiation of human BMSCs, amplifying RANKL production, and ultimately promoting osteoblast growth.[50] Moreover, the NT-3, binding with its receptor TrkC, induces the upregulation of BMP-2 and TGF-β1 expression, facilitating the osteogenic differentiation of rat BMSCs (Table 1).[51]

Furthermore, Sema3A, a guidance molecule in axonal pathfinding, has been identified as another key regulator of bone resorption and formation, coupling osteoblasts and osteoclasts during bone metabolism (Table 1).[52] The binding of Sema3A to the Nrp-1 receptor can activate the Wnt/β-catenin signaling pathway in osteoblasts and enhance osteogenic differentiation of BMSCs with osteogenesis-related gene expression.[53] Moreover, the neuron-derived Sema3A not only promotes normal nervous system development but also participates in regulating vascular invasion, further contributing to bone formation.[54,55]

The autonomic nervous system exerts significant influence on bone metabolism, precursor cell differentiation, extracellular matrix mineralization, and tissue remodeling processes, primarily through different signal transduction pathways regulated by neurotransmitters and neuropeptides.[56] Sympathetic innervation, characterized by the release of NE, exerts dual effects on bone homeostasis (Table 1). By activating α-adrenergic receptors at low concentrations, the NE enhances DNA synthesis in the rat BMSCs, thereby promoting osteoblast proliferation.[57]

Table 1. Key molecules, biological processes, and corresponding signaling pathways of nerve–bone crosstalk

Neuroactive molecules	Receptors	Processes	Outcomes	Signaling pathways	References
Leptin	Hypothalamus	Upregulating osteoblast β2 receptor and BMSC leptin receptor expression; enhancing the NE level in the hypothalamus.	Interfering with bone mass regulation; suppressing bone formation.	Wnt	13,38,85,86
5-HT	Sympathetic nerve	Suppressing sympathetic nerve activity.	Inhibiting bone resorption and stimulating bone formation.	--	40
Sema3A	Neuropilin-1 (Nrp-1)	Activating RANKL receptor for promoting osteogenic gene expression of osteoblasts and BMSCs.	Regulating bone resorption and bone formation; participating in neural and vascular formation.	Wnt/β-catenin	52,53,87
NGF	TrkA	Upregulating TGF-β, BMP-9, and VEGF gene expression; suppressing SMAD and p38-MAPK expression; promoting BMSC survival and osteoblast differentiation; restoring sensory neuron activity.	Innervation, vascularization, and bone regeneration.	NGF/TrkA	43-47
BDNF	TrkB	Increasing integrin β1 expression; promoting BMSC proliferation and osteoblast differentiation.	Bone fracture healing and bone regeneration.	ERK1/2, Akt	50,88
NT-3	TrkC	Increasing expression of BMP-2 and TGF-β1.	Enhanced osteogenic differentiation of BMSCs.	ERK1/2, Akt	51
NE	Adrenergic receptors (α-AR and β-AR)	Activating α-adrenergic receptors (α-AR) at low NE levels to enhance BMSC proliferation; inhibiting osteoclast growth by reducing β-AR at high NE level.	Inhibition of bone resorption and acceleration of bone formation.	ERK1/2, PKA, RANKL/ RANK/OPG	13,59,89-91
ACh	Muscarinic receptor (mAChRs) and nicotinic receptor (nAChRs)	Promoting osteoblast proliferation.	Bone mass regulation and osteocyte amplification.	--	61-63
VIP	Intestinal peptide receptor type 1 (VPAC1)	Decreasing BMSC proliferation; promoting osteoblast differentiation and osteoclast apoptosis.	Maintaining bone metabolism and accelerating bone regeneration.	TGF-β, BMP, SMAD	92
NPY	G-protein coupled receptor (GPCR)	Downregulating osteogenic gene expression by reducing cAMP and NE levels.	Interfering bone metabolism.	--	65,68-69
SP	Neurokinin-1 receptor (NK1R)	Promoting BMSC migration, osteoblast differentiation, and matrix mineralization.	Regulation of bone formation and bone resorption.	β-adrenergic receptor (AR), BMP, ERK1/2, Wnt/β-catenin	70-72,93-95
CGRP	Calcitonin receptor-like receptor (CLR)	Inhibiting osteoclast growth and promoting osteoblast differentiation.	Bone remodeling and reduction of bone resorption.	Wnt/β-catenin, PPARγ, p38-MAPK, Hippo/Yap, RANKL/OPG	75,76,77-79

Note: This table provides a comprehensive overview of the key molecules, dynamic processes, and corresponding signaling pathways of nerve–bone crosstalk. Each entry provides detailed information regarding the specific neurotransmitters or growth factors, the corresponding action sites or receptors, the biological processes experienced, the outcomes achieved, the signaling pathways involved, and relevant citations for further reference.
Abbreviations: ACh: Acetylcholine; AR: Adrenergic receptor; BDNF: Brain-derived neurotrophic factor; BMP-2: Bone morphogenetic protein 2; BMP-9: Bone morphogenetic protein 9; BMSCs: Bone marrow-derived mesenchymal stem cells; CGRP: Calcitonin gene-related peptide; 5-HT: Serotonin; NE: Norepinephrine; NGF: Nerve growth factor; NPY: Neuropeptide Y; Nrp-1: Neuropilin-1; NT-3: Neurotrophin-3; Sema3A: Semaphorin 3A; SP: Substance P; TGF-β: Transforming growth factor beta; Trk: Tyrosine kinase; VEGF: Vascular endothelial growth factor; VIP: Vasoactive intestinal peptide.

Conversely, at higher concentrations, NE preferentially binds to β-adrenergic receptors (β-AR), leading to elevated RANKL expression and reduced bone formation, thus benefiting osteoclast growth and bone resorption.[13,58] Moreover, the NE inhibits human BMSC proliferation through ERK1/2 and PKA phosphorylation pathways.[59] Vasoactive intestinal peptide (VIP), a neuropeptide secreted by the sympathetic nerves accompanying with the NE secretion, exerts a complex regulatory role in bone metabolism (Table 1). While inhibiting BMSC proliferation, the VIP stimulates osteogenic differentiation in these cells by activating diverse signaling cascades involving TGF-β and BMPs, ultimately promoting bone remodeling and regeneration.[60] In contrast, the parasympathetic nervous system exerts anti-inflammatory effects, preserving bone mass balance by counteracting sympathetic activity and increasing osteoclast apoptosis to reduce bone resorption.[13] ACh, a primary neurotransmitter of the parasympathetic system, interacts with various receptors to enhance osteoblast proliferation and improve bone mass (Table 1).[61-63]

On the other hand, peptidergic innervation originating from the sensory nerves exerts direct regulatory control over bone formation through the release of neurogenic factors, in which some key peptides such as NPY, SP, and CGRP are involved (Table 1).[60,64] The NPY, known for its diverse physiological roles, interacts with Y1 receptors on osteoblasts, influencing cellular functions related to bone metabolism, and is implicated in modulating the actions of the NE.[65-67] Experimental evidence suggests that the NPY exerts inhibitory effects on osteoblast functions by reducing cAMP levels, impairing mineralization processes, hindering osteoblast differentiation of MSCs, promoting lipogenesis, and inducing apoptosis in these cells.[65,68,69] In contrast, the SP primarily interacts with the neurokinin 1 receptor, stimulating osteoblast activity and promoting osteogenesis.[70] Notably, the SP inhibits chondrogenic differentiation and terminal differentiation of chondrocytes through β-AR signaling pathways during endochondral ossification.[71] Moreover, the SP facilitates the migration of MSCs toward osteogenic niches, also enhancing their proliferation, differentiation, and matrix mineralization in a concentration-dependent manner, thereby accelerating bone remodeling and maturation.[72,73] The CGRP, another important peptide, influences bone remodeling by regulating local blood flow through vascular actions and direct modulation of intraosseous cellular activities.[74] By binding to calcitonin-like receptor (CLR), the CGRP inhibits osteoclast generation and differentiation through intricate signaling pathways, leading to reduced bone resorption.[75,76] Simultaneously, the CGRP also promotes the migration of MSCs toward osteogenic niches, enhances osteogenic differentiation, and inhibits MSC apoptosis.[77-79]

Apart from neurotrophic factors, neurotransmitters, and neuropeptides, the intricate interplay between glial cells and their associated substances plays a pivotal role in orchestrating bone regeneration processes. Beyond their traditional roles in neural functions, the glial cells, particularly SCs, have emerged as key regulators of bone repair involving a multitude of intricate pathways. SCs exhibit a remarkable capacity to support nerve regeneration and augment the proliferation and differentiation of osteoblasts following bone injury, thereby contributing significantly to the restoration of bone integrity.[80] Upon injury, SCs undergo a dynamic phenotypic shift characterized by the downregulation of myelin-associated glycoprotein expression and the upregulation of glial fibers and NGF levels, indicative of a myelin-regenerating phenotype.[81] These cells actively participate in shaping an anti-inflammatory microenvironment by modulating key signaling pathways such as MAPK, TNF, and Rap1. Notably, SC-derived exosomes have emerged as promising therapeutic agents for bone repair, as evidenced by their ability to enhance the migration, proliferation, and differentiation of BMSCs, found by Wu *et al.*[82] Furthermore, SC precursors (SCPs) exhibit remarkable plasticity, capable of differentiation into diverse skeletal progenitor cells and mature osteocytes, thereby contributing to bone regeneration and repair.[83] The paracrine factors, secreted by the SCP and directly working on osteoclast differentiation, play a pivotal role in orchestrating ossification processes, modulating inflammatory responses, and influencing vascular development, collectively fostering a conducive environment for bone healing. Moreover, recent studies have unveiled a neuron-derived small extracellular vesicle (sEV) pathway by which the enriched microRNA promoted bone regeneration and post-traumatic brain injury.[84] These sEVs, particularly the fibronectin 1 on their surface, can target key proteins within bone tissues, facilitating bone healing by modulating the expression of critical transcription factors such as FOXO4 and CBL proteins through miR-328a-3p and miR-150-5p molecules, respectively.[34]

3. Several strategies for building an innervated osteogenesis microenvironment by functional biomaterials

As concluded from above, innervation and early neural development are critical factors for the osteogenesis and bone formation processes that are characterized by neuropeptides, neurotransmitters, neurotrophic factors, and other neural cell-derived messengers. Therefore, in terms of current advances in bone regeneration, the integration of specific neuromodulation cues into biomaterials has attracted interest toward bone organoid construction and refractory bone defect repair.

Regrettably, the general properties of biomaterials often present significant differences in neuronal and osteoblast development. For example, neuronal differentiation is feasible when a soft biomaterial matrix with stiffness lower than 1 kPa is used, whereas osteoblast differentiation often happens on the stiff biomaterial surface with rigidity much higher than 10 kPa.[96] To optimize the outcome of bone regeneration, the crosstalk between innervation and bone development should be enhanced by introducing additional neurotrophic cues for biomaterial advancement. Within this section, several major approaches to orchestration between neurogenesis and osteogenesis processes, mainly including bioactive inorganic mineral scaffold, neurotrophic factor release, and neural cell-derived exosome conjugation, are listed as follows and described according to the recent advances.

3.1. Bioactive inorganic minerals

Bone tissues are naturally mineralized architectures assembled by oriented collagen fibers and inorganic mineral crystals. Thus, the maintenance of bone metabolism, robustness, and homeostasis partially depends on the content and crystallinity of the inorganic minerals. Among all the natural minerals in skeleton systems, calcium phosphate (CaP) crystals are the most common; therefore, CaP-based inorganic minerals such as hydroxyapatite, tricalcium phosphate (TCP), brushite, and bioactive glass have been chosen as artificial bone biomaterials applied in bone tissue engineering.[97,98] Benefited from abundant chelation spaces of phosphate anions, some trace elements with neuromodulation and osteoinductive activities, including Mg^{2+}, Zn^{2+}, and Cu^{2+}, could be introduced into the crystal lattice of the CaP-based minerals (Figure 3), for developing the novel osteoinductive scaffolds and tissue-engineered bone organoids.[99] Once released from the scaffolds, these bioactive cations diffuse into the intracellular space of osteocytes and are involved in the intracellular signaling pathways related to sensory nerve activation and osteogenesis functions. Whitlockite ($Ca_{18}Mg_2(HPO_4)_2(PO_4)_{12}$), for instance, is a bioactive mineral in human bone constitutions, with the stimulus of Mg element to SC migration and the release of NGF, BDNF, and VEGF.[100,101] It is also evidenced by Huang *et al.*[102] that the sustained release of Mg^{2+} from scaffolds significantly promotes early innervation, vascularization as well as bone wound healing in a rat femoral condyle defect. Moreover, the addition of the Zn element to the whitlockite further facilitates the rapid innervation within the bone regeneration process by promoting neural cell adhesion, SC migration, and paracrine, possibly benefiting from the activation of sensory nerves and the inhibition of sympathetic activity.[103] In light of the advantages of the bioactive inorganic minerals in innervated bone regeneration, the tissue-engineered bone organoid matrixes can be modified by various trace elements to construct a more appropriate microenvironment for enhanced osteogenic differentiation and bone development.

3.2. Releasing neurotrophic factors from biomaterials

In comparison with the bioactive inorganic mineral scaffolds, the loading and controlled release of NGFs seem to be a more common and efficient osteogenesis cue for the osteoinductive biomaterials through the early neurogenesis process. Stimulating the release of neurotrophic factors from biomaterials represents a key approach in the field of innervated bone regeneration research. As a well-studied neurotrophic factor mentioned above, the NGF has been widely discovered in the skeleton system containing bone marrow, periosteum, trabecula, and cortical bone,[104,105] and has been incorporated in silk fibroin scaffolds with sustained release of NGF for improving peripheric neural cell adhesion, axonal growth in the Haversian canal, and activation of osteogenetic signaling pathways such as MEK/ERK pathway.[106,107] Previous studies have demonstrated that the cocktail combination of BMP-2 and NGF significantly accelerated bone healing with increased expression in osteoblast differentiation and, more importantly, better maintained the balance between osteoblasts and osteoclasts for inhibiting excessive ossification compared with those outcomes with the administration of single factor.[108,109] By a similar signaling pathway to that of the NGF, the BDNF also showed the ability to facilitate osteogenesis and bone formation both *in vitro* and *in vivo*, as evidenced by obvious upregulation in osteogenesis marker osteocalcin and various neurogenesis markers containing microtubule-associated protein 2, glial fibrillary acidic protein, neural/glial antigen 2, and β-tubulin III.[90] To further enhance the innervation and vascularization within the osteogenesis microenvironment, the bioactive inorganic minerals mentioned above have been employed to load and sustainably release the neurotrophic factors with a delayed half-life period in the host. For example, our group has developed the Laponite nanosheets abundant in Mg^{2+} as a vehicle for NGF delivery and found that the NGF-Laponite complex obviously improves the osteogenic activity of BMSCs by stimulating CGRP secretion of sensory neurons and enhancing communication between the BMSCs and sensory neurons (Figure 4).[110] Moreover, we have analyzed the major mechanism of the crosstalk between innervation and osteogenesis processes, indicating that the CGRP-mediated nerve–bone crosstalk would be a potential approach for facilitating innervated bone regeneration.

3.3. Exosome-mediated regeneration of innervated bone

As evidenced in the previous section, SCs, pivotal glial cells of the peripheral nerves in the skeleton system, are

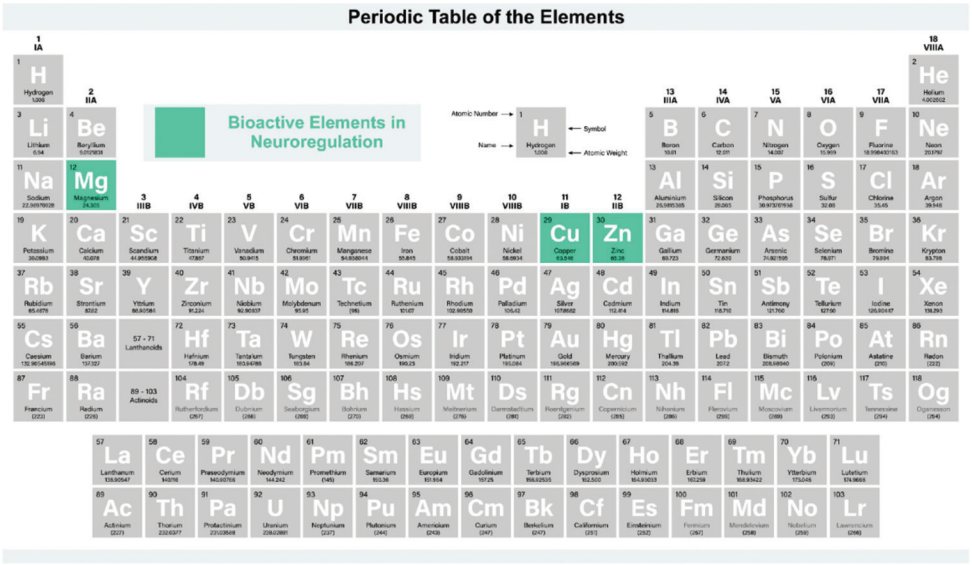

Figure 3. The trace elements with neuromodulatory and osteoinductive activities. Reprinted with permission.[101] Copyright © 2023, Chinese Medical Association.

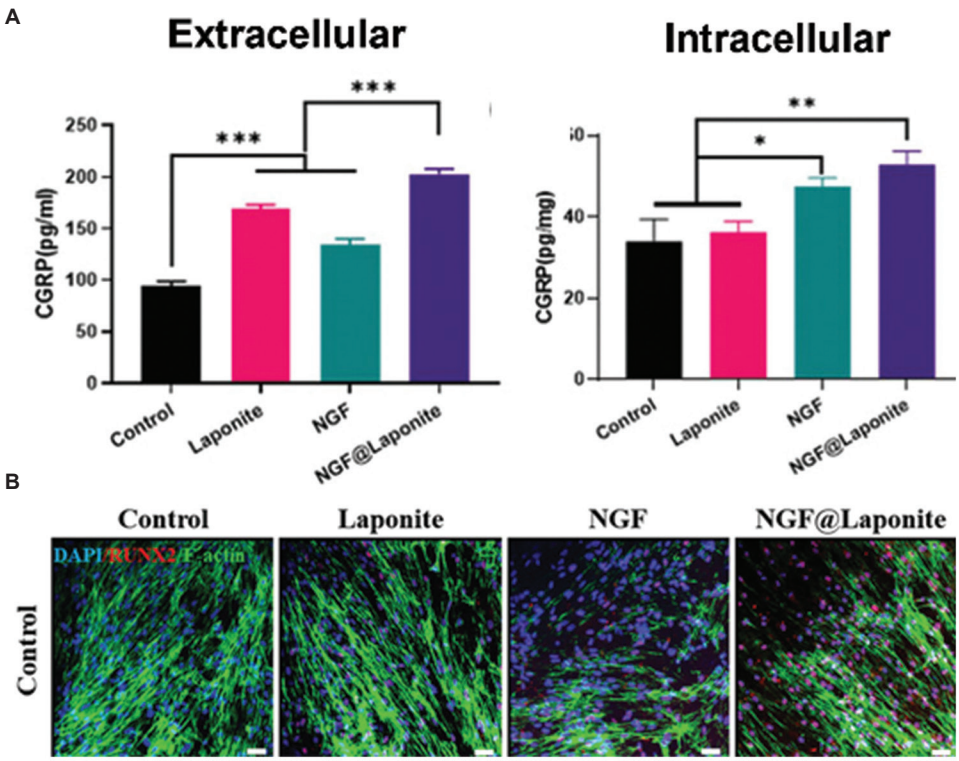

Figure 4. NGF administration by laponite vehicles promotes CGRP secretion of neurons and osteogenic differentiation of BMSCs. (A) ELISA results of CGRP secretion of DRG neurons with the treatment of NGF@Laponite (*$P < 0.05$, ** $P < 0.01$, ***$P < 0.001$). (B) Immunofluorescent labeling of osteogenic marker Runx2 in BMSCs with the treatment of NGF@Laponite. Scale bar: 50μ. Reprinted with permission.[113] Copyright © 2021, Wiley-VCH GmbH.

Abbreviations: BMSCs: Bone marrow-derived mesenchymal stem cells; CGRP: Calcitonin gene-related peptide; DRG: Dorsal root ganglion; ELISA: Enzyme-linked immunosorbent assay; NGF: Nerve growth factor.

deeply involved in the orchestration of peripheral nerve regeneration and bone wound healing.[111-113] A recent study has unveiled that the SCs can rapidly reprogram and dedifferentiate into a repair phenotype with excessive factor secretion after bone injury, indicating that the activation and maintenance of the repair SC phenotype

is a promising strategy for developing and optimizing biomaterials with superior osteoinductive capacity.[114] New advancements also showed that the SCs respond to external stimuli with biomaterial-mediated interventions, like endogenous electric field formation, and express more neurogenic proteins to stimulate sensory nerve activity and axonal outgrowth, further fostering vascularization and newborn bone development.[115-117] Given the critical roles of the SCs within bone tissue development and regeneration, a novel approach of loading the SCs and their derivatives into the bone biomaterials and the osteo-organoids has emerged as an innovative strategy to repair the refractory bone defects.

Exosomes, also known as extracellular vesicles from the paracrine pathway, are considered a promising therapy for bone regeneration because abundant biological factors are included in the exosomes. Especially, the SC-derived exosomes, as an important crosstalk vehicle between sensory neurons and osteocytes in the periosteum and Haversian systems, have been used to improve the osteogenesis ability of MSCs while promoting vascularization and innervation within the bone lesions (Figure 5).[118] Moreover, it has been demonstrated that the anti-inflammatory property of the SC-derived exosomes markedly facilitated macrophage polarization toward the M2 phenotype rather than the M1 phenotype, contributing to an anti-inflammatory microenvironment for osteocyte growth.[119] Therefore, the exosomes are usually engineered into a sustained-release agent, in which the neurotrophic factors stored in the exosomes can be released from biomaterials to stimulate regeneration of the innervated bone tissues. Apart from the SCs, MSCs also produce exosomes with

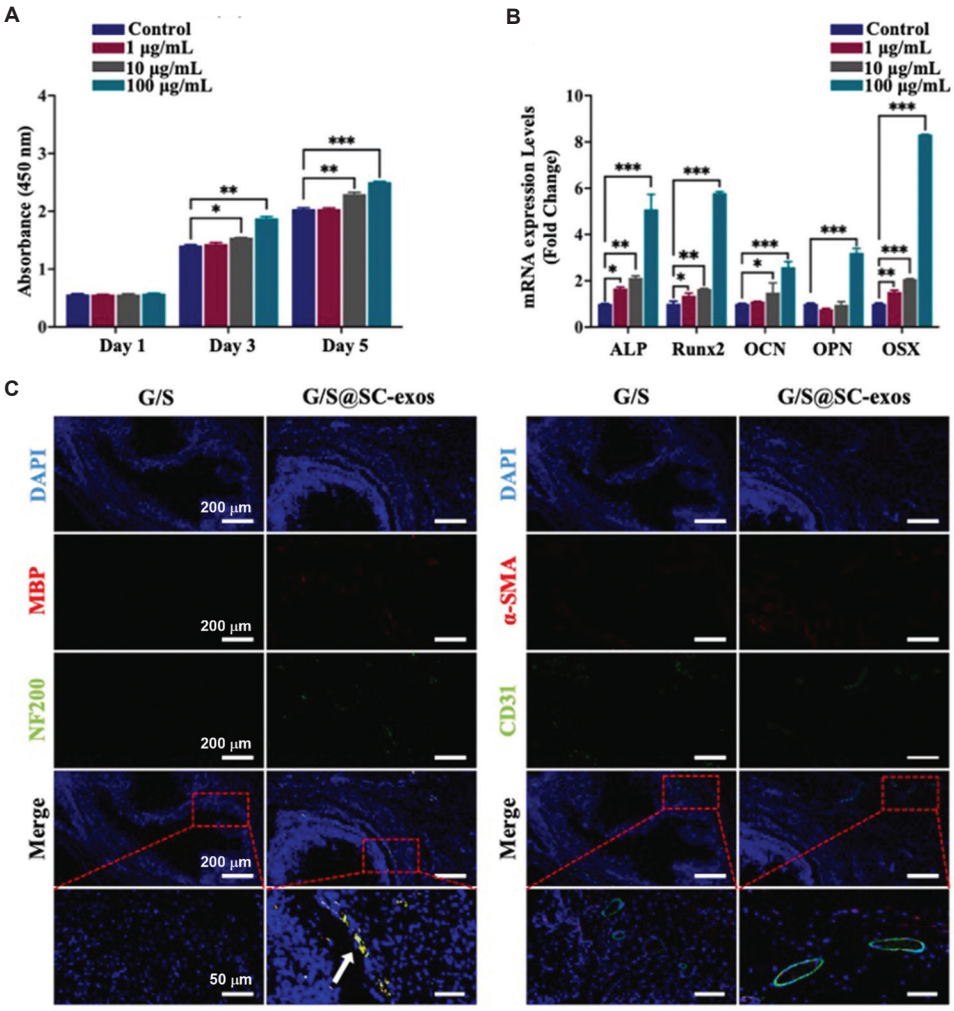

Figure 5. The SC-derived exosomes exhibit the ability in bone regeneration with vascular and neural reconstruction. (A) Cell viability of BMSCs after exosome treatment at 1, 3, and 5 days. (B) qPCR analysis of osteogenesis-related gene expression following treatment with exosomes in different concentrations (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). (C) Immunofluorescent staining of vascularization markers (a-SMA, CD31) and nerve fiber markers (MBP, NF200) on a rat skull defect model. Reprinted with permission.[121] Copyright © 2023, KeAi Publishing.
Abbreviations: qPCR: Quantitative polymerase chain reaction; SC: Schwann cells.

tissue repair potential, not only showing excellent anti-inflammatory ability but also facilitating the migration of SCs and the CGRP secretion of DRG neurons, thus contributing to an elevated osteogenic differentiation efficiency of MSCs.[120]

Different from the bioactive inorganic minerals and the neurotrophic factors, the exosome-mediated nerve–bone crosstalk features intricate interaction mechanisms underlying bone regeneration, as well as diverse release approaches of neuroactive substances. On the one hand, the NGFs released from the exosomes bind to the surface receptors on osteocytes, neural cells, and vascular endothelial cells and activate the corresponding signaling pathways. On the other hand, the exosomes also can be endocytosed into the targeted cells, and the loading microRNA can be translocated toward nucleus regions for regulating protein synthesis and cell differentiation. Therefore, the exosomes often exhibit long-term bone regeneration with a delayed release period and abundant target pathways. Moreover, Lian *et al.*[120] modified the MSC-derived exosomes by stimulating MSCs with the NGF, and the modified exosomes show an excellent potential in accelerating neurogenesis and osteogenic differentiation, representing a unique strategy to improve therapeutic outcomes in comparison with bioactive inorganic minerals and neurotrophic factors. Meanwhile, the intricate interaction mechanisms of the exosome-mediated nerve–bone crosstalk are still not fully understood, presenting huge challenges to further clinical translation.

4. Fabrication of bone organoids with enhanced nerve–bone crosstalk

Bone organoids, mainly containing stem cell spheroids and their extracellular matrixes, represent a promising regeneration therapy for healing refractory bone defects, whereas vascularization and innervation formation constitute an uphill battle for osteo-microenvironment remodeling in the osteo-organoid technology innovation and clinical translation. Responding to these challenges, the 3D co-culture of osteoblast, osteoclast, hematopoietic-associated, and other cells is often preformed for osteo-organoid formation through intramembranous or endochondral osteogenesis processes, thus customized into diverse organoid types, such as callus organoids, woven bone organoids, and trabecular bone organoids. Although restricted in a microscopic space, the 3D culture of cell spheroid-based bone organoids still presents the robust osteogenetic ability under biochemical and biomechanical stimuli;[121,122] for instance, dental pulp stem cells prefer to form spheroid-like microtissues on an agarose gel substrate and differentiate toward osteoblast lineage with cannabidiol treatment.[123] With the advancement in biofabrication technology, particularly 3D bioprinting, bone organoids with bone-like microstructure and osteogenic microenvironment features can be fabricated by cell-laden 3D bioprinting, as well as multicellular 3D bioprinting, characterized by not only an assembly of osteoblast spheroids into a larger-scale bone microtissue but also integration between osteocytes, vascular endothelial cell, and neural cells with more close contacts. The bioinks serving as extracellular matrices of the 3D-bioprinted osteo-organoids should provide the biochemical or biomechanical cues mentioned above for facilitating neural growth inside the bone microtissues. In this section, the recent advances of the 3D-bioprinted osteo-organoids are reviewed and emphasized, highlighting, especially, the activation of innervation by 3D-bioprinted scaffolds and the development of multicellular 3D-bioprinted bone microtissues with enhanced nerve–bone crosstalk.

4.1. Cell-laden 3D bioprinting for promoting skeletal nerve restoration and bone regeneration

3D bioprinting, also called additive manufacturing, is a promising approach for the fabrication of tissue-engineered osteo-organoids, benefitted from its abilities in rapid prototyping, high-density cell integration, and heterogeneous architecture manufacturing. Recent studies have presented the 3D bioprinting strategies and therapy applications of tissue-engineered bone microtissues; among them BMSC-laden 3D-bioprinted constructs represent a common choice for tissue-engineered bone transplantation and regeneration.[124] Wang *et al.*[125] have fabricated microfilament-based scaffolds with different geometric structures using 3D electrostatic printing and found the preferred microfilament patterns with an angle of 90° not only promoted osteogenic differentiation of BMSCs but also stimulated expression of vascular and neural growth factors for neovascularization and innervation formation. Another study also observed the enhanced vascularized, neurogenic, and osteogenic marker expression in a rabbit femoral defect model after implantation of a 3D-printed polyhedron-like bioceramic scaffold, demonstrating the superiority of 3D bioprinting over traditional tissue engineering approaches.[126] However, newborn tissues arising from the BMSCs encapsulated in the 3D-bioprinted constructs are subject to inadequate metabolism due to lack of vascularization, and their osteogenic differentiation is also limited due to neurotrophic factor stimulus constraints. As mentioned above, both the activation of sensory neurons and the inhibition of sympathetic nerve activity contribute to the enhanced osteogenic differentiation of BMSCs and bone fracture healing. It has been demonstrated that the sensory nerve stimulators (NGF, CGRP) and the sympathetic nerve inhibitors (nifedipine and propranolol) can be loaded in and released from 3D-bioprinted gelatin/

sodium alginate scaffolds, consequently promoting osteogenic differentiation of the BMSCs encapsulated in the bioprinted scaffolds, and improving bone metabolism, homeostasis and neurovascularization.[110,127,128] Furthermore, the exosome-loaded 3D-bioprinted scaffolds can show a similar neuromodulatory effect, as evidenced by our recent research that following introduction into the BMSC-laden 3D-bioprinted constructs, the SC-derived exosomes facilitated *in vitro* osteogenic differentiation of the BMSCs and *in vivo* bone regeneration with elevated vascularization and innervation levels (Figure 6).[118] Moreover, the exosomes derived from the neural stem cells (NSCs) that were activated by traumatic brain injury presented robust capacities in accelerating osteogenic differentiation of BMSCs and bone fracture healing and were loaded into a 3D-printed TCP/hydrogel scaffold for activation of vascularized bone regeneration.[129]

4.2. Multicellular 3D-bioprinted bone microtissues capable of neurovascularization

It is widely recognized that another unique advantage of 3D bioprinting is the fabrication of multicellular constructs, through which different cell types can be precisely assembled, like how they are arranged in heterogeneous microtissues.[130] With multicellular 3D bioprinting technology, live neural cells, such as NSCs and SCs, can be loaded into BMSC-free or BMSC-laden 3D-bioprinted constructs, forming BMSCs co-culture systems capable of innervation by recruiting BMSCs and promoting their differentiation toward osteoblast lineage. With treatment of the NSC-loading 3D-bioprinted constructs, for example, a rat cranial bone defect repair results revealed that blood vessel and nerve ingrowth were enhanced by neuronal regeneration and axonal outgrowth in the bone injury lesion, consequently providing an appropriate microenvironment for guiding bone regeneration.[131] Different from the NSC-laden 3D-bioprinted constructs, the SCs and the BMSCs are often printed into the innervated bone organoids, in which the bioinks are a key factor for the regulation of osteogenesis and neurogenesis. A study from the Chinese Academy of Sciences has reported a 3D-printed tree-like TCP bioceramic scaffold for developing the co-culture systems of BMSCs and SCs, and the SC co-culture significantly promoted the BMSC adhesion and proliferation on the scaffold surface, which were influenced by the biophysical microstructures of the tree-like bioceramics.[132] They also found in another research that 3D-bioprinted BMSC/SC co-culture systems presented robust osteogenic efficacy, regardless of the *in vitro* or *in vivo* assays used, and nanowire-like calcium silicate minerals were included in the multicellular bioinks for supporting formation of the innervated bone microtissues,[133] providing convincing evidence that the multicellular bone organoids offer an innovative strategy for tissue-engineered bone regeneration and represent a viable clinical therapy in future.

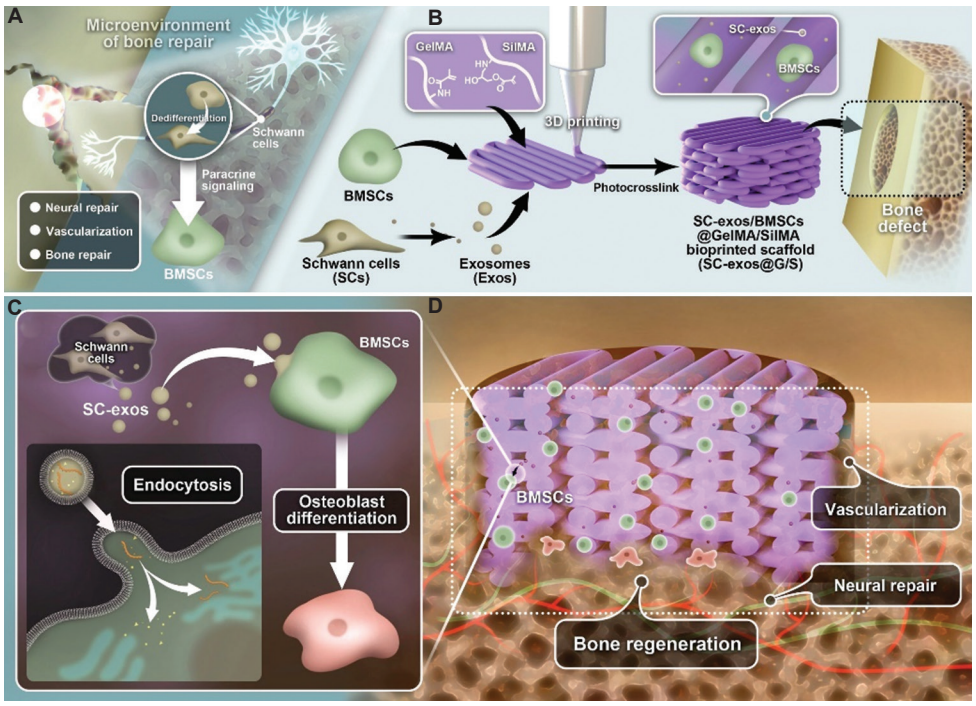

Figure 6. (A-D)BMSC-laden 3D-bioprinted constructs facilitate vascularized and innervated bone regeneration through neurogenesis cues. Reprinted with permission.[121] Copyright © 2023, KeAi Publishing.
Abbreviations: BMSCs: Bone marrow-derived mesenchymal stem cells; Exos: Exosomes; SCs: Schwann cells.

5. Conclusion and perspectives

Recent advances in the field of bone regeneration are grounded more in the neurovascularization of tissue-engineered bone implants and bone organoids. Nevertheless, the crosstalk mechanisms between neurogenesis and osteogenesis remain obscure as compared to what is known about vascularized bone regeneration, posing a major challenge in biomaterial development for bone tissue engineering and regenerative medicine. The first part of this review offers a detailed summary of the neuromodulatory molecules and the corresponding signaling pathways within skeleton development and bone wound healing processes, describing the innervation and the crosstalk mechanisms within the osteogenesis microenvironment. With the introduction of recent developments of biomaterials with neuromodulatory effects, several major biomaterial strategies, including bioactive inorganic mineral doping, neurotrophic factor release, and neural cell-derived exosome conjugation, and the innovative 3D bioprinting technologies, which promote vascular, neural growth and bone regeneration in bone injury repair and bone organoid development processes, are presented. Although bone organoids provide a promising therapy for refractory bone defect repair, some existing intractable issues and challenges need to be addressed for future clinical application.

5.1. Lack of strategies for regulating central nerve–bone crosstalk

In numerous studies focusing on the roles of skeletal peripheral nerves containing sensory nerves and sympathetic nerves in bone health and regeneration, the stimuli to sensory neurons are often regarded as an important approach for the rational design and enhanced osteogenic function of the current biomaterials. Moreover, brain-derived neuroactive substances are also involved in communication between the skeleton and nervous systems, maintaining tissue homeostasis for bone injury healing. Without feasible strategies, the modulation of brain-derived nerve activity and central nerve–bone crosstalk is still a key challenge for biomaterial development and should be further investigated in depth for possible clinical application.

5.2. Lack of non-invasive neuromodulation strategies for promoting innervated bone regeneration

Neurons can sense extracellular biophysical environments, such as light, stress, and electromagnetic fields, and secrete more neurotransmitters and NGFs under the stimuli. Correspondingly, the functional biomaterials should respond to the external physical signals, generating heat or electricity effects for stimulating neurogenesis and osteogenesis. For instance, near-infrared-light responsive carbon nitride nanosheets not only released the NGF from the silk fibroin scaffolds but also produced endogenous electricity under irradiation of the near-infrared-light, significantly accelerating the activation of neurogenesis along with osteogenesis.[134] Such a smart, responsive biomaterial provides a novel, non-invasive neuromodulation strategy with improved tissue regeneration efficiency that is of vital importance for regenerative medicine.

5.3. Lack of standardized protocols for bone organoid preparation, preservation, and transplantation

Bone organoids represent an emerging biofabrication technology and a promising regeneration therapy. Despite involvement in partial reproduction of vascular and neural structures and mimicry of bone tissues' essential functions, the current bone organoids should be engineered into more sophisticated architectures, and their preparation and preservation methods should be standardized to guide autogenous bone graft replacement in future.

Despite coverage on the nerve–bone crosstalk mechanisms and their applications in tissue engineering-based bone regeneration, the current review may contain less-rigorous descriptions and lack a discussion of certain key studies and evidence due to incomplete literature selection, which is possibly attributed to the strict adherence to publication years and term combinations during literature.

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Conflict of interest

The authors declared no conflicts of interest.

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Ethics approval and consent to participate

Not applicable.

Consent for publication

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Availability of data

Not applicable.

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