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Chapter

Traditional Chinese Medicine Therapy for Targeting Osteoblastogenesis

Yanqiu Liu

Abstract

Osteoblasts are derived from bone marrow mesenchymal stem cell (BMSC) precursors, which differentiate into mature osteoblasts and mediate bone formation. This process is called osteoblastogenesis. A deficiency in osteoblastogenesis of BMSCs can result in bone-related diseases including osteoporosis. Thus, developing drugs for targeting osteoblastogenesis from BMSCs has become one of the therapeutic strategies for osteoporosis. In China, kidney-nourishing Chinese herbal drugs such as ER-Zhi-Wan have been believed to be potential for treating osteoporosis through targeting osteoblast proliferation and differentiation. The key pathways for regulating osteoblastogenesis include canonical and noncanonical Wnt pathway, semaphorin-mediated pathway, and MAPK-mediated BMP2-Smad pathway. Some natural products have been confirmed to regulate more than one pathway and exert multi-target effect through the use of one compound or combined use of more than two compounds, such as wedelolactone and oleonuezhenide. In addition, tissue engineering provides a promising strategy in the field for targeting osteoblastogenesis. New types of biomaterials including hydroxyapatite (HAp) combined with Chinese medicine can exert enhanced effect on osteoblastogenesis and provide new therapy for treating osteoporosis.

Keywords: targets, osteoblastogenesis, traditional Chinese medicine, Wnt pathway, semaphorins, biomaterials

1. Introduction

The bone is a dynamic organ, capable of regenerating and remodeling throughout the lifetime. Bone remodeling is a process, which is mainly balanced by osteoblast-mediated bone formation and osteoclast-mediated bone resorption. Bone marrow mesenchymal stem cells (BMSCs) are osteoprogenitors, which differentiates into maturated osteoblasts and mediated bone formation, therefore maintaining the balance of bone remodeling. The process of osteoblast proliferation and differentiation from BMSCs is called osteoblastogenesis, which plays a key role for osteogenesis. A decrease in osteoblastogenesis of mesenchymal stem cells is observed to result in bone-related diseases, such as osteoporosis [1, 2]. Stimulation of proliferation and osteoblastogenesis from BMSCs becomes a therapeutic strategy for osteoporosis. A large number of factors have been implicated in regulating osteoblast differentiation, such as the Wnt family [3, 4]. Canonical Wnt signaling is crucial for regulation of osteoblast development including osteoblast proliferation, differentiation, and

survival. Activation of the canonical Wnt signaling pathway involves recruitment of a complex including LRP5/6 and GSK3β, stabilization of β-catenin, regulation of transcription factors such as runx2, and activation of Wnt target genes [5]. This pathway is active in BMSCs, and therefore many signaling molecules are developed as drug targets such as GSK3β and LRP5/6. Noncanonical Wnt signaling pathway is mediated by Wnt5a, which activates downstream pathways including Wnt/Ca^{2+}/PKC, small GTPase Rho, and JNK pathways [6]. Consequently, transcription factors, such as AP1 family [7], are activated, and survival-related gene expression is induced. The anabolic agents, such as parathyroid hormone (PTH), are developed to treat osteoporosis through enhancing osteoblastogenesis. However, administration of $PTH_{1–34}$ for a long time can increase bone resorption, resulting in bone neurosis [8]. Therefore, research is currently focusing on drugs that can simultaneously regulate bone resorption and bone formation and could thus develop a new class of dual-action therapeutic agents for osteoporosis [9, 10].

According to traditional Chinese medicine theory, "kidney-nourishing" herbal drugs are commonly believed to have the ability of nourishing bones and therefore are used to treat osteoporosis. Traditional Chinese herbs have the characteristics of multi-components and multi-target; thus the development of bifunctional agents from traditional Chinese herbs is promising. Many potential compounds isolated from "kidney-nourishing" herbal drugs have been found to enhance osteoblastogenesis [11]. We focus on a traditional Chinese prescription, called Er-Zhi-Wan, which consists of Ecliptae herba and *Fructus Ligustri Lucidi*. Extract of Er-Zhi-Wan has been reported to increase bone volume and enhance bone formation. We determine the components by using HPLC-MS method and screen the active compounds. Wedelolactone isolated from Ecliptae herba is firstly reported to inhibit osteoclastogenesis and simultaneously enhance osteoblastogenesis. In ovariectomized mice, administration of wedelolactone prevented ovariectomy-induced bone loss by enhancing osteoblast activity and inhibiting osteoclast activity. At the molecular level, wedelolactone altered several key signaling pathways. Wedelolactone facilitated osteoblastogenesis through activation of Wnt/GSK3β/β-catenin signaling pathway, which led to the activation of runx2 and the expression of downstream genes. Simultaneously, wedelolactone inhibited osteoclastogenesis through inhibition of RANKL/RANK/NF-κB pathway, resulting in suppression of c-Fos/NFATc1 activation and osteoclast marker gene expression. Although wedelolactone can treat osteoporosis with the characteristics of bifunctional activity, wedelolactone is not perfect. At the high dose, wedelolactone can trigger cytotoxicity against BMSCs. Therefore, we propose that components from *Fructus Ligustri Lucidi* could alleviate wedelolactone-induced cytotoxicity, since it is believed that the synergy effect contributes for the improved therapeutic efficacies.

Several compounds from *Fructus Ligustri Lucidi* are determined to have the ability to enhancing osteoblastogenesis. Among them, oleonuezhenide is found to increase bone mineralization induced by wedelolactone. Additionally, high dose of wedelolactone-induced cytotoxicity in BMSCs was relieved by addition of oleonuezhenide, and these BMSCs protected by oleonuezhenide maintained osteoblastic activity. These data further confirm that oleonuezhenide enhances wedelolactone's action on osteoblast differentiation and activity through Wnt/CK2α/β-catenin pathway and prevents wedelolactone-induced cytotoxicity through Wnt5a/CK2α/ERK pathway, indicating that combination of different compounds generates multi-target effect. This might contribute for the efficacy for the mixture of different herbal drugs.

In addition to the development of new type of drugs for targeting osteoblastogenesis, tissue engineering provides a promising strategy in the field for osteogenesis from BMSCs, which aims to induce new, BMSC-driven, bone regeneration

and has increased the possibility of engineering bone in vitro to treat bone defects particularly in vivo [12]. As alternatives polymeric biomaterials are applied in clinical practice since the 1960s; their popularity is related to the ease of preparation of various products in complex shapes and a wide range of physical and mechanical properties. Hydroxyapatite (HAp) is a major mineral component of calcified tissues including bones. Synthetic HAp has been extensively used as an important material for bone substitute, owing to its excellent osteoinductive properties. However, most HAp is considered to be weak in osteoinductive ability, which may impact the repair capacity for bone defects. Thus, the combined effect of HAp, growth factors, or osteoinductive agents such as natural products on osteoblastogenesis might be promising. We investigate the action of incorporation of wedelolactone and HAp nanoparticles with different shapes and sizes on osteoblastogenesis from BMSCs. First, HAps are constructed by a rodlike shape with different particle sizes. HAp-1 combined with wedelolactone induced a higher ALP activity with different degrees, suggesting that combination of biomaterial and compounds contributes for osteoblastogenesis and thus can be used as therapeutic strategy for osteoporosis. Overall, this chapter will discuss recent findings regarding osteoblastogenesis and its related therapeutic strategies.

2. Osteoblastogenesis and its related therapies

2.1 The cellular and molecular mechanism of osteoblastogenesis

Osteoblasts arise from bone marrow mesenchymal stem cell (BMSC) precursor, which differentiates into maturated osteoblasts and mediated bone formation. The maturated osteoblasts synthesize dense, cross-linked collagen and specialized proteins in much smaller quantities, including osteocalcin and osteopontin, which compose the organic matrix of bone. This organic matrix forms a strong and dense mineralized tissue—the mineralized matrix.

Along with osteoblasts, osteoclast breaks down bone matrix. The balance of bone formation and bone resorption maintained bone homeostasis [13]. However, the balance tends to be negative with age, particularly in postmenopausal women, often leading to a loss of bone serious enough to cause fractures, which is called osteoporosis. A decrease in osteoblastogenesis of mesenchymal stem cells can be observed in osteoporosis [2]. Reduced proliferation and osteoblastic differentiation of BMSCs were reported to associate with the reduction of healing capacity such as impairment of bone formation in osteoporotic patients. Therefore, stimulation of proliferation and osteoblastogenesis from BMSCs becomes a therapeutic strategy for osteoporosis.

There are several signaling pathways involved in osteoblastogenesis. BMP (bone morphogenetic protein) signaling is a fundamental pathway that mediates osteoblast differentiation [14]. BMP signaling is mediated through type I and type II BMP receptors. After binding to BMP ligands, BMP receptors formed a complex. This dynamic interaction leads to signal transduction through either Smads or mitogen-activated protein kinases (MAPKs) which further activates the transcription of specific target genes involved in osteoblastic differentiation and bone formation [15]. Several MAPKs have been identified, including extracellular signal-regulated kinases (ERKs), c-Jun N-terminal protein kinase (JNK), and p38 MAPK. These three types of MAPKs are essential components of the signal transduction machinery that occupy central positions in this differentiation process [16]. Following activation of MAPK signaling during this differentiation process, BMP2/Smad signaling is activated.

Canonical and noncanonical Wnt signaling pathways play key roles in the regulation of osteoblast development including enhancement of osteoblast proliferation as well as differentiation [17]. The canonical Wnt/GSK3β/β-catenin signaling is a key pathway for regulating bone formation and contributing to osteoblastic differentiation. Canonical Wnt pathway involves the formation of a complex consisting of Wnt1, 3a proteins, Frizzed, LRP5/6, and GSK3β. A crucial step in transducting the Wnt signal is to destroy the cytoplasmic GSK3β complex by inducing GSK3β phosphorylation and subsequently, inhibits β-catenin phosphorylation, thereby stabilizing β-catenin. The accumulated β-catenin thus enters the nucleus and activates the expression of the Wnt target genes. These target genes include marker genes for osteoblastogenesis. Alkaline phosphatase is a membrane-anchored protein that is a characteristic marker expressed in large amounts at the apical (secretory) face of active osteoblasts. Other marker genes, including SP7 (which encodes osterix) and Bglap (encoding osteocalcin), were markedly increased in their expression during the osteogenic differentiation. Runx2 is the master osteogenic transcription factor that takes part in the process of osteoblast maturation. Runx2 is also found to transduce Wnt signaling for mediating osteogenic differentiation of BMSCs [18]. It can act as crosstalking regulator between Wnt signaling pathways and others that enhance osteoblastogenesis. The canonical Wnt signaling pathway is active in BMSCs, and therefore many signaling molecules are developed as drug targets such as GSK3β and LRP5/6.

Noncanonical Wnt signaling pathway is mediated by Wnt5a, which activates downstream pathways including Wnt/Ca^{2+}/PKC, small GTPase Rho, and JNK pathways [19]. Noncanonical Wnt signaling pathway has been reported to regulate many cellular processes, including gene expression, cell proliferation, and apoptosis. Wnt5a can interact with Fz receptor and activates the cytoplasmic protein Dvl2 through casein kinase 2α (CK2α), which induces the activation of downstream molecules such as RhoA and ERK1/2. CK2 is a key regulator both in canonical and noncanonical Wnt signaling pathways [20, 21]. CK2α is its catalytic subunit. CK2α can induce nuclear translocation of β-catenin and thereby precluding degradation mediated by the proteasome. CK2α can also induce disheveled activation and therefore acts as a switch to define distinct branches of noncanonical Wnt signaling pathways. Although CK2α was developed as targets for embryogenesis, neuronal differentiation, and myogenic differentiation, the role of CK2α for osteoblastogenesis is still unclear.

Semaphorins are a family of cell-surface or soluble proteins that are able to regulate cell-cell interactions as well as cell differentiation, morphology, and function. In the mammalian system, 20 semaphorins have been identified and fall into 5 classes (semaphorins 3–7) that are characterized by particular structural properties [22]. Among them, Sema3A play a key role in coupling of osteoblastogenesis and osteoclastogenesis. Sema3A is produced by osteoblasts and has been identified as a potent and direct inhibitor of osteoclast formation from osteoclast precursor cells. Distinct from other coupling factors, Sema3A promotes osteoblast differentiation from BMSCs (the precursor of osteoblasts), indicating a dual function role in which it inhibits osteoclastogenesis and enhances osteoblastogenesis [23]. The Sema3A signaling pathway is activated through binding with its cell-surface receptor composed of an Nrp1 and plexinA1 protein complex, which functions as a signal-transducing subunit [24]. This complex induces different downstream signaling molecules in osteoclasts and osteoblasts, resulting in different regulatory effects on differentiation. Therefore, regulation of the Sema3A pathway in osteoclasts and osteoblasts would be promising for the bone remodeling balance and be helpful for the development of therapeutic agents.

In addition to Sema3A, several other semaphorins play a role in osteogenesis. They can be expressed on osteoclast or osteoblast. Sema3A and Sema3E are

produced from osteoblasts, while Sema4D and Sema6D are expressed by osteoclasts. Sema7A can be expressed in both osteoblasts and osteoclasts. The role of semaphorin family proteins in osteoblast and osteoclast is different [25–27]. Sema3E are produced from osteoblasts, while Sema4D and Sema6D are expressed by osteoclasts. Sema7A can be expressed in both osteoblasts and osteoclasts. The role of semaphorin family proteins in osteoblast and osteoclast is different. Sema7A is reported to be expressed in osteoblasts and promoted the osteoblast migration [28]. In addition to Sema7A, expression level of Sema3E from mouse osteoblasts was reported to be increased by PTH and 1, 25-(OH)2 D3 treatment [29]. The effects of semaphorins are mediated by plexins, a group of nine transmembrane receptors that can be subdivided into four classes, plexins A–D. The semaphorin-plexin system has an important role in regulating bone cell function. Therefore, regulating the balance of semaphorin family protein levels and semaphorin-mediated signaling pathway might balance bone remodeling through enhancing osteoblastogenesis and simultaneously inhibiting osteoclastogenesis.

2.2 Chinese herbal drugs for targeting osteoblastogenesis

2.2.1 Traditional Chinese medicine theory

Traditional Chinese medicine (TCM) is a style of traditional medicine built on a foundation of more than 2500 years of Chinese medical practice that includes various forms of herbal medicine, acupuncture, massage (tui na), exercise (qigong), and dietary therapy [30] but recently is also influenced by modern Western medicine.

One of the basic theories of TCM is zàng-fǔ theory. The term zàng refers to the five entities, including the heart, liver, spleen, lung, and kidney, while fǔ refers to the six yang organs, including the small intestine, large intestine, gallbladder, urinary bladder, stomach, and Sānjiaō [31]. Among them, the kidney is considered to be related to bones. As distinct from the Western medical definition of kidneys, the TCM concept is more a way of describing a set of interrelated parts than an anatomical organ. The main functions of the kidney are to strengthen bones, dominate human growth and development, produce marrow to fill up the brain, etc. (**Figure 1**).

According to the Chinese medicine kidney theory, many kidney-nourishing herbal drugs can strengthen bones; therefore they are used for treatment of bone-related diseases such as osteoporosis. Enhancement of osteoblastogenesis might be one of the mechanisms of action of these herbs. A famous Chinese doctor named

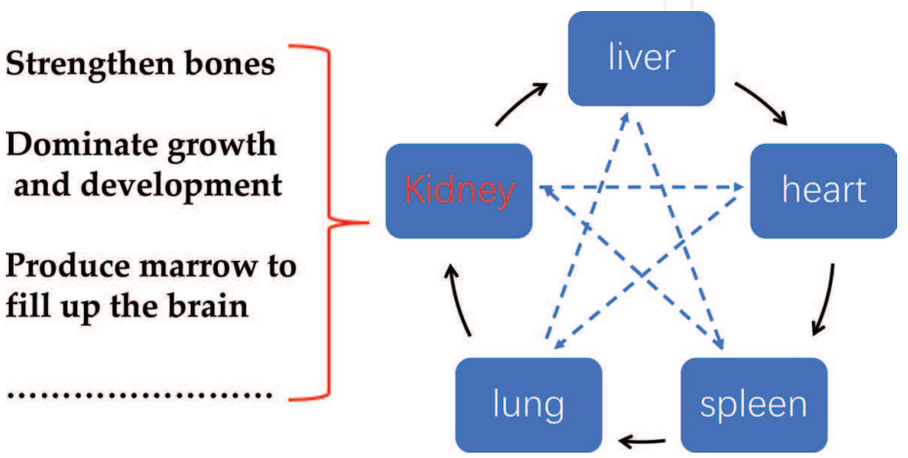

Figure 1.
The relationship of zàng-fǔ Chinese medicine theory and strengthen bones.

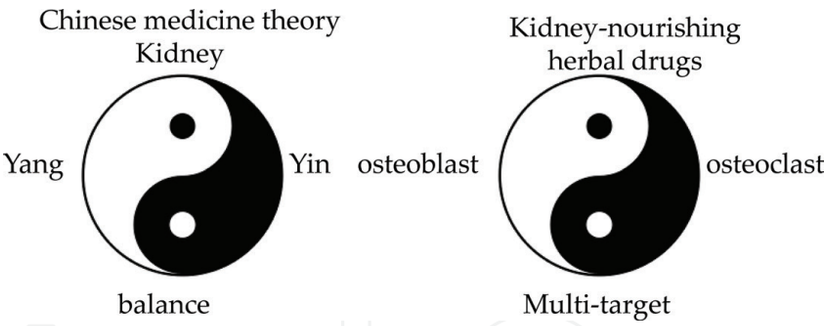

Figure 2.
Different regulated roles of kidney yin and yang in osteoblastogenesis. Kidney yang and yin might alter osteoblast and osteoclast function and enhance osteoblastogenesis and inhibiting osteoclastogenesis.

Zhang Jie Bin (approximately 1563–1640) wrote "there are two kidneys, (kidney yin and yang), with the gate of vitality between them" (**Figure 2**). The difference between kidney yin and kidney yang on strengthening bones is still unclear, but the different mechanisms of action of kidney yin and yang on osteoblastogenesis might partially explain the difference.

2.2.2 Chinese herbal drugs that enhance osteoblastogenesis and/or inhibiting osteoclastogenesis

Many kidney-tonifying herbal drugs are found to regulate osteoblastogenesis. Some are kidney-yang herbal drugs, including *Herba Epimedii*, *Taxus yunnanensis*, *Rhizoma Drynariae*, etc. Some are kidney-yin herbal drugs, including *Eclipta* herbal and *Fructus Ligustri Lucidi*. *Herba Epimedii* is a commonly used Chinese medicine as "kidney yang" herbs for thousands of years. It contains active components such as flavonoids and phytosteroids. Total flavonoids of *Herba Epimedii* are suggested to enhance osteoblast proliferation and differentiation and to be potential candidates for treating osteoporosis [32]. It includes icariin, epimedin A, epimedin B, epimedin C, icariside II, icaritin, etc. Although icariin is a principal flavonoid glycoside in *Herba Epimedii*, icariside II and icaritin, two hydrolytic metabolites in vivo as well as present in *Herba Epimedii*, showed higher activity of osteoblast proliferation and differentiation [33, 34] (**Table 1**).

Ecliptae herba, also known as "Mo-Han-Lian," is the aerial parts of *Eclipta prostrata* L. (Asteraceae), which have antiosteoporotic effect [45, 46]. Wedelolactone is a compound isolated from Ecliptae herba. Although ethyl acetate extract of Ecliptae herba and wedelolactone did not change BMSC proliferation, the extract and wedelolactone enhance BMSC differentiation toward osteoblasts. BMSC incubation with wedeloloactone results in an increase in the activity of alkaline phosphatase (ALP), a marker enzyme for maturated osteoblasts, in a dose-dependent manner. Also, mineralization level and calcium deposits increased accordingly in response to wedelolactone. At the molecular level, wedelolactone directly inhibited GSK3β activity and enhanced the phosphorylation of GSK3β and thereafter stimulated the nuclear translocation of β-catenin and runx2. The expression of osteoblastogenesis-related marker gene including osteorix, osteocalcin, and runx2 was increased. In ovariectomized mice, administration of wedelolactone prevented ovariectomy-induced bone loss by enhancing osteoblast activity and promoting new bone formation [47].

Distinct with other osteogenic agent, such as $PTH_{1–34}$, wedelolactone exert dual function role in enhancing osteoblastogenesis and simultaneously inhibiting osteoclastogenesis. For the anabolic agent, parathyroid hormone (PTH) a concomitant

Compounds or extracts	Herbal drugs or plants	Validation	Refs.
Icarrin, icariside II, icaritin	*Herba Epimedii*	Enhance osteoblast proliferation and differentiation	[34]
Wedelolactone	Ecliptae herba	Promote osteoblast differentiation and bone formation	[35, 36]
Specnuezhenide, ligustroflavone, salidroside, and Gl3	*Fructus Ligustrum lucidum*	Stimulate osteoblast proliferation and bone formation by BMP2 and runx2 activation	[37, 38]
Puerariae radix extract	*Puerariae radix*	Play a role in osteoblastic bone formation; induces osteoblastic differentiation markers such as ALP, OCN, OPN, and Col I and mineralization in SaOS-2 cells	[39]
Tetrahydroxystilbene glucoside	*Fallopia multiflora*	Promote osteoblast differentiation	[40]
Total flavonoids	*Rhizoma Drynariae*	Enhance osteoblast activity through BMP2/Smad pathway	[41]
Aqueous extract	*Angelica sinensis*	Stimulate proliferation and ALP activity of OPC-1	[42]
Extract	*Salvia miltiorrhiza*	Stimulates ALP activity in MC3T3-E1 cells	[43]
Extract	*Astragalus membranaceus*	Promote new bone formation on periodontal defects in vivo	[44]

Table 1.

Cellular and molecular targets for different herbal drugs with the ability of enhancing osteoblastogenesis.

increase in bone resorption can be observed. These drawbacks of the current therapies might be attributed to one target for these drugs that fail to uncouple bone degradation and formation: they stimulate or inhibit both processes at the same time. Research is currently focusing on drugs that can simultaneously regulate bone resorption and bone formation. Wedelolactone might be potential for the development of new class of drugs for treating osteoporosis. Further, the dual function of wedelolactone might be attributed to multi-target action on osteogenesis.

In addition to Wnt/GSK3β/β-catenin pathway activation by wedelolactone, we found that the semaphorin 3A pathway, as the upstream of Wnt/GSK3β/β-catenin pathway, was activated. Wedelolactone can increase mRNA expression of Sema3A in BMSCs and blocking Sema3A activity with its antibody reversed wedelolactone-induced alkaline phosphatase activity in BMSCs. Further, wedelolactone enhanced binding of Sema3A with cell-surface receptors, including neuropilin (NRP)1 and plexinA1. In addition, nuclear accumulation of β-catenin, a transcription factor acting downstream of wedelolactone-induced Sema3A signaling, was blocked by the Sema3A antibody. For the osteoclasts, a 9 day incubation fraction of conditioned media obtained from wedelolactone-treated bone marrow mesenchymal stem cell (BMSC) significantly inhibited tartrate-resistant acid phosphatase (TRAP) activity in RANKL-stimulated osteoclastic RAW264.7 cells. Conditioned media and wedelolactone promoted the formation of plexinA1-Nrp1, but conditioned media also caused these sequestration of the plexinA1-DNAX-activating protein12 (DAP12) complex and suppressed the phosphorylation of phospholipase C (PLC)γ2. These data suggest that wedelolactone promoted osteoblastogenesis through production of Sema3A, thus inducing the formation of a Sema3A-plexinA1-Nrp1 complex

and β-catenin activation. In osteoclastic RAW264.7cells, wedelolactone inhibited osteoclastogenesis through sequestration of the plexinA1-DAP12 complex, induced the formation of plexinA1-Nrp1 complex, and suppressed PLCγ2 activation [48].

Semaphorin family proteins exert different roles in wedelolactone-enhance osteoblastogenesis and wedelolactone-inhibited osteoclastogenesis. In addition to Sema3A, osteogenic medium(OS)-reduced Sema7A mRNA expression and OS-enhanced Sema3E mRNA expression in BMSCs were reversed by wedelolactone, but OS-reduced Sema3B mRNA expression had no change. Although there is evidence of the role of Sema3B in bone remodeling [49], OS treatment decreased Sema3B mRNA expression. Wedelolactone and Sema3B antibody did not affect ALP activity. Sema3B is reported to inhibit the proliferation and induce apoptosis in various types of cancers. Whether Sema3B has a role in BMSC proliferation and apoptosis is needed to be further studied. Wedelolactone enhanced the binding of Sema7A with plexinC1 and Beta1, but addition of Sema7A antibody partially blocked the binding triggered by wedelolactone. At the same time, addition of Sema4D antibody to wedelolactone-treated osteoclastic RAW264.7 cells showed a more significant decrease in TRAP activity and bone resorption pit formation. Wedelolactone inhibited the production of Sema4D and formation of Sema4D-PlexinB1 complex. Overall, wedelolactone inhibited the production of Sema4D and formation of Sema4D-PlexinB1 complex in RAW264.7 cells, thereafter inhibiting osteoclastogenesis. At the same time, wedelolactone enhanced osteoblastogenesis through promoting the production of Sema7A and Sema7A-PlexinC1-Beta1 complex formation in BMSCs. These results suggested that wedelolactone enhanced osteoblastogenesis but inhibited osteoclastogenesis through altering semaphorin family proteins [50].

BMP signaling pathways have a critical role in bone-formation process, and their effects can be mediated by Smad signaling. Among BMP family proteins, BMP2 has been reported to be essential for inducing bone formation [51]. Several studies have reported that MAPK signaling including JNK, ERK, and p38 pathways are involved in osteoblastogenesis [52]. p38 MAPK is required for osteoblast differentiation and induction of osteogenic marker genes. Also, p38 activation has been observed in lactoferrin-treated MC3T3-E1 cells. However, there is evidence that osteoblast differentiation is stimulated by activation of ERK and JNK, but not by activation of p38 MAPK [53]. Wedelolactone increased phosphorylation of extracellular signal-regulated kinases (ERKs), c-Jun N-terminal protein kinase (JNK), and p38 in BMSCs. Phosphorylation of mitogen-activated protein kinases (MAPKs), ERK, and JNK started to increase on day 3 of treatment, and p38 phosphorylation was increased by day 6 of treatment. Expression of bone morphogenetic protein (BMP)2 mRNA and phosphorylation of Smad1/5/8 was enhanced after treatment of cells with wedelolactone for 6 and 9 days. The addition of the JNK inhibitor SP600125, ERK inhibitor PD98059, and p38 inhibitor SB203580 suppressed wedelolactone-induced alkaline-phosphatase activity and bone mineralization. Increased expression of BMP2 mRNA and Smad1/5/8 phosphorylation was blocked by SP600125 and PD98059, but not by SB203580. Our findings confirmed that wedelolactone enhanced osteoblastogenesis through induction of the JNK- and ERK-mediated BMP2-Smad1/5/8 pathway [54].

Wedelolactone is the derivation of coumarin, which can target estrogen receptor and exert estrogenic activity. Wedelolactone also was found to be docked onto the crystal structure of GSK3β through electrostatic or hydrophobic interactions. Therefore, wedelolactone might exert multi-target effect and induce the signaling network for enhancing osteogenesis (**Figure 3**). However, wedelolactone at high dose has cytotoxicity against BMSC survival. Also, the concentration of wedelolactone into the blood is lower, which limited the application for treatment of osteoporosis in clinic. We suppose that other components combined with wedelolactone can exert synergetic effect.

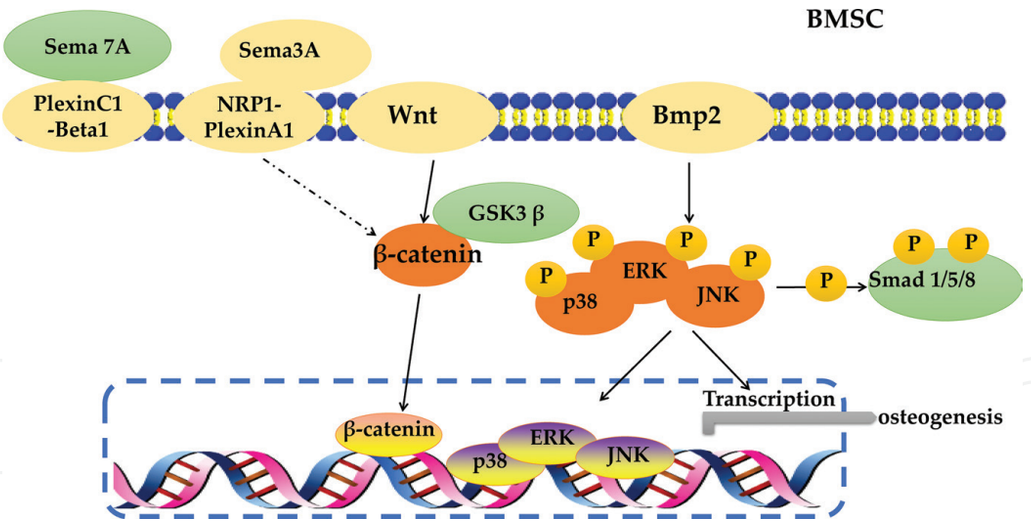

Figure 3.
Involvement of signaling network in wedelolactone-induced osteoblastogenesis. Wedelolactone induced Sema7A, 3A-mediated signaling pathway, activated downstream of β-catenin nuclear translocation, and promoted BMP2/Smad1/5/8 pathway activation, resulting in enhancement of osteoblastogenesis.

Er-Zhi-Wan is composed of Herba Ecliptae and *Fructus Ligustri Lucidi*, which is a famous traditional Chinese formulation firstly recorded in "Yi Bian" written in Ming Dynasty, which possesses the actions of tonifying the liver and kidney yin, nourishing body's essential fluid, and arresting hemorrhage. Er-Zhi-Wan extracts have been reported to prevent osteoporosis and inhibit osteoclast differentiation [55]. Er-Zhi-Wan containing serum inhibited proliferation and differentiation of preosteoclastic RAW264.7 cells. Therefore, we screened the synergetic components from *Fructus Ligustrum Lucidum*.

Fructus Ligustrum Lucidum (FLL), also known as Nu-Zhen-Zi, is the fruit of *Ligustrum lucidum* Ait., which has been used in traditional Chinese medicine for over 1000 years to nourish the liver and the kidney and thereafter strengthen the bones. In modern research, FLL is reported to possess anticancer, antidiabetic, anti-inflammatory, and heptoprotective effects. The crude FLL extract is reported to modulate the turnover of bone and the calcium balance in ovariectomized rats [56]. It also shows that ethanol extract improves calcium balance and bone metabolism in aged female rats through enhancing bone mineralization process [57]. The predominant components isolated from FLL include flavonoids, triterpenes, phenylethanoid glycosides such as salidroside, and secoiridoid glucosides such as specnuezhenide and ligustroflavone. Salidroside, specnuezhenide, and Gl3 are reported to increase osteoblast activity in osteoblast-like UMR106 cells. Aqueous extract of FLL activates ERα/β-mediated gene transcription, but the isolated compounds are inactive [58]. Salidroside is shown to promote the proliferation of BMSCs and increase the expression and secretion of stem cell factor (SCF) [59]. However, the cellular action of FLL and its compounds regulating bone metabolism is still unclear. In our study, FLL extract and five compounds from FLL were investigated to affect alkaline phosphatase (ALP) activity of bone marrow mesenchymal stem cells (BMSCs). FLL and its five components, including salidroside, specnuezhenide, nuezhenide G13, oleonuezhenide, and ligustroflavone, facilitated BMSC differentiation toward osteoblasts partially through BMP/LPR6/runx2 pathway.

Further, the combined effects of wedelolactone and various doses of compounds from *Fructus Ligustri Lucidi*, including oleonuezhenide, salidroside, and oleanolic acid, on osteoblastogenesis were evaluated. The combination of oleonuezhenide and wedelolactone was found to exert a synergistic effect on osteoblast differentiation. Wedelolactone at 6 μM and oleonuezhenide at 9 μM enhanced osteoblast

differentiation and bone mineralization. The enhanced effect was more potent when bone marrow mesenchymal stem cells were treated with a combination of wedelolactone and oleonuezhenide. Osteoblastogenesis-related marker genes including osteocalcin, runx2, and osterix were upregulated in the presence of 6 μM wedelolactone and 9 μM oleonuezhenide. At the molecular level, oleonuezhenide did not affect GSK3β phosphorylation induced by wedelolactone but elevated casein kinase 2α (CK2α) expression, resulting in β-catenin and runx2 nuclear translocation. The addition of 4,5,6,7-tetrabromo-N,N-dimethyl-1H-benzimidazol-2-amine (DMAT), a CK2α inhibitor, blocked oleonuezhenide-induced alkaline phosphatase (ALP) activity, and simultaneously suppressed β-catenin nuclear accumulation, induced by treatment with a combination of oleonuezhenide and wedelolactone. In addition, 30 μM wedelolactone-induced cytotoxicity in bone marrow mesenchymal stem cells was relieved by 9 μM oleonuezhenide. These bone marrow mesenchymal stem cells were protected by oleonuezhenide and maintained osteoblastic activity. Oleonuezhenide increased Wnt5a and CK2α expression. Wedelolactone-reduced extracellular signal-regulated kinase (ERK) phosphorylation was reversed by oleonuezhenide. However, the addition of DMAT reduced ERK phosphorylation induced by oleonuezhenide. Taken together, these data demonstrate that 10 μM oleonuezhenide enhanced the effects of 6 μM wedelolactone on osteoblastogenesis, by GSK3β- and CK2α-mediated β-catenin activation. Thus, wedelolactone-induced cytotoxicity was prevented through CK2α-mediated ERK activation. This combined effect of wedelolactone and oleonuezhenide on osteoblastogenesis may be contributed to understanding the efficiency of Er-Zhi-Wan on curing bone-related diseases, such as osteoporosis.

2.3 Combined therapy by using biomaterials and drugs

Tissue engineering provides a promising strategy in the field for osteoblastogenesis from BMSCs, which aims to induce new, BMSC-driven, bone regeneration and has increased the possibility of engineering bone in vitro to treat bone defects particularly in vivo [60–62]. As alternatives polymeric biomaterials are applied in clinical practice since the 1960s. Their popularity is related to the ease of preparation of various products in complex shapes, their moderate price, and a wide range of physical and mechanical properties. Biocomposite scaffolds are generally used for bone tissue engineering to act as a temporary matrix and provide a suitable environment for cell proliferation, differentiation, and extracellular matrix (ECM) deposition until it is restored by new natural tissue over the desired time. Various biomaterials are designed to engineer bone tissue. The bone tissue engineering scaffolds possess non-cytotoxicity, a high surface to volume ratio, abundant porosity for the transport of nutrients and regulatory factors, interconnectivity of pores for neovascularization at the site of new tissue regeneration, and osteoconductive and osteoinductive properties. Most of these material properties are satisfied by poly(L-lactic acid)-co-poly (ε-caprolactone) (PLACL), which is a copolymer of PCL and PLLA and was found to be potential synthetic polymer for bone regeneration therapy. Osteoblasts cultured on biocompatible poly(L-lactic acid)-co-poly (ε-caprolactone)-silk fibroin-hydroxyapatite-hyaluronic acid (PLACL-SF-HAp-HA) showed a significantly higher level of proliferation and increased osteogenic differentiation and mineralization [63]. Promising polymeric biomaterials however often do not fulfill the necessary requirements for the production of suitable bone implants. One of the challenges to overcome is the proliferation of cells on the implant surface [64] which could be achieved by mimicking the natural 3D bone structure with a composite of organic polymer and inorganic components.

Hydroxyapatite (HAp) is a major mineral component of calcified tissues including bones. Synthetic HAp has been extensively used as an important material for bone substitute, owing to its excellent osteoinductive properties [65]. Synthetic HAp [Ca10(PO4) 6(OH)2) has been extensively used as an implant material for bone substitute, owing to its excellent osteoinductive properties. Calcium phosphate biomaterials (HAp-TCP) with appropriate 3D geometry are able to bind and concentrate endogenous bone morphogenetic proteins in circulation, may become osteoinductive, and can be effective carriers for bone cells. Different characteristics and sizes of HAp are developed to be generally used for bone tissue engineering, which acts as a temporary matrix and provides a suitable environment for cell proliferation, differentiation, and extracellular matrix (ECM) deposition. However, HAp is considered to be weak in osteoinductive ability, which may impact the repair capacity for bone defects. Growth factors play an important role in the process of bone formation; some scaffolds have been developed as delivery carriers for growth factors and showed great bone repair ability. However, considering the high production cost and limited active period, the clinical application of exogenous growth factors is restricted. We proposed that natural products combined with HAp might have enhanced effect on osteoblastogenesis. Three kinds of HAps constructed by a rodlike shape with particle size of 25 nm (HAp-1), 37 nm (HAp-2), and 33 nm (HAp-3) did not affect BMSC survival but induced activity of alkaline phosphatase (ALP), a marker enzyme for osteoblastogenesis. The rodlike HAp might have distinct properties for osteoblast differentiation [66]. Additionally, particle size is a very important influencing factor on activity of HAp samples. The smallest particle has the best activity might be due to its easier contact of the target in the cell. The O-H groups abundantly located in the surface of HAp-1 crystal might facilitate the hydrogen bond interaction between the HAp-1 and the protein of the cell, which is in accordance with cell activity data. The structure and activity relationship analyses give us some instruction to design new HAp samples with smaller size and more hydroxyl group to improve activity.

Interestingly, the combination of HAp-1 and wedelolactone exhibited a higher and more prolong time for ALP activity, indicating that HAp-1 and wedelolactone exerted synergetic effect on ALP activity. Recent studies have demonstrated that due to the excellent specific surface area, micro-/nano-hybrid structured HAp (micro-nano HAp) granules could be applied as carriers of drug delivery systems to enhance osteogenic ability [67]. HAp-1 treatment resulted in a more significant increase in the number of ALP staining-positive BMSCs and maintained an extended time for the increased number of ALP staining-positive BMSCs. The extended time for enhanced ALP activity in the presence of HAp-1 and wedelolactone indicates that HAp-1 might have the ability of carrying wedelolactone and subsequently sustained release of wedelolactone from the HAp-1. HAp-1 combined with wedelolactone induced a higher ALP activity for a longer time than HAp-2 and HAp-3 and also increased the bone mineralization level. Osteoblastogenesis-related marker gene expression including osteorix, osteocalcin, and runx2 were increased after BMSCs were treated with HAp-1. In conclusion, HAp, which is a major mineral component of calcified tissues including bones, with different sizes generated different effects on osteoblastogenesis. HAp-1 combined with wedelolactone exerted an enhanced effect on osteoblastic differentiation, mineralization, and osteoblastogenesis-related marker gene expression, which has potential for treating osteoporosis.

3. Conclusion

Targeting osteoblastogenesis has become a promising therapeutic strategy for treatment of osteoporosis. Several pathways including canonical and noncanonical

Wnt pathway, semaphorin pathway, and BMP/Smad pathway play a critical role in regulating osteoblastogenesis. Regulating the biological network for enhancing osteoblastogenesis and simultaneously inhibiting osteoclastogenesis might develop new type of multi-target drug for treating osteoporosis. The kidney-nourishing Chinese herbal drugs have the potential since these herbs contain many components and thus exert synergetic effect. The multi-target mechanism of Er-Zhi-Wan, a prescription record since Ming Dynasty in China, is confirmed by study of combined effect of wedelolactone and oleonuezhenide. Further study of these herbs as well as screening the active components might find novel potential drugs. Further, the development of tissue engineering biomaterials and combination with Chinese herbal drugs might generate the superior therapeutic effect.

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Conflict of interest

The authors have declared that there is no conflict of interest.

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