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ScienceDirect

Journal of Magnesium and Alloys 10 (2022) 3327–3353

www.elsevier.com/locate/jma

Review

Magnesium alloys for orthopedic applications:A review on the mechanisms driving bone healing

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Received 1 September 2022; received in revised form 13 November 2022; accepted 25 November 2022
Available online 16 December 2022

Abstract

Magnesium (Mg) alloys have attracted a wealth of attention in orthopedic fields for their superior mechanical properties, degradability, and excellent biocompatibility. Consistently, to resolve the issues on rapid degradation, more studies are dedicated to the researches on the composition design, preparation and processing, surface modification, the degradation modes of Mg alloys. Nevertheless, the mechanisms by which Mg alloys promote bone healing remain elusive. This review gives an account of specific mechanisms on Mg alloys promoting bone healing from four aspects, immunomodulatory, angiogenesis, osteogenesis and regulation of osteoclast function. We highlight the regulation of Mg alloys on the functional status and interactions of numerous cells that are involved in bone healing, including immune cells, osteogenic-related cells, osteoclasts, endothelial cells (ECs), nerve cells, etc., and summarize the signaling pathways involved, with the aim to provide the basis and support on future investigation on mechanisms on Mg alloys driving bone regeneration. More importantly, it provides a rationale and a general new basis for the application of Mg alloys in orthopedic fields.

Keywords: Magnesium alloys; Bone regeneration; Immunomodulation; Angiogenesis; Osteogenesis.

1. Introduction

The incidence of musculoskeletal disorders has increased dramatically in recent years, such as fractures, large segmental bone defects, osteoarthritis [1]. However, there are still huge problems and challenges in the clinical treatment of these conditions [2]. The ensuing medical and socioeconomic burden has become a serious public health problem [1]. The development and utilization of biomaterials have made great contributions to solving this condition [3]. Stainless steel, titanium (Ti) and its alloys, and cobalt-chromium (Co-Cr) alloys are traditional orthopedic implants widely used in clinical practice. They have sufficient mechanical strengths and good biocompatibility [4]. However, these inert biomaterials still have drawbacks in their application. Metal particles or ions released by them during corrosion or wear can lead to serious complications such as poisoning, allergies, etc. [5]. At the same time, their elastic modulus does not match well with bone tissue. The resulting stress shielding will eventually lead to implant loosening and bone loss [4]. Furthermore, a second surgery is often required to remove the implant after bone tissue healing [4]. In contrast, biodegradable biomaterials can progressively degrade at implant sites. In this case their removal after the bone tissue healing is

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https://doi.org/10.1016/j.jma.2022.11.014

no longer necessary [6]. Moreover, material degradation not only produces small molecules to regulate the microenvironment of bone regeneration but also allows the ingrowth of new bone tissue [6]. Development of degradable orthopedic implant materials with excellent mechanical properties and biocompatibility will hold great promise for the treatment of bone injuries and diseases [6]. Therefore, biodegradable polymers have attracted much attention as new orthopedic implant materials [7]. At present, a variety of degradable polymer materials have been approved by the US Food and Drug Administration (US FDA) and are widely used in orthopedics [6],[7]. These degradable polymers include natural polymers Collagen-graft^{TM} (HA/TCP/bovine collagen), OssiMend^{TM} (porous bone mineral with collagen), synthetic complexes poly (L-lactic acid) (PLLA), poly (glycolic acid) (PGA), polycaprolactones (PCL), etc. [6],[8]. However, due to insufficient mechanical strength, these polymers only have great potential for application at low load-bearing fracture sites [9]. Furthermore, some intermediate degradation products have been shown to induce long-term inflammatory responses, which are not conducive to bone regeneration [9]. These limitations have forced researchers to search for better-performing biodegradable orthopedic implant materials.

Mg and its alloys have been extensively studied in the field of orthopedic implants over the past 20 years due to their superior biodegradability, mechanical properties and biocompatibility [10]. First, Mg possesses appropriate elastic modulus that is close to bone tissue, which can reduce stress shielding to avoid bone loss and guide bone regeneration well [10]. Second, Mg alloys can be gradually degraded and produce little adverse host reactions after implantation [11]. In addition, complete degradation of the implants after bone tissue healing avoids secondary removal surgery [10]. More importantly, Mg is a crucial element for bone growth [12]. It has been widely demonstrated to significantly contribute to bone regeneration and repair, showing great application potential in the field of orthopedics [13]. At the same time, the discovery of its ability to promote angiogenesis and immune regulation provides a great advantage for its application as orthopedic implants [12],[14]. Currently, the clinical application of Mg alloys has made remarkable progress. Mg alloys are designed as screws [15],[16], plates [17], and even 3D scaffolds [18] for fracture fixation or bone defect repair. In 2013, the first Mg screw MAGNEZIX® used in hallux valgus fracture came out and was certified by Communauté Européenne (CE) [15]. Subsequently, Mg-Ca-Zn system (RESOMET®) performed well in clinical tests of hand fracture treatment and was approved for clinical use by Korean Food and Drug Administration (KFDA) in 2016 [16]. In China, high-purity Mg screws were developed and have been successfully used in the treatment of avascular necrosis of femoral head [19]. Moreover, recently, Shanghai Jiao Tong University developed a new patented Mg-Nd-Zn-based alloy (Jiaoda BioMg, called JDBM), which has shown great clinical application potential in *in vivo* and *in vitro* experiments, especially in large animal models, as bone plates, screws and even porous scaffold materials [20]. Although these clinical developments are gratifying, the rapid degradation of Mg alloys is still a huge obstacle to its orthopedic application [21]. The rapid degradation results in premature loss of the mechanical integrity and support of Mg alloys. Excessive release of hydrogen (H_2), metal ions and formation of high alkaline environment are harmful to tissue cells and hinder new bone regeneration and osseointegration [17],[22]. To solve this problem, a great number of studies have focused on the composition design, preparation and processing, surface modification, the degradation modes, *in vitro* corrosion testing and osteogenic performance verification of Mg alloys [23–25].

However, it cannot be overlooked that good biological interaction between the host tissue and the implant is the key to the success of the implant [26]. The shaping of the microenvironment of the implant site and the regulation of the functional state of the host cells by Mg alloys are issues worthy of attention and research. The exploration of the interaction and the underlying mechanisms between Mg alloys and the host after implantation will provide more theoretical basis for the optimization of Mg alloys and promote the application of Mg alloys in orthopedic treatment. Unfortunately, although an increasing number of studies have focused on the effect of Mg alloys on bone regeneration-related cells, the comprehensive regulation of Mg alloys on host cell function during bone regeneration and the underlying mechanisms remain unclear [27].

This review comprehensively summarizes the current research progress on the mechanism of Mg alloys promoting bone regeneration and repair. We elaborate from four aspects: immunomodulatory, angiogenesis and osteogenesis and regulation of osteoclast function. The regulation effect and potential mechanism of Mg alloys on the functions of various cells involved in bone healing were mainly discussed. It aims to provide basic support and direction for future exploration of the mechanism of Mg alloys driving bone healing. More importantly, it provides more theoretical basis and optimization ideas for the application of Mg alloys in orthopedics.

2. Bone healing process after injury

Bone healing is a complex and dynamic process, including three consecutive and partially overlapping phase of inflammation, repair, and remodeling [28]. After bone injury, injured cells, immune cells, and other resident cells including ECs and osteoblasts release pro-inflammatory mediators to recruit more immune cells to initiate an inflammation response. Under the action of immune cells and inflammatory factors, the tissue debris and bacteria at the injury sites are removed, providing a favorable environment for subsequent angiogenesis and bone regeneration [28],[29]. Repair-related cells such as fibroblasts, mesenchymal stem cells (MSCs), and vascular precursor cells are recruited for initial proliferation and differentiation, enabling bone healing to enter the repair phase [29],[30]. In addition, angiogenic factors expressed by macrophages, osteoblasts, and ECs induce the ingrowth of blood vessels, thus promoting tissue repair [31]. Notably, the most important feature of the repair phase is the transforma-

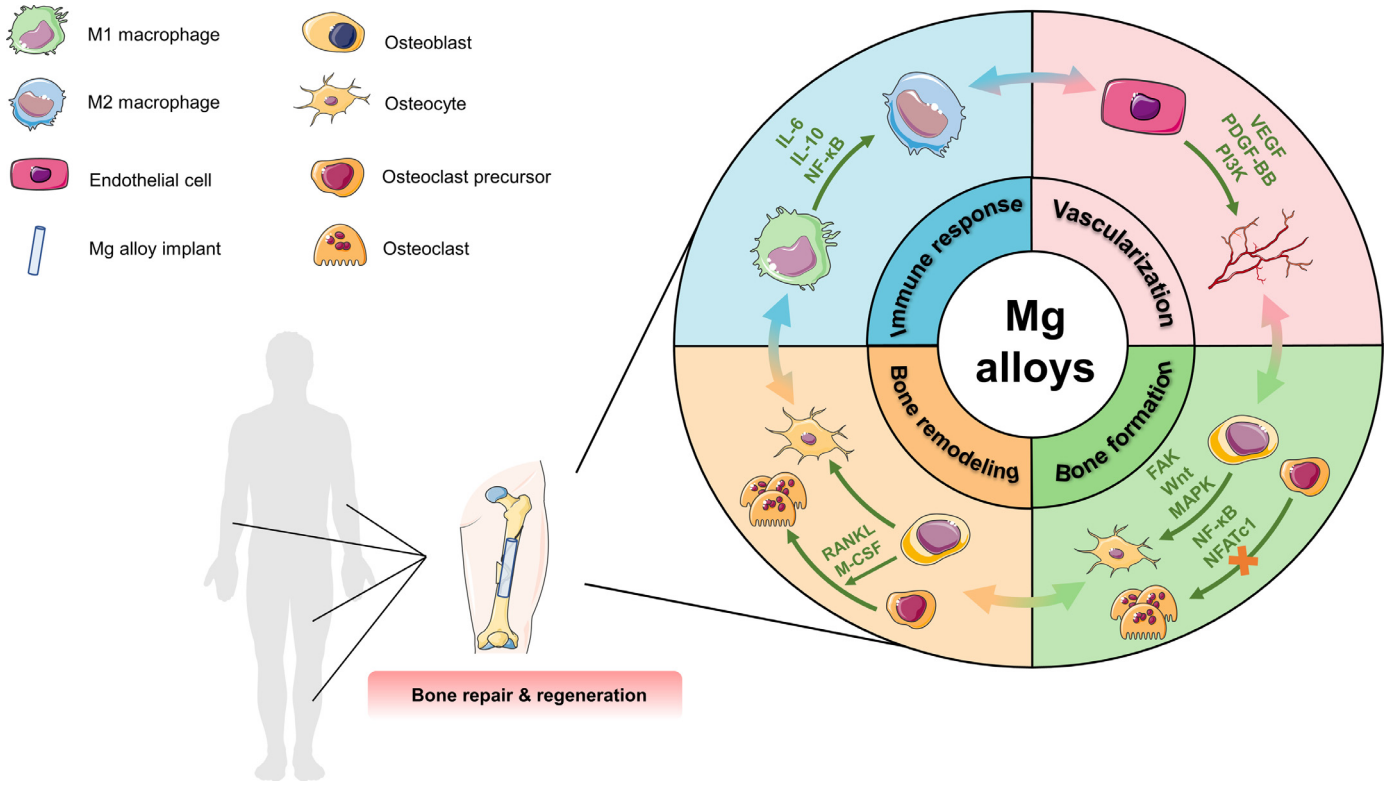

Fig. 1. Schematic illustration of Mg alloys on participating immune response, angiogenesis, osteogenesis and regulation of osteoclast function as bone implants to drive bone healing.

tion of microenvironment toward an anti-inflammatory state at injury sites. Macrophage polarization towards an M2 anti-inflammatory phenotype is thought to be essential for anti-inflammatory microenvironment shift [32]. Under the action of M2 macrophages, anti-inflammatory mediators and various growth factors, osteoblast-related cells began to dominate the bone repair phase [33]. They recruit, proliferate, differentiate, and form callus through intramembranous ossification or endochondral ossification. Ultimately, the callus structure undergoes bone remodeling under the coordination of osteoclasts and osteoblasts to form a mature lamellar bone structure, and the healing of the damaged bone is achieved [29],[34].

By reviewing the process of bone healing, it is not difficult to find that various cells of different lineages, including immune cells, vascular cells, osteoblasts, osteocytes, and osteoclasts, are involved in regulating bone healing orderly [31],[33],[35]. Compared with traditional inert biomaterials, Mg alloys can not only promote osteogenesis, but also regulate immune microenvironment, revascularization and bone remodeling of the injury site, which are crucial for successful bone healing. This is mainly due to Mg alloys show regulatory effects on all cells mentioned above [12],[36],[37] (Fig. 1). Therefore, Mg alloy interacts with various cells in various ways at different phases of bone healing, in order to achieve comprehensive regulation of bone healing. In the following, we describe the mechanisms by which Mg alloys modulate immune responses, angiogenesis, osteogenesis, and osteoclast function during bone regeneration and repair.

3. Mg alloys modulate immune responses favored osteogenesis and angiogenesis

3.1. Mg alloys are involved in the inflammatory activation at the implantation sites

3.1.1. Protein adsorption initiates immune response

After the *in vivo* implantation of biomaterials, the initial event is protein in the blood and interstitial fluid being adsorbed on the surface of biomaterials [38]. The protein absorption layer forms hemostatic clot through the activation of coagulation cascade on the biomaterial surface, which is regarded as transient provisional matrix of cellular adhesion and migration. Then, the hemostatic clot regulates the adhesion, migration, and proliferation of immune cells to initiate the inflammatory response [38]. During this process, fibrinogen (Fg) is proposed as a key mediator to prime immune response for inducing platelet activation and hemostatic clot formation [39]. Mg and alloying elements in Mg alloys are corroborated to affect the process of Fg adhesion [39],[40]. Wang et al. investigated the Fg adsorption behavior in Mg-Zn binary alloy, discovering that the anchoring residues with high degree of positive charge on the surface of Mg atom facilitated Fg adsorption in Mg alloys with low Zn content, such as Mg-1Zn,

Mg-2Zn and Mg-3Zn alloy. In contrast, the decreased anchoring residues on the surface suppressed its adsorption when Zn content was high, such as Mg-5Zn and Mg-10Zn alloy [39]. Furthermore, it is demonstrated that the species of alloying elements in Mg alloys affects Fg adsorption as well. As was shown in the previous study, Fg preferentially adsorbed on the second phases containing Y, Ce or Nd, while the ones with Zn element inhibited its adsorption. This may due to the surface of the former ones is prone to form active corrosion sites that favor protein adsorption [40].

3.1.2. The activation of innate immune system: the recruitment of neutrophils and the phagocytosis of macrophages

After protein adsorption, neutrophils are recruited to the implantation sites within hours, thereby priming acute inflammatory response [35]. Cipriano et al. confirmed, for the first time, that Mg^{2+} degraded from Mg-Zn-Sr alloy could regulate the recruitment and adhesion of inflammatory cells after vascular cellular adhesion molecule-1(VCAM-1) was upregulated, which was a key factor of ECs to initiate immune response [41]. During this process, the attractive effects of Mg^{2+} on neutrophils may be regulated by IL-8 secretion via p38 signaling pathway [42]. After activated, neutrophils induce the production of chemokines in surrounding tissues, so as to stimulate the activation of monocytes, macrophages, immature dendritic cells (DCs) and lymphocytes, for further immune response [35].

Monocytes migrate to the site of injury through adherence factors and differentiate into macrophages in acute inflammation phase [43]. Monocytes and macrophages play a central role in the inflammatory phase and contribute to establishment of a more sustained immune response [28]. In the study of Xie et al., the implantation of JDBM alloy in a rabbit femur model brought inflammatory cell infiltration and TNF-α high secretion near the bone-implant interface in the early stage (day 5 after operation). Further experiments *in vitro* confirmed that a high concentration of Mg^{2+} (about 43.3 mM) significantly promoted the M1 macrophage polarization. The expression of TNF-α and inducible nitric oxide synthase (iNOS) in macrophages was up-regulated, while the expression of Arg-1 (M2 phenotype marker) was inhibited. At the same time, the phagocytic ability of polarized M1 macrophages was also improved under the action of a high concentration of Mg^{2+} [44]. It is reasonable to speculate that the high concentration of Mg^{2+} produced by the early rapid degradation of Mg alloys can promote the early inflammatory reaction by enhancing the polarization of M1 macrophages and improving their phagocytosis. From this perspective, moderate early rapid degradation of Mg alloy is desirable, which is beneficial to the early debridement of the implant microenvironment. However, it is difficult to confirm the above speculation because the measurement of Mg^{2+} concentration at the implantation site cannot be realized. In any case, this suggests that when designing Mg alloys with different degradability, we should pay attention to the effect of the early rapid degradation of Mg alloys on the early inflammatory reaction at the implant site. Furthermore, macrophages are able to phagocytose the second phase which in turn affects the degradation of Mg alloys. Liu et al. cocultured macrophages with $Mg_{17}Al_{12}$ particles with an average size of 10 μm, which has the similar structure and size with the $Mg_{17}Al_{12}$ second phases in Mg-Al-Zn alloy. It was observed by scanning electron microscope (SEM) that macrophages grasped the $Mg_{17}Al_{12}$ particles through the extended filopodia, triggering particle internalization, and then, the particles were processed in the endo/lysosomal compartments [45]. Nonetheless, both particles in large size and in excess lead to the failure in the uptake of macrophages, thus forming foreign-body giant cells (FBGCs). FBGCs release profibrotic growth factors to promote fibroblast activation and proliferation [46], which subsequently leading to excessive fibrotic capsule and ultimate engraftment failure of biomaterials [35]. This suggests that controlling the size and amounts of second phase particles in Mg-based implants is crucial for easily digestion by macrophages as well as ensuring the proper operation of immune system [45].

3.1.3. The activation of adaptative immune system: the maturation of dendritic cells and the activation of T lymphocytes

In addition to innate immune systems, adaptive immune systems are also involved in the host response to biomaterials. Antigen-presenting cells (APCs), especially DCs, are the key factors to bridge the innate and adaptive immune systems [47]. They phagocytose antigens and present them to T cells. Subsequently, T cells are activated as Th1 or Th2 cells, which enables active immune defense [47]. Interestingly, the activated T cells drive macrophages toward an M1 or M2 phenotype, thus promoting tissue healing by modulating macrophage polarization [48],[49]. As calcium antagonists, Mg regulates cellular immune response related to antigen processing by influencing calcium homeostasis and intracellular free Ca^{2+} concentration [50], including promoting immature DCs transformation to the mature ones [51] and the activation of T lymphocytes [52]. It was found that antigen stimulation tended to evoke a rapid influx of Mg^{2+} in T lymphocytes. The mutation of the selective Mg^{2+} transporter Mg^{2+} transporter 1 (MagT1) abolished Mg^{2+} as well as Ca^{2+} influx simultaneously, which further weakened the response of T cells towards antigen presentation [52]. This suggests that the balance between Mg and Ca plays a pivotal role in specific immune response [53]. Furthermore, Kanellopoulou et al. discovered that during T cell response, Mg^{2+} enhanced T cell activation and exerted immunomodulatory roles by directly regulating the specific kinase active site. Reduced Mg^{2+} specifically impaired TCR signal transduction [54]. Similarly, Wang et al. demonstrated that nanoparticles containing Mg promoted cell-mediated Th1 immunity as well as antibody-mediated Th2 immunity *in vitro* [47], which also confirmed the promotion of Mg on T lymphocytes activity.

3.2. Mg alloys promote an anti-inflammatory immune microenvironment by regulating M2 macrophage polarization

As mentioned above, the early rapid degradation of Mg alloys seems to promote the M1 macrophage polarization to participate in the early inflammatory reaction. Although activating M1 macrophages at the early stage is key to the initiation of regeneration, the long-term presence of M1 macrophages tends to damage tissues and triggers chronic inflammation due to the over-secretion of pro-inflammatory cytokines. The timely end of inflammatory reaction is directly related to the outcome of the implant [55]. Therefore, the effect of the peak value and duration of early rapid degradation of Mg alloy on the early inflammatory reaction is noteworthy. The early inflammatory reaction of bone healing usually lasts around a week [28], so it may be appropriate to limit the duration of early rapid degradation of Mg alloy *in vivo* to one week. The transformation of macrophages into M2 phenotype after the early inflammatory response subsided is crucial, as it is beneficial to create an anti-inflammatory microenvironment for tissue regeneration [56]. Interestingly, Mg alloys have been proven to have the potential to regulate the polarization of M2 macrophages [12],[57–59], in which low-concentration Mg^{2+} and [60],[61] a weak alkaline environment [57] play a central role. The activation of M2 macrophages can additionally contribute to matrix mineralization [62] and tissue vascularization [55], thus realizing rapid tissue regeneration with high quality [56],[63]. Therefore, the different degradation rates of Mg alloy in different periods may promote its dual regulation of the M1 phenotype and M2 phenotype of macrophages (Fig. 2). Timely conversion of the degradation curve of Mg alloy will be the key to effectively exerting its bone immunomodulation function, which needs special attention in alloy design.

3.2.1. Effects of Mg alloys on M2 macrophage polarization

Mg alloys were previously reported to have potential ability to regulate M2 macrophage polarization [12],[57–59] (Table 1). In the study of Kim et al., Mg-Si-Ca alloy extract was confirmed to significantly inhibit the expression of pro-inflammatory cytokines 1L-6, IL-1β and TNF-α in macrophage, suggesting the inhibition of M1 macrophages [57]. Costantino et al. found that Mg-10Gd and Mg-2Ag alloy extracts exacerbated mostly the M2 profiles of macrophage while inhibiting the M1 one. The extracts gradually increased the IL-1ra/IL-1β ratio and the expression of IL-10 over time, thereby promoting the formation of tissue regeneration-promoting states [58]. Similarly, the Mg-Si-Ca alloy extract was shown to promote M2 macrophage polarization thereby regulating the expression of cytokines [12]. Rahmati et al. implanted Mg-Zn-Ca alloy into rat femur, and further confirmed the promotion of Mg alloy on M2 macrophage polarization *in vivo*. They found that M2 macrophages were significantly increased at the implantation site, while the proportion of M1 macrophages was significantly reduced after 10 days [59]. It coincides with the immune regulation of normal bone healing which enters an anti-inflammatory and tissue regeneration state after a week of acute inflammation.

The regulation of M2 macrophage polarization by Mg alloys is inseparable from the effect of Mg^{2+}. Interestingly, the modulation of macrophage polarization by Mg^{2+} was shown to be concentration-dependent. As mentioned above, high concentrations of Mg^{2+} favor the M1 macrophage polarization and inflammatory response. Conversely, low concentrations of Mg^{2+} are thought to promote anti-inflammatory responses. In the study of Hu et al., 120 mg/L Mg^{2+} reduced the gene expression of TNF-γ, IL-6 and IL-1 in macrophages by inhibiting the activation of NF-κB [60]. Sun et al. observed that less than 70 mg/L of Mg^{2+} concentration favored maximizing M2 macrophage polarization, as well as maintaining cell viability [61]. This concentration-dependent modulation of macrophage polarization can be fully exploited in Mg alloy implants. In the early stage of Mg alloy implantation, relatively rapid degradation leads to higher Mg^{2+} concentration, which induces and promotes an inflammatory response. After the early rapid degradation, the degradation of Mg alloys tends to be stable, and the release of Mg^{2+} at an appropriate concentration promotes the M2 macrophage polarization to play an anti-inflammatory regulatory role and promote the smooth progress of the bone healing process [44].

Besides, the role of the alkaline environment and H_2 release on macrophages cannot be ignored. In the study of Kim et al., pure Mg and Mg alloy extracts without pH control significantly inhibited the expression of IL-6, TNF-α and IL-1β in macrophages. However, this effect was weakened when the pH of the extracts was neutralized, suggesting that the alkaline pH inhibited the selection of pro-inflammatory cytokines in macrophages [57]. In the review of others, H_2 were summarized to reduce the expression of inflammatory factors including TNF-α, IL-6, IL-1β, CCL-02, IL-10, TNF-γ, IL-12, CAM-1, HMGB-1, PGE-2 and nuclear factor-κB (NF-κB) [68]. This anti-inflammatory effect of H_2 will be conductive to the shaping of bone regeneration environment. However, there are few studies on the effects of the alkaline environment and H_2 release on the polarization and function of macrophages, especially in the field of Mg alloys.

3.2.2. Interaction between Mg alloy-activated M2 macrophages and MSCs

As was shown in the previous studies, M2 macrophages, cooperating with MSCs were proved to promote tissue regeneration [62] as well as bone repair [69]. Recently, it has been demonstrated that the cooperative relationships between MSCs and macrophages are enhanced with the regulation of Mg alloys [12]. Mg alloys convert the macrophages from the M1 phenotype to the M2 phenotype to suppress inflammation. Subsequently, M2 macrophages secrete anti-inflammatory cytokines and pro-regenerative factors to form a favorable bone immune microenvironment, thereby promoting the osteogenic differentiation of MSCs [70–72]. It was observed in numerous studies that with the aid of Mg, M2 macrophages upregulated the expression of BMP-2, BMP-6 and TGF-β, which

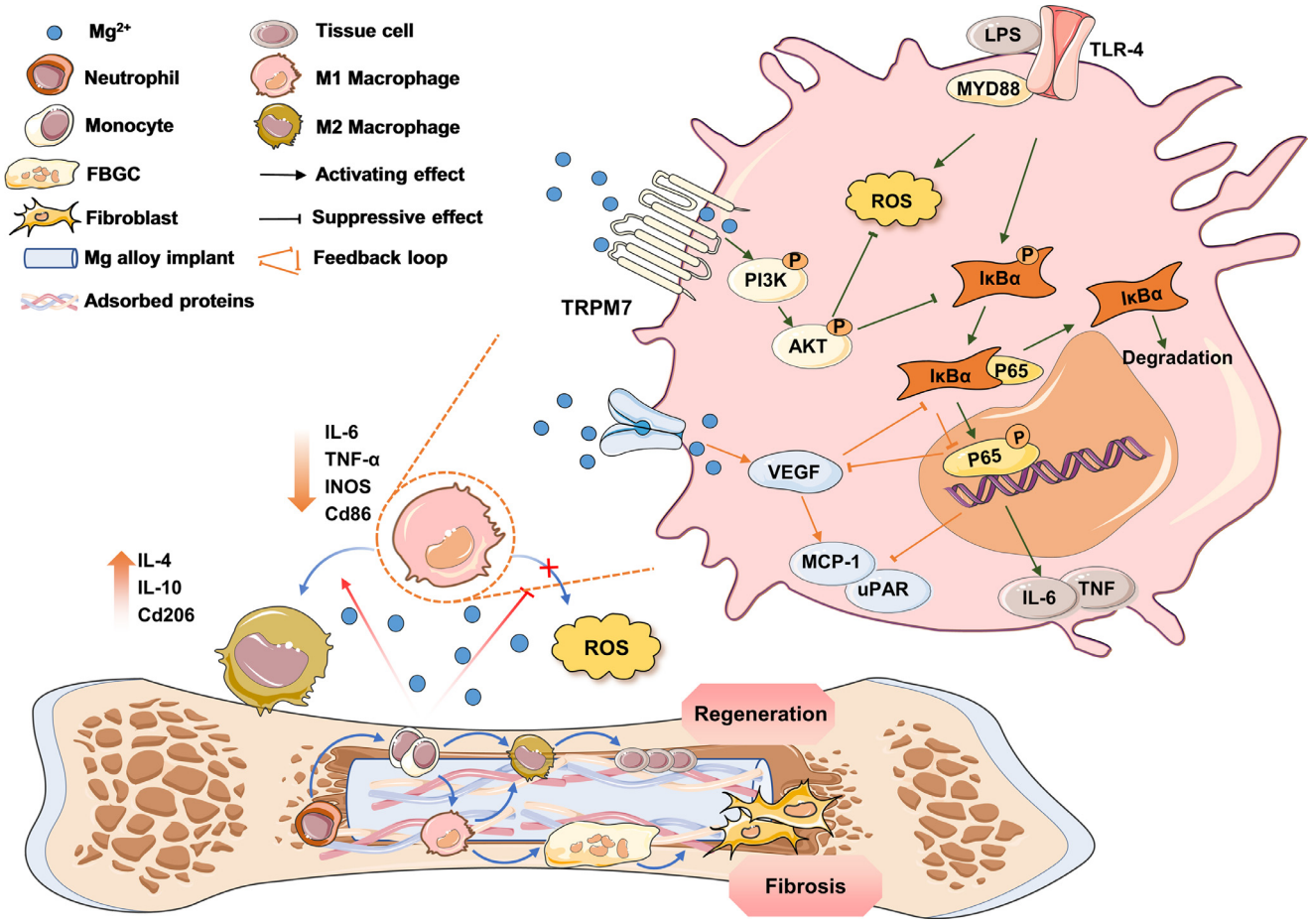

Fig. 2. Schematic diagram of Mg alloys promoting M2 macrophage polarization to establish an anti-inflammatory microenvironment which is favor to bone healing. In the early stage of implantation, Mg alloys tend to trigger immune response, including protein adsorption, inflammatory cell recruitment, monocyte differentiation, macrophage M1 phenotype polarization and phagocytosis, etc. Then, relatively low concentrations of Mg^{2+} generated by stable degradation inhibits M1 phenotype, and promotes the phenotypic switch to M2, which leads to tissue regeneration instead of fibrosis. Mg^{2+} enters M1 macrophage and activates the TRPM7-PI3K-AKT1 signaling pathway, then scavenges intracellular ROS based on TLR-4-MYD88-NF-κB signaling pathway. The excellent immunomodulatory effects of Mg alloys can be further enhanced through a feedback loop between VEGF and TLR-4/ NF-κB pathway.

supported MSCs differentiation and osteoblasts mineralization [63],[71],[73]. Wang et al. and Zhang et al. further confirmed that the positive role of these signaling factors upon MSC osteogenesis could be influenced by oncostatin M (OSM) and gp130, or BMP/SMAD signaling pathway [63],[74]. In the study of Li et al., Mg-Si-Ca alloy extract significantly promoted the M2 macrophage polarization. The expression of anti-inflammatory cytokines (IL-10, IL-1RA), chemokines CCL5 and BMP2 was enhanced. The conditional medium harvested from the alloy extract stimulated macrophages significantly increased the osteogenic potential of hMSCs (the expressions of COL1A1, ALP, Runx2, osteopontin (OPN), etc. were all up-regulated) [12]. Furthermore, Zhao et al. discovered that JDBM scaffold dampened macrophage-induced inflammatory profile, while inducing chondrocyte differentiation of MSCs [64]. This is probably because proinflammatory factors released from M1 macrophages suppress MSCs chondrogenesis. Mg might promote an enabling environment for chondrogenesis, by reversing the adverse effects of M1 macrophage-induced inflammation [60]. This osteoimmunomodulation mediated by Mg^{2+} has been proven to significantly promote the regeneration of new bone *in vivo*. In the study of Li, et al., the implantation of bandages carrying magnesium oxide nanoparticles on the surface of femur cortical periosteum increased the number of CD206 (+) M2 macrophages and brought about significantly enhanced new bone regeneration. Injection of clodronate liposome to kill macrophages leaded to decreased Col1a1 (+) osteoblasts and new bone volume proving that Mg^{2+} can activate bone regeneration by regulating macrophages [75].

3.2.3. The critical role of Mg alloy-activated M2 macrophages in angiogenesis

Recently, some studies on macrophages interacting with biomaterials indicate that M2-polarized macrophages induced by Mg^{2+} tend to trigger angiogenesis and wound healing [48],[59]. Hou et al. found that Mg-Zn-Y-Nd alloy regulated the macrophage phenotypic switching from M1 to M2 phenotype, which further accelerated ECs proliferation and CD31 expression, thereby improving endothelization [55]. Similarly,

Table 1
Immunomodulatory mechanisms of Mg alloys.

Biomaterials	Cell line	Regulatory effect on index/signaling pathway	Immunomodulatory function on cells	Refs.
Mg-Nd-Zn-Zr alloy (JDBM)	MSCs and macrophages	P65/NF-κB signaling (-) ROS (-)	Transforms macrophages from the M1 to M2 subtype.	[64]
	THP-1 macrophages	TRPM7-PI3K-AKT1 signaling pathway (+) ROS, TNF, IL-6 (-) TLR-4-MYD88-NF-κB/MAPK signaling pathway (-)	–	[65]
	Vascular smooth muscle cells (VSMCs) and macrophages	–	Converts macrophages into M2-type cells. The proliferation, migration, and proinflammatory phenotypic switching of VSMCs are inhibited.	[66]
Mg-Zn-Y-Nd alloy	Macrophages and smooth muscle cells (SMCs)	The pathway of scale-endothelial-NO-SMCs/ macrophage regulation (-)	–	[67]
	Macrophages and ECs	CD31, CD206 (+) TNF-α (-)	Regulates macrophages to switch to M2 phenotype. Improves endothelization.	[55]
Mg-5Ca-1Zn alloy	RAW 264.7 cells	TNF-α, IL-1β, IL-6 (-)	–	[57]
Mg-0.45Zn-0.45Ca (ZX00 alloy)	Macrophages	CD206 (+)	Stimulates macrophage polarization at the implant-bone interface 5 and 10 days.	[59]
Mg-2Ag and Mg-10Gd alloy	Human promonocytic cells (U937 cells)	IL-1ra, IL-10 (+) MCP1, IL-8 (-)	Exacerbates the M2 profiles of macrophage while inhibits the M1 one.	[58]
Mg-10Gd alloy	MSCs and macrophages	BMP6, OPN (+) TNF-α, IL-1β (-)	Stimulates the osteogenic differentiation of human umbilical cord perivascular cell (HUCPV), influenced by OSM and gp130, or a SMAD-related signaling pathway.	[63]

Rahmati et al. implanted Mg-0.45Zn-0.45Ca alloy into the rat femora, finding the endothelial migration and angiogenesis as well. This is probably due to the growth factors (dominated by vascular endothelial growth factor, VEGF) released by M2-polarized macrophages [59]. In the studies of and Li et al., Zhang et al. and Li et al., Mg-mediated M2 macrophage polarization and consequent upregulation of VEGF secretion was observed as well [12],[73],[74]. After the combination of VEGF and its receptor, a downstream signaling cascade is activated, which promotes angiogenesis [73].

3.3. The immunomodulatory signaling pathway during bone healing with implantation of Mg alloys

The anti-inflammatory effects of Mg alloys can be mediated by the following mechanisms, including eliminating intracellular reactive oxygen species (ROS) level, activating PI3K-AKT1 signaling pathway, regulating Toll-like receptor (TLR), inhibiting NF-κB/mitogen-activated protein kinase (MAPK) signaling pathway, etc. [59],[61],[65],[76] (Table 1).

Oxidative stress is an essential mechanism leading to inflammatory. As ROS level increases, the immune cells tend to get dysfunctional [77]. Therefore, suppressing production of excess ROS after implantation seems to be important for inhibiting the inflammatory cascade and reducing tissue damage [78]. As a natural calcium antagonist, Mg avoids the activation of oxidative stress under calcium-dependent intracellular regulation, thereby exerting antioxidant effects [55]. In the study of Bai et al., Mg alloy-coated Ti implants exhibited higher level of superoxide dismutase (SOD) and total antioxidant capacity (TAC) in comparison with Ti implants, suggesting Mg alloys possess outstanding ability to resist oxidative stress [78]. Similarly, Mg^{2+} degraded from JDBM alloys result in a significant decrease of ROS induced by lipopolysaccharide (LPS) [64],[65], thus effectively mitigating inflammatory.

Recent advances have suggested a crucial role of PI3K/AKT pathway in maintaining the integrity of immune system, for its significant enhancement on endogenous anti-inflammatory capacity [79]. A recent study of Su et al. indicated that the activation of PI3K/AKT signaling pathway was the pivotal mechanism of Mg^{2+} inhibiting LPS-induced inflammatory responses [80]. Nonetheless, when investigating the anti-inflammatory mechanisms of Mg alloys, it was AKT1 rather than AKT that performed anti-inflammatory actions. Jin et al. substantiated for the first time that TRPM7-PI3K-AKT1 signaling pathway was the critical target for immune modulation of Mg alloys [65]. The transient receptor potential cation channel subfamily M member 7 (TRPM7)

is a predominant Mg^{2+} channel in mammalian cells, serving a critical role in regulating macrophage function [66]. By inducing TRPM7 gene expression silencing, AKT1 but not AKT phosphorylation in $MgCl_2$ was down regulated. Moreover, AKT1 was proved to be an important mediator to promote the M2 macrophage polarization [65]. These findings provide the basis for immunomodulatory effects of TRPM7-PI3K-AKT1 signaling pathway in Mg alloys.

TLRs are pattern-recognition receptors (PRRs) of immune cells, mainly existing in macrophages and DCs. They assist in the recognition of foreign pathogens and particles, and initiate innate immune responses during foreign body reaction [77]. It was previously reported that high-purity Mg and trace Mg^{2+} downregulated TLR-induced inflammatory cytokines in a NF-κB-dependent manner [60],[74],[81]. The study of Sugimoto et al. further suggested that $MgSO_4$ with a concentration of 60 mg/L appeared to be effective to inhibit TLR, thus reducing the production of IL-6 and TNF-α [82]. Recently, the anti-inflammatory properties of Mg alloys by regulating TLR signals have also been confirmed. Jin et al. discovered that Mg^{2+} degraded from JDBM dampened LPS-induced inflammatory reactions through downregulating the expression of TLR-4 and MYD88 [65].

Due to a number of inflammatory stimuli focused on NF-κB signaling pathway, NF-κB is considered as a key molecule in the regulation of inflammatory response [64]. Previous researches have confirmed that Mg^{2+} in MgSO4 and magnesium isoglycyrrhizinate solution inhibited inflammatory by decreasing NF-κB nuclear translocation and phosphorylation [60],[83],[84]. As detailed above, the suppression of TLR-NF-κB signaling pathway is demonstrated to be one of the major anti-inflammatory mechanisms of Mg^{2+} [74]. It is proved that Mg^{2+} decreases TLR-mediated cytokine production by upregulating IκB-α and downregulating NF-κB p65 [82]. To explore the anti-inflammatory mechanism of Mg alloys, Jin et al. cocultured THP-1-dervied macrophages with JDBM samples, 15%JDBM extracts and 7.5 mM $MgCl_2$, respectively. They discovered that Mg^{2+} suppressed the inflammatory responses through decreasing the activity of TLR-4-MYD88-NF-κB/MAPK signaling pathway, via deactivating IKK-α/β, IKβ-α, P65, P38 and JNK by phosphorylation [65]. What is more, except for suppressing the inflammatory pathway directly, Mg^{2+} appears to promote M2 macrophage polarization after inhibiting the NF-κB pathway, thereby exerting an indirect anti-inflammatory effect [[61],[64],[74]]. As is reported, anti-inflammatory effect of Mg^{2+} by inhibiting NF-κB pathway is also related to the antagonism on L-type calcium channels [50]. This is because Ca^{2+} enhances the expressions of pro-inflammatory cytokines via the NF-κB transcription factor [60],[73]. Interestingly, Xia et al. found that Mg^{2+} suppressed NF-κB pathway and induced high expression of VEGF simultaneously [81]. Moreover, they demonstrated a feedback loop between VEGF and TLR4/NF-κB which achieved direct and indirect anti-inflammatory effects. At the same time, high levels of the inflammatory chemotaxis factors MCP-1 and uPAR, which used to be stimulated by VEGF, were suppressed by TLR4/NF-κB instead, thus enhancing the anti-inflammatory effects. These findings suggest the feedback mechanism of VEGF in TLR-4-NF-κB signaling pathway [81].

4. Underlying mechanisms of Mg alloys in different stages during angiogenesis

The role of Mg alloys in promoting the revascularization and the angio-osteogenesis coupling of the defect site in the process of bone repair has been discovered gradually [12],[14],[59],[85],[86]. However, there are relatively few studies on the regulate effects of Mg alloy orthopedic implants on ECs behavior. The underlying angiogenesis mechanisms and pathways are also rarely studied. This section summarizes the regulate effects of Mg alloy orthopedic implants on ECs behavior and the underlying angiogenesis mechanism in different stages during angiogenesis (Table 2 and Fig. 3).

4.1. Appearance of factors to stimulate angiogenesis

Angiogenesis-related factors, such as hypoxia-inducible factor (HIF) [87],[97], VEGF [98], are upregulated under the combined action of hypoxia and alloy degradation to initiate the angiogenic process after implantation of Mg alloys. HIF is an important transcription factor that promotes angiogenesis by promoting VEGF transcription under hypoxia [88]. Yoshizawa et al. reported that 10 mM $MgSO_4$ enhanced the expression of HIF-2α which then promoted the expression of VEGF in BMSCs [87]. In the study of Gao et al., the application of Mg coating on porous Ti6Al4V scaffold significantly increased the expression of HIF-1α and VEGF in human umbilical vascular endothelial cells (HUVECs) [88]. This phenomenon is most likely caused by the influx of Mg^{2+} into HUVECs. This is confirmed by the study of Yu et al. They found that the introduction of Mg^{2+} to the Ti surface significantly upregulated the expression of MagT1 on the surface of HUVECs to promote Mg^{2+} influx, leading to the stimulation of VEGF transcription by activating HIF-1α [99]. VEGF is an important growth factor involved in the process of angiogenesis. After binding with its receptor on ECs, VEGF drives the mitogenic and chemotactic responses of ECs, resulting in instability and decomposition of junctions between ECs [89]. Next, VEGF acts as a signal to stimulate ECs proliferation, migration and tube formation [100], thereby playing a crucial role in almost the entire angiogenesis process that follows [97]. In the study of Xu et al., Mg degradation products at concentrations of 2 to 8 mM promoted the expression of VEGFA and VEGFB under hypoxic conditions [90]. Wang et al. confirmed that the medium with pH 7.0–7.6 significantly promoted the expression of VEGFA in human dermal microvascular endothelial cells (HDMECs) [101]. In the study of Zhao et al., pure Mg screws fixation significantly increased the expression of VEGF around the implant in patients with osteonecrosis of the femoral head who received vascularized bone graft. The results showed better therapeutic effects, such as great bone regeneration and mineralization with a low bone flap displacement and femoral

Table 2
The signaling pathway involved in the angiogenic effect of Mg alloys.

	Cell line	Animal models	Index/Signal pathway	Ref.
$MgSO_4$	hBMSCs	NA	Expression of HIF-2α, VEGF (+)	[87]
Mg coating on Ti6Al4V	HUVECs	NA	Expression of HIF-1α, VEGF (+)	[88]
Zn/Mg-Ti	HUVECs	NA	Expression of MagT1(+) Expression of HIF-1α, VEGF (+)	[89]
Purity Mg	HUVECs	NA	Expression of VEGFA, VEGFB, MMP-13 (+)	[90]
Mg-CPS	HUVECs	NA	Expression of VEGF (+) AKT signaling pathway	[91]
$MgCl_2$	HUVECs	NA	Expression of MMP-2 and MMP-9 (+) Wnt/β-catenin signaling pathway	[92]
Mg-Cu	HUVECs	Rat	Expression of FGFR, ACVRL1 and TIE-1 (+)	[86]
Mg-Zn-Mn alloy	HUVECs	NA	Expression of MMP-2, FGF (+) FGF-PI3K-AKT signaling pathway	[93]
Ca-P-coated Mg-Zn-Gd scaffold	HUVECs, trigeminal neurons	Rat canine large-sized orbital bone defect model	Expression of VEGF in HUVECs (+) Expression of CGRP in trigeminal neurons (+) Serum CGRP (+)	[85]
Mg^{2+}	Trigeminal neurons	NA	Expression of CGRP in trigeminal neurons (+) Mg^{2+}/Trpv1/CGRP	[85]
Purity Mg	NA	Rat femoral segmental defect DO model	CGRP-FAK-VEGF signaling pathway	[94]
$MgCl_2$	HUVECs, MC3T3-E1 cells	NA	Expression of PDGF-BB in HUVECs and MC3T3-E1 cells (+)	[95]
$MgSO_4$	ECs	Transient leaky vessel model in mice	Expression of VE-cadherin, occluding, and zonula occludens-1, ZO-1(+) TRPM7/MagT1- S1P1- Rac1, AKT, ERK1/2 signaling pathway	[96]

"(+)" represents upregulated or promoted, NA: not applicable.

head collapse rate [19]. However, high Mg concentration and pH value are detrimental to VEGF expression. Wu et al. observed that when the content of MgO was within 10 wt%, the secretion of VEGF in Mg-$Ca_5(PO_4)_2SiO_4$ (Mg-CPS) bioceramics extracts was elevated. Nonetheless, when the content of MgO reached 15%, the VEGF secretion of HUVECs decreased due to the action of excess Mg^{2+} and hydroxide [91].

4.2. Degradation of vascular basement membrane

The premise of the formation of new blood vessels is the degradation of basement membrane [98]. The main biomolecules during this process include matrix metalloproteinases (MMPs), which are responsible for digesting the basement membrane and liberating ECs from the vascular wall for their diffusion and migration [102]. Study by Pan et al. showed that Mg^{2+} significantly increased the expression of MMP-2 and MMP-9 [92]. And in the study of Li et al., 6.25% Mg-Zn-Mn alloy extract significantly improved the expression of MMP-2 [93]. Moreover, Xu et al. found that MMP-13 was upregulated under the action of 2 mM and 4 mM Mg degradation products [90]. MMPs also promote the sprouting of ECs by releasing angiogenesis-related factors bound to the matrix (such as FGF, VEGF) [103]. At present, there is few studies of Mg alloys on promoting the sprouting of ECs through this pathway, which can be further studied in future.

4.3. Endothelial cell proliferation, migration, and tube formation

4.3.1. The effect of Mg alloys on endothelial cell proliferation, migration, and tube formation

After the basement membrane is degraded and ECs are liberated, ECs proliferate, migrate and transform into tip cells or stalk cells with different functions and shapes under the action of various angiogenic signals to realize the formation of new endothelial branches. Subsequently, tube formation stage begins, during which solid branches are transformed into lumens [98]. Mg plays a concentration-dependent role in promoting endothelial cell proliferation, migration and tube formation [88],[90],[91],[93],[95]. Liu et al. confirmed that the proliferation of HUVECs can be significantly promoted when the concentration of Mg^{2+} reached 1 mM. And when the Mg^{2+} concentration increased between 1 and 5 mM, the cell proliferation also increased significantly. However, Mg^{2+} concentrations higher than 10 mM negatively affected angiogenic factors (VEGF, PDGF-BB) secretion and tube formation of HUVECs. Mg^{2+} concentrations above 20 mM even affected the survival of HUVECs [95]. Li et al. also found a similar trend. It showed that 6.25% Mg-Zn-Mn alloy extracts significantly enhanced HUVEC proliferation and tube formation, while 25%, 50%, and 75% Mg-Zn-Mn alloy extracts only moderately enhanced HUVEC tube formation and suppressed proliferation of HUVEC [93]. Interestingly, Xu et al. used 5% O_2 to simulate the hypoxic microenvironment at the injury site. They found that Mg extract at concentrations of 4 to 8 mM significantly upregulated HUVEC proliferation under

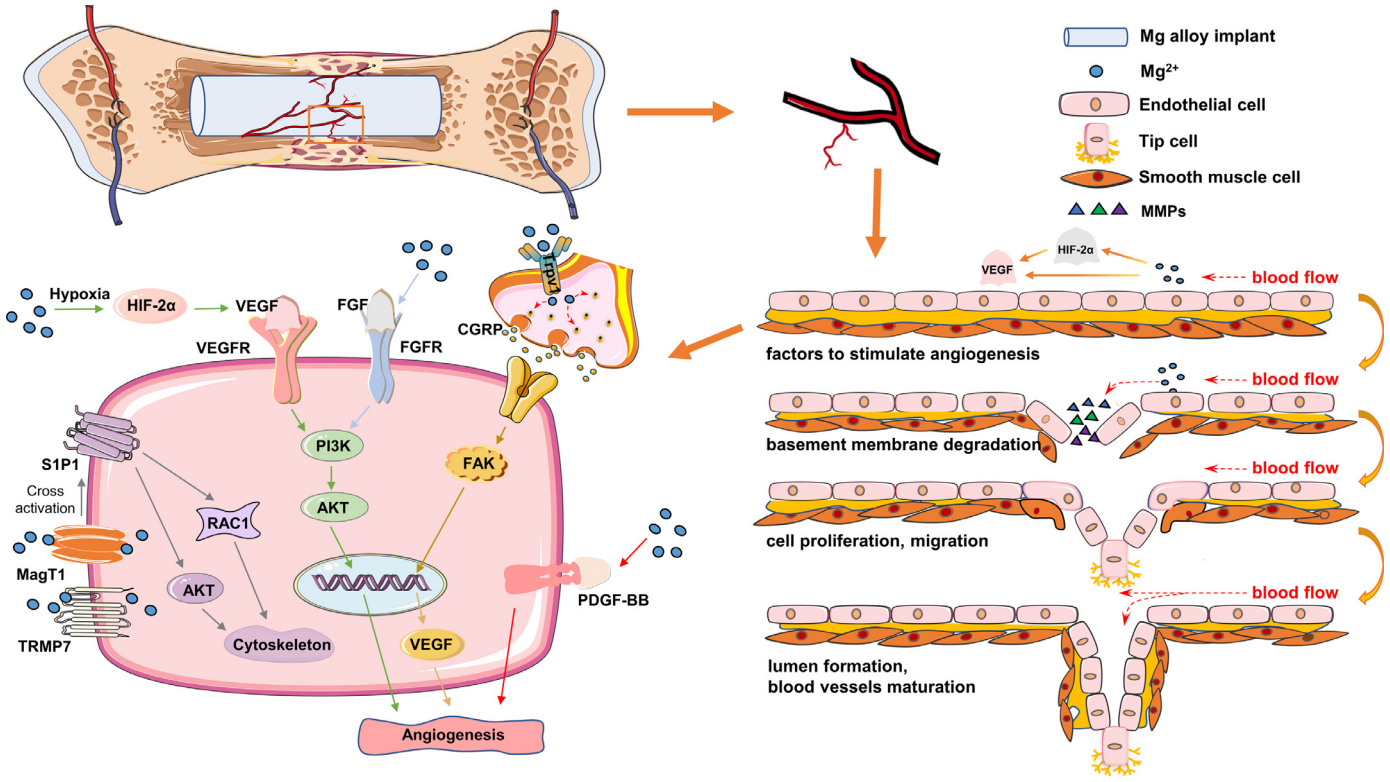

Fig. 3. Mg^{2+} degraded from Mg alloys on the underlying mechanism of angiogenesis in bone healing. Angiogenesis-related factors (HIF, VEGF) are upregulated under the combined action of hypoxia and Mg^{2+} to initiate the angiogenic process after implantation of Mg alloys. Then Mg^{2+} participates in the regulation of MMPs on basement membrane degradation. Endothelial cell proliferation, migration and tube formation are promoted under the action of Mg^{2+}. Finally, the vascular lumens form to achieve blood circulation. Under hypoxia, Mg^{2+} upregulates the expression of VEGF by stimulating HIF-2α. PI3K/AKT signaling pathway activates through VEGF and FGF. TRPM7 and MagT1 cross-activate S1P1 in response to Mg^{2+}. Then S1P1 promotes cytoskeletal reorganization by RAC1 and AKT. Moreover, Mg^{2+} upregulates the expression and release of CGRP by entering nerve endings through Trpv1. CGRP-FAK-VEGF signaling pathway is activated to participate in angiogenesis. The up-regulation of the expressions of PDGF-BB by Mg^{2+} also plays an important role in angiogenesis.

hypoxic conditions but not under normoxia [90]. Furthermore, pH value has an effect on cell function. Wang et al. confirmed that the culture mediums with pH 7.4–7.6 were more suitable for the proliferation, migration and tube formation of HDMECs than the acidic environment. However, when the pH further rose to 7.8, this effect will be weakened [101]. Wu et al. found that the existence of Mg^{2+} partially reversed the over-alkaline adversity. However, when the pH value of Mg-doped bioceramic extract is too high (up to 9.64), it caused a negative influence on proliferation and tube formation of HUVECs [91].

4.3.2. Underling mechanisms of Mg alloys regulating endothelial cell behavior

What are the potential mechanisms through which Mg^{2+} regulates the function and state of ECs? First, the transformation of ECs into tip and stem cells is regulated by the Notch signaling pathway [104]. However, the role of Mg^{2+} in this process has not been studied. Second, Zhu et al. demonstrated that MagT1 and TRPM7 appear to be the major transporters for extracellular Mg^{2+} in ECs. MagT1 and TRPM7 play important roles in the functional regulation of ECs. Inhibition of MagT1 or TRPM7 will significantly affect proliferation and migration of ECs [96]. More importantly, several signaling pathways were confirmed to be involved in Mg-mediated proliferation, migration and tube formation of ECs. Pan et al. confirmed that the Wnt/β-catenin pathway in HUVECs was activated under the action of Mg^{2+} and might be involved in cell migration and invasion [92]. In the study of Zhang et al., Mg^{2+}-containing silicate bioceramics extracts inhibited the expression of Arp2/3 complex inhibitors in HUVECs to upregulate Arp2/3 complexes. As a regulator of actin polymerization, the highly expressed Arp2/3 complex promoted the migration of HUVECs [105]. In addition, in the study of Liu et al., the Mg-Cu alloy extract significantly up-regulated the expression of FGFR. They speculated that the ionic components in the extract might directly act on FGFR or indirectly act on FGFR by promoting the expression of FGF to promote the activation of intracellular related cellular pathways, especially PI3K/AKT pathway [86]. Then, the study of Li et al. confirmed this conjecture. They found that Mg-Zn-Mn alloy extract up-regulated the expression of FGF. FGF acted on FGFR to activate the intracellular PI3K/AKT signaling pathway to promote the tube formation of HUVECs [93]. Activation of PI3K/AKT signaling pathway is considered a key factor in endothelial cell motility, migration and tube formation [106]. Wu et al. also confirmed the activation of PI3K/AKT signaling pathway mediated by Mg^{2+}. Interest-

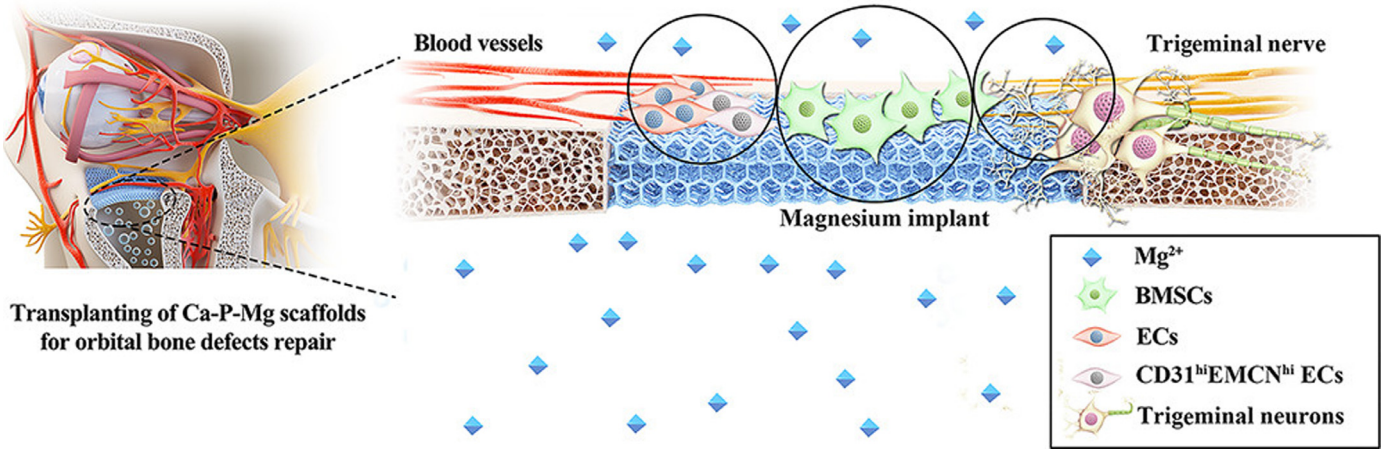

Fig. 4. The schematic diagram shows that Ca-P-coated Mg-Zn-Gd scaffolds implanted in an orbital bone defect model trigger trigeminal neurons via Trpv1 to produce the neuropeptide CGRP, which promotes angiogenesis and osteogenesis [85].

ingly, they found that the expression of VEGF, which also plays an important role in endothelial cell migration, was inversely correlated with the activation of the PI3K/AKT signaling pathway. They suspected that this might be due to the high expression of VEGF promoted by appropriate Mg^{2+} concentration initiated the negative feedback regulation on the PI3K-AKT pathway. In contrast, high concentration Mg^{2+} gradually promoted the activation of the PI3K/AKT signaling pathway, but lost the positive effect on the expression of VEGF. Thus, the PI3K/AKT signaling pathway was less affected by VEGF and remained active [91].

4.3.3. Neurons and their secreted neuropeptides are involved in Mg-mediated angiogenesis

Notably, sensory nerves and their secreted neuropeptide calcitonin gene-related peptide (CGRP) were confirmed to play an important role in the promotion of endothelial cell migration and tube formation by Mg^{2+} [85],[94]. Zhang et al. demonstrated that Ca-P-coated Mg-Zn-Gd scaffolds significantly promoted the expression of the serum CGRP in a canine large-sized orbital bone defect model. Inhibition of CGRP significantly down-regulated the number of new blood vessels at the injured site, suggesting the important role of CGRP in Mg alloys-mediated revascularization of the injured site [85]. Further, they confirmed that Mg^{2+} entered trigeminal neurons through transient receptor potential vanilloid subtype 1 (Trpv1) to promote the synthesis and release of CGRP, thereby promoting angiogenesis (Fig. 4) [85]. Implanting Mg intramedullary nails in a rat femoral segmental defect DO model, the research team further verified the elevation of CGRP and its role in angiogenesis under the action of Mg^{2+} [94]. They found that CGRP concentrations ranging from 10^{-10} to 10^{-8} M dose-dependently promoted endothelial cell migration and tube formation [94]. Next, this pro-angiogenic effect was confirmed to be achieved by promoting the phosphorylation of focal adhesion kinase (FAK) and then activating the expression of VEGF. Thus, Mg^{2+}-mediated activation of the CGRP-FAK-VEGF signaling axis played an important role in angiogenesis in bone defect sites [94].

4.3.4. PDGF-BB enhances angio-osteogenesis coupling under the action of Mg^{2+}

Additionally, platelet-derived growth factor-BB (PDGF-BB), a key factor in angiogenesis, was also confirmed to be significantly up-regulated under the induction of Mg^{2+} [95]. Liu found that Mg^{2+} concentrations ranging from 1 to 5 mM promoted the expression of PDGF-BB in HUVECs in a dose-dependent manner. However, when the Mg^{2+} concentration reached 10 mM, the expression of PDGF-BB showed a downward trend [95]. In addition to ECs, Mg^{2+} have also been showed to promote PDGF-BB secretion by preosteoblast MC3T3-E1. PDGF-BB secreted by MC3T3-E1 cells effectively promoted HUVECs vascularization, thereby promoting angio-osteogenesis coupling [95]. Indeed, PDGF-BB has long been recognized as a regulator of H-type vessel formation which is critical for angio-osteogenesis coupling [107]. Moreover, it has confirmed that preosteoclasts are also the source of PDGF-BB secretion. PDGF-BB secreted by preosteoclasts promotes angiogenesis through FAK-PI3K/AKT signaling pathway and bone regeneration through sphingosine-1-phosphate (S1P) signaling pathway [107]. However, no experiments have demonstrated whether this effect of preosteoclasts is enhanced in the presence of Mg alloys or Mg^{2+}. Considering that Mg alloys implantation will inhibit the differentiation of preosteoclasts and increase the number of preosteoclasts, whether the above-mentioned roles and pathways are activated during Mg alloys implantation is worth investigating.

4.4. Maturation and stability of blood vessels

After the lumen is established, VEGF and Rho-associated protein kinase (ROCK) drive the vascular lumen to expand [108],[109], with the beginning of blood perfusion, oxygen and nutrients circulation in the vessels, as well as the downregulation of VEGF expression [104]. Subsequently, the

newly generated vasculature is corrected by remodeling and pruning [110]. The maturation and stabilization of neovascularization and the maintenance of the existing vasculature are later events in the angiogenesis process [104]. Liu et al. found that Mg-Cu alloy extracts up-regulated the expressions of the endothelial receptor tyrosine kinase TIE-1 and activin receptor-like kinase ACVRL1 on the plasma membrane of HUVECs. They speculated that the ionic components of the extract might directly activate these two endothelial receptors, or indirectly act and activate the endothelial receptors by inducing the expression of angiogenic factors. Activated receptors activate intracellular signaling pathways that play a role in angiogenesis [86]. According to the confirmed knowledge, TIE-1 and ACVRL1 can maintain the stability of established blood vessels [111] and participate in TGF-β signaling pathway to regulate blood vessel maturation [112], respectively. Therefore, it is reasonable to speculate that Mg^{2+} are involved in the regulation of vascular stability and maturation through their effects on TIE-1 and ACVRL1.

4.5. Endothelial cell barrier

The tight junction structures between adjacent ECs contribute to maintenance of vascular integrity [104]. Except for promoting ECs migration and proliferation, Mg^{2+} also plays a remarkable role in modulating endothelial barrier integrity. TRPM7 and MagT1 cross-activate sphingosine1-phosphate1 (S1P1) in response to Mg^{2+}. Activated S1P1 then activates Ras-related C3 botulinum toxin substrate 1 (RAC1), AKT and ERK1/2, leading to endothelial cytoskeleton reorganization and enhancement of junctional proteins (VE-cadherin, occluding, and zonula occludens-1, ZO-1) expression to form a tight endothelial barrier [96]. Moreover, barrier-stabilizing mediators are also important regulatory targets for Mg^{2+} to augment endothelial cellular integrity. It has been shown that Mg^{2+} enhances the expression of FGF, AKT, angiogenin (Ang) and endothelial nitric oxide synthase (eNOS) to improve endothelial barrier functions [96]. Lacking FGF signal results in enhanced VE-cadherin phosphorylation, thus disrupting the binding to p120-Catenin, which is detrimental to the robust intercellular connections [96]. Ang, together with its TIE receptor, is considered as an important regulator of maintaining vascular homeostasis. Their combination reinforces the vascular stability, following AKT-mediated eNOS phosphorylation [113]. Nonetheless, the specific signaling pathway of most barrier-stabilizing mediators is not well understood. Thus, detailed intracellular mechanisms remain to be explored.

5. Effects of Mg alloys on osteoblast-related cells and their underling osteogenic mechanisms

5.1. The effect of Mg alloys on the function and state of osteoblast-related cells

The promoting effect of Mg alloys on the adhesion, spreading, proliferation and differentiation of osteogenic-related cells has been widely reported [45],[57],[114–127]. In particular, Mg alloys have been shown to have positive effects on the expression of the early osteogenic markers ALP, the osteogenic regulators RUNX2 and Osterix (OSX), the intermediate and late osteogenic markers bone sialoprotein (BSP), OPN, osteocalcin (OCN), collagen Col-I and BMP2, and calcium deposition [12],[27],[115],[128–133]. This is mainly attributed to Mg^{2+} degraded from alloys, the effects of which has been confirmed in the study of Mg-containing biomaterials and magnesium salt solutions [57],[99],[134–141]. The effect of Mg^{2+} on osteogenic-related cells is obvious concentration-dependent [137],[142]. But there are differences in the susceptibility of different cell lines to Mg^{2+} concentration [140],[142],[143]. Generally, when the concentration of Mg^{2+} is between 5 mM and 20 mM, it is more favorable for the proliferation of osteoblast-related cells, including MSCs, periosteum-derived cells and osteoblasts [140],[142–144]. High Mg environment significantly inhibited cell proliferation and survival [144–146]. As for osteogenic differentiation, the susceptibility and response of different cells to Mg^{2+} varies greatly (Table 3). Lin et al. reported that 2.5 to 5 mM Mg^{2+} was optimal for osteogenic differentiation of rBMSCs [146]. The study by Wang et al. showed that 3 to 6 mM Mg^{2+} obviously promoted the expression of ALP, RUNX2, and OCN in MG 63 cells [143]. Yoshizawa et al. demonstrated that 5 to 10 mM Mg^{2+} had the most pronounced effect on the osteogenic differentiation and extracellular matrix mineralization of hBMSCs [87]. However, this effect was significantly weakened when the Mg^{2+} concentration reached 20 mM [87]. In contrast, Zhang et al. and He et al. reported that the osteogenic differentiation of hFOB1.19 cells and periodeum-derived cells could still be significantly promoted under the effect of 20 mM Mg^{2+} [142],[147].

In addition to Mg^{2+}, the effects of pH and H_2 release on osteoblast-related cells cannot be ignored. The bone defect site is a relatively hypoxic and acidic microenvironment in the early stage. Relatively acidic microenvironment has been shown to significantly attenuate osteoblast ALP expression and extracellular matrix mineralization thereby greatly inhibiting bone formation [149],[150]. Conversely, a weak alkaline microenvironment can promote osteoblast survival and stimulate osteoblast differentiation [151–153]. The studies by Pan et al. and Galow et al. both confirmed that the expression of osteogenic markers in osteoblasts was significantly increased when the pH value was around 8.5 [152],[153]. Recently, Chen et al. found that the alkaline microenvironment (about pH 8.5) formed by Mg-Ga layered double oxide (LDO) nanosheets on the surfaces of alkali-heat-treated titanium (AT) implants promoted the autophagic activity and induced osteogenic differentiation of MSCs [154]. However, too high pH value can adversely affect osteoblast-related cells and the susceptibility of different cells to pH is slightly different [155]. The study of Tan J et al. confirmed that the medium with pH>8.5 inhibited the proliferation of rBMSCs, and the medium with pH<9.0 could promote the proliferation of MC3T3-E1 cells [155]. Therefore, the weak alkaline microenvironment brought about by

Table 3
Effects of different concentrations of Mg^{2+} on the osteogenic differentiation of osteoblast-related cells.

Source of Mg^{2+}	Mg^{2+} concentration	Cell line	Effect		Refs.
$MgCl_2$	1.6, 2.5, 5, 10, 20 mM	rBMSCs	ALP and OCN expression	<10 mM: (+); 2.5, 5 mM: most significantly (+)	[146]
$MgSO_4$	0.8, 5, 10, 20 mM	hBMSCs	Extracellular mineralization	5, 10 mM: most significantly (+)	[87]
$MgSO_4$	10 mM	hBMSCs	Extracellular mineralization; ALP expression	10 mM: significantly (+)	[148]
$MgCl_2$	0.8,1.8,5,10, 20 mM	Periosteum-derived cells	Extracellular mineralization; RUNX2, BSP and OCN expression	5, 10, 20 mM: significantly (+)	[142]
$MgSO_4$	0.8, 2, 5, 10, 20, 40 mM	hFOB1.19	Extracellular mineralization; ALP expression	5, 10, 20 mM: significantly (+); 40 mM: (-)	[147]
$MgCl_2$	1, 3, 6 mM	MG63	ALP, RUNX2 and OCN expression	3 mM: significantly (+) 6 mM: most significantly (+)	[143]
$MgCl_2$	10 mM	MC3T3-E1	Extracellular mineralization; ALP expression	10 mM: significantly (+)	[95]

the moderate degradation of Mg alloys should have a positive effect on osteoblast-related cells. On the contrary, the over alkaline environment brought about by rapid degradation will be unfavorable to the osteogenic performance of Mg alloys. This has been confirmed in researches related to Mg alloys [57],[86],[119],[121],[122],[156]. Notably, it has been reported that the positive effect of Mg^{2+} on osteoblast-related cells can offset the negative effect of high pH, thereby relatively improving the tolerance of osteoblast-related cells to the over alkaline environment [91],[147]. In the study by Zhang et al., the proliferative viability of hFOB1.19 osteoblasts decreased significantly when the pH of the medium reached 9 or 10. The addition of 5 mM Mg^{2+} protected cells from the interference of high alkaline environment and partially restored viability [147]. As for H_2, the current understanding is that the release of excess H_2 caused by the rapid degradation of alloys will lead to the formation of air cavities and affect cell adhesion that impair bone regeneration [157–159]. However, the effects of different concentrations of H_2 on osteoblast-related cells and their underlying mechanisms have not been revealed.

5.2. The signaling pathway involved in the osteogenic effect of Mg alloys

5.2.1. Integrin dependent cell adhesion initiates the transformation of cell function

As we all known, the adhesion of osteoblasts on the implant surface is mediated by integrins [160]. Osteoblast-related cells achieve efficient adhesion and anchoring through the binding of integrins on the cell membrane to extracellular matrix proteins adsorbed to the implant surface [160]. Mg alloys can promote the expression of integrins to promote cell adhesion on the alloy surface [12],[120],[130]. In the study of Zhao et al., both Mg-1Zn alloy and Mg-1Zn-0.5Sn alloy showed a promotion effect on the expression of integrin $\alpha 1$ and integrin $\beta 1$ [120]. Mg-1Ca-xSr (x = 0.2, 0.5, 1.0, 2.0 wt.%) alloys have also been confirmed to significantly increase the expression of integrin $\alpha 5$ and integrin $\beta 1$ on the surface of MC3T3-E1 cells [130]. In addition, Li et al. found that Mg-1.0Ca-0.2Si alloy extract stimulated the expression of integrin $\alpha 3$, integrin $\alpha 4$, integrin $\alpha 5$, integrin $\beta 1$, and integrin $\beta 3$ on the surface of hMSCs [12]. Notably, the upregulation of cell-surface integrin expression by these alloys did not occur until alloy samples were co-cultured with cells for many days or in extracts obtained from prolonged soaking of samples [12],[120],[130]. This may be because after the rapid degradation period, the appropriate concentration of metal ions and the weak alkaline microenvironment generated by the slow degradation of Mg alloys are favorable for the expression of integrins. Indeed, integrins, as a family of α/β heterodimeric adhesion metalloprotein receptors, whose function is highly dependent on and regulated by divalent cations [161]. Mg^{2+} have been confirmed to play a key role in the function of integrins [145],[162],[163]. Particularly, Mg^{2+} can significantly upregulate the expression of integrin $\alpha 1$ and integrin $\beta 1$ genes in BMSCs, human bone-derived cells (HBDCs) and other cells to promote the initial adhesion and spreading of cells [99],[162]. Moreover, The promoting effect of alkaline environment on the expression of integrin $\alpha 2$, integrin av and integrin $\beta 1$ has been confirmed in the study of Wang et al. [151].

The regulation of cell adhesion to implant surfaces by integrins is only the starting point for the dynamic transition of osteogenesis-related cell functions. The ensuing activation of integrin-dependent and integrin-independent complex intracellular signaling pathway networks will orderly regulate the proliferation and differentiation of osteoblast-related cells and ultimately achieve successful bone regeneration [160],[164]. In the study of Li et al., Mg-0.2Si-1.0Ca alloy extracts were shown to enhance phosphorylation of FAK, a key hub in the signaling cascade from integrin activation to osteogenic differentiation. Then its downstream pathways AKT-glycogen synthase kinase-3β (AKT-GSK-3β) and extracellular signal-regulated kinase1/2-p38 MAPKs (ERK1/2-p38 MAPKs) pathway are activated [12]. It has been confirmed that the ERK1/2-p38 MAPKs pathway promotes the osteogenic differentiation of hBMSCs by targeting the Runx2 gene [165].

Meanwhile, the increase in the phosphorylation level of GSK-3β means that the Wnt signaling pathway is also activated and contributes to osteogenic differentiation [166]. The activation of these cascades is most likely mediated by integrins [12]. Similarly, Li M et al. found that the Mg-1Ca-2.0Sr alloy extracts significantly increased the expression of integrin α5 and β1 of MC3T3-E1 cells and rapidly induced the activation of ERK1/2 in MC3T3-E1 cells, suggesting that Mg-1Ca-2.0Sr alloy may promote bone regeneration through activation of ERK1/2 MAPK pathway via integrins [130]. Notably, a recent study have shown that the alkaline environment also involved in the up-regulation of integrins and the activation of FAK signaling pathway to promote the osteogenic differentiation of BMSCs [151].

5.2.2. Mg^{2+} mediates the activation of MAPK signaling pathway driving osteogenesis

Indeed, the MAPK signaling pathway, including ERK1/2 signaling, p38 signaling, and JNK signaling, has be regarded as one of the critical signaling pathways involved in osteogenic differentiation [87]. Including the evidence mentioned above, many studies have indicated that the MAPK pathway plays a key role in the process of Mg alloys promoting bone regeneration [12],[27],[130],[143]. Wang et al. confirmed that the Mg-Zn alloy extracts might significantly promote the osteogenic differentiation of BMSCs by activating MAPK signaling pathway including ERK1/2 signaling, p38 signaling, and JNK signaling [27]. Mg^{2+} plays an important role in this effect [143].The activation of the MAPK signaling pathway by Mg^{2+} can be achieved in many integrin-independent ways in addition to the integrin-dependent pathway mentioned above. Lin et al. and Wang et al. respectively found that Mg^{2+} activates the ERK MAPK pathway and p38 MAPK pathway by influx into the cytoplasm, thereby stimulating the osteogenic differentiation [27],[146]. Li et al. revealed that Mg^{2+} contributed to the activation of p38 MAPK signaling pathway by regulating the expression of BMPs receptor BMPR2. And the magnesium-induced high expression of long noncoding RNA (lncRNA) LOC103691336 plays a key mediating role [167]. LOC103691336 competitively binds to miR-138–5p, resulting in the weakening of miR-138–5p binding to BMPR2 and thereby releasing its inhibitory effect on BMPR2. The up-regulated BMPR2 will activate the downstream p38 MAPK signaling to participate in the osteogenic differentiation of BMSCs [167]. Mg^{2+} has also been verified to upregulate Runx2 transcription in BMSCs by targeting ERK1/2 and p38 MAPK pathways via miR-16 [168]. Notably, in the study of Li et al., the Mg-1Ca-2.0Sr alloy extracts only activated ERK1/2 signaling, while JNK and p38 pathway proteins were not significantly up-regulated [130]. It is consistent with the study by Lin et al. who found that intracytoplasmic Mg^{2+} selectively activated the MAPK/ERK pathway [146]. However, in the study of Li et al. [12] and Wang et al. [27], p38 signaling and JNK signaling were also confirmed to be involved in the osteogenic differentiation of Mg alloys. It remains to be verified whether it is caused by the experimental operation and due to differences in the cell types used or the composition of Mg alloys.

5.2.3. Activation of classical and nonclassical Wnt pathways driving osteogenesis by Mg^{2+}

The Wnt signaling pathway, especially the canonical (Wnt/β-Catenin) signaling pathway, is one of the most critical signaling pathways in the process of bone regeneration and repair [166]. Whether it contributes to osteogenic effect of Mg alloys is the focus of researchers. Zhao et al. implanted a JDBM alloy in an osteochondral defect model of the femoral condyle non-weight-bearing area in rats and co-injected the Wnt/β-catenin signaling pathway inhibitor ICRT3. The results showed that the application of ICRT3 significantly reduced the new bone mass and inhibited the expression of osteogenic genes Runx2, Bmp2, suggesting that the Wnt/β-catenin signaling pathway plays a key role in the process of JDBM alloy promoting bone defect repair [169]. Moreover, the study of Xia et al. confirmed the activation of Mg-3.5Li-0.5Ca alloy on the Wnt/β-Catenin signaling pathway [131]. Compared with Ti and pure Mg extracts, Mg-3.5Li-0.5Ca alloy extracts significantly reduced the level of GSK-3β, a key member of the β-catenin destruction complex in the cytoplasm of hBMSCs. Thereby, the expression of β-catenin and its nuclear translocation were significantly upregulated. As a result, the expression of Wnt signaling pathway-related genes Lef-1, Tcf-1 and Axin2 increased, regulating the expression of downstream target genes that play an important role in osteogenic differentiation [131]. Wang et al. further confirmed that it was Mg^{2+} that mediated the activation of Wnt/β-catenin signaling pathway by Mg alloys [143]. In addition, Hung et al. confirmed that the major ligand of Wnt signaling, Wnt3a, was significantly up-regulated by $MgSO_4$ treatment, leading to accumulation and nuclear translocation of β-catenin in hBMSCs to activate the transcription of downstream target genes Lef-1 and Dickkopf-1(Dkk-1) [148]. Interestingly, in a distraction osteogenesis (DO) rat model, pure Mg implants were found to activate the alternative Wnt pathway. In addition to the up-regulated expression of the ligand Wnt5b protein and the membrane protein receptor FZD, the expression of YAP and Runx2 was also increased [37]. It has been confirmed that alternative Wnt signaling promoted the high expression of Tead in the nucleus by activating YAP/TAZ and promoting its nuclear translocation, thereby upregulating the expression of Runx2 and other osteogenic genes to promote osteogenesis [170]. Therefore, Mg may also promote osteogenic differentiation through the alternative Wnt signaling pathway [37]. However, puzzlingly, the combined application of pure Mg implants in a DO rat model did not activate the Wnt/β-catenin signaling pathway [37]. It is unknown whether the special environment of DO or other factors including H_2 release and changes in Mg^{2+} concentration during the degradation of pure Mg leads to this result.

These *in vivo* and *in vitro* evidences confirmed the activation of Wnt signaling pathway by Mg alloys. As the key signal pathway of bone homeostasis and bone repair, Wnt is a crucial target for pharmacological agents to accelerate

bone repair. Monoclonal antibodies against the Wnt antagonists Sclerostin, Dkk-1 and Midkine have been demonstrated to have therapeutic potential to enhance bone repair in preclinical studies [171],[172]. The activation of the Wnt signaling pathway of Mg alloys will effectively support its potential in bone disease treatment, provide temporary support for bone defects and avoid the application of additional drugs.

5.2.4. Activation of other well-known osteogenesis-related signaling pathways driving osteogenesis by Mg^{2+}

Moreover, other well-known osteogenesis-related signaling pathways have also been confirmed to be involved in the process of Mg alloys or Mg^{2+} in promoting osteogenesis. In the study of Li et al., Mg-1Y and/or Mg extracts were found to activate Smad-dependent signaling pathways by upregulating the expression of TGF-β family members BMP2 and TGF-β1 [173]. In detail, the up-regulated BMP2 or TGF-β1 in the extract forms a complex with type I and II serine/threonine kinase receptors to activate receptor mediated Smad-/mothers against decapentaplegic(R-Smad). Phosphorylated R-Smads form complexes with Smad4 and translocate into the nucleus to interact with key transcription factors to promote osteogenesis and inhibit adipogenic differentiation of hMSCs [173]. And Mg^{2+} have been confirmed to enhance the expression of BMPR2, resulting in an increase in the activity of the downstream Smad signaling pathway [167]. In fact, the application of BMPs in the treatment of bone diseases is widely explored and accepted [174]. Moreover, in recent years, extensive studies have attempted to modify implants to carry BMPs to achieve better bone regeneration and repair [175]. On the contrary, Mg alloys may achieve the desired bone therapeutic effect through effective regulation of endogenous BMPs without the need of additional bioactive factors. Furthermore, in the DO rat model, the hedgehog pathway was confirmed to be the upstream signaling hub for activation of the non-canonical Wnt signaling pathway. Mg implantation can activate the hedgehog pathway by binding to Patched1 (Ptch) protein and stabilizing its structure. Therefore, the inhibitory effect of Ptch protein on smoothened (Smod) is weakened, resulting in the up-regulation of glioma associated oncogene homolog 1 (Gli1) and Gli2. Ultimately, Gli1 and Gli2 promote the expression of Wnt5b and activate the alternative Wnt signaling pathway [37].

5.2.5. Mg-mediated specific patterns of osteogenesis-related signaling pathway activation

As important Mg^{2+} membrane transporters, TRPM7 and MagT1 have attracted much attention for their roles in Mg^{2+}-mediated osteogenesis. Diaz-Tocados et al. confirmed the critical role of TRPM7 in the regulation of osteogenesis-related cell behavior by Mg^{2+}. They found that inhibition of TRPM7 with 2-APB resulted in significant downregulation of osteogenic marker genes Runx2, Osx and Ocn, decreased ALP activity, and decreased matrix calcification. Moreover, 2-APB significantly inhibited the expression of cell proliferation markers Cyclin D1 and PCNA [176]. In fact, the role of TRPM7 in the regulation of osteogenesis-related cell behavior by Mg^{2+} may be multifaceted and complex. On the one hand, TRPM7 promotes the intracellular transport of Mg^{2+}, which is the premise for the realization of the intracellular effect of Mg^{2+}. Diaz-Tocados JM found that the nuclear translocation of the Notch intracellular domain (NICD) will be increased after TRPM7 mediated the influx of Mg^{2+} in undifferentiated MSCs. The activated Notch signaling pathway is involved in the regulation of cell proliferation and osteogenic differentiation [176]. Moreover, Hou et al. demonstrated that intracytoplasmic Mg^{2+} competed with Ca^{2+} for binding sites in calmodulin (CaM), resulting in a shutdown of CaM activity. In this way, the inhibition of CaM on calmodulin-dependent protein kinase4 (CaMK4) function was relieved. Activated CaMK4 upregulated the phosphorylation level of the transcription factor CREB1. The increased p-CREB1 promoted the transcription and expression of OPN by binding to the promoter of OPN and made the cells transition to an osteogenic differentiation state. In the rat femur model, the implantation of high-purity Mg screws brings good osseointegration. Injection of an inhibitor of CaMKIV, however, significantly down-regulated the expression of OPN and p-CREB, which eventually led to the formation of massive cavities around the screw and poor osseointegration. Therefore, the successful bone regeneration induction of Mg alloys *in vivo* is closely related to CaM/CaMKIV/ CREB1 pathway [177]. On the other hand, in addition to mediating the inflow of Mg^{2+} into cells, TRPM7 also has protein kinase activity [178]. Zhang et al. suggested that Mg^{2+} activated intracellular PI3K/AKT signaling pathway by acting on TRPM7 [147]. Activation of the TRPM7/PI3K signaling pathway increased the expression levels of RUNX2 and ALP, leading to a significant increase in osteogenic activity of osteoblast cells [147]. Furthermore, the TRPM7/PI3K signaling pathway was also involved in the migration of osteoblasts by up-regulating the expression of migration-related genes MMP-2, MMP-9, and VEGF [147]. Incidentally, the function of TRPM7 was confirmed to be regulated by cytokines such as EGF, VEGF, PDGF, etc. [178]. The activation of cytokines and their crosstalk with TRPM7 after Mg alloy implantation is a matter of concern.

The role of MagT1 in bone healing, especially in vascularized bone regeneration, has been demonstrated. Embryonic bone development and osteogenic differentiation of BMSCs are accompanied by up-regulation of MagT1 [146]. Notably, Mg^{2+} released from biomaterials have been shown to promote the expression of MagT1 in rBMSCs [99]. In the study of Lin et al., Mg^{2+} at concentrations below 10 mM significantly up-regulated the expression of MagT1 in rBMSCs, and concentrations from 2.5 to 5 mM were optimal. And this concentration-dependent MagT1 expression is consistent with the osteogenic differentiation ability of BMSCs. Furthermore, they confirmed that the upregulated MagT1-mediated influx of Mg^{2+} was closely related to the activation of the intracellular ERK1/2 pathway. Deletion of MagT1 significantly downregulated ERK phosphorylation and resulted in restricted osteogenic differentiation of rBMSCs [146]. Reviewing the above description of the function of MagT1 in angiogene-

sis, it is not difficult to understand the role of MagT1 in the regeneration of vascularized bone. It is also confirmed by the successful vascularized bone regeneration results of the Mg-enriched 3D culture system in a rat critical-sized cranial defects model [146].

5.2.6. The role of ECs and neurons in Mg-mediated osteogenesis

As mentioned above, the interaction between endothelial cells and osteogenesis-related cells promotes the angio-osteogenesis coupling. PDGF-BB, which is expressed by ECs and pre-osteoblasts under the induction of Mg^{2+}, not only plays an important role in angiogenesis, but also regulates the function of osteoblast-related cells. Wang et al. demonstrated that Mg implantation upregulated PDGF-BB expression in a rabbit anterior cruciate ligament reconstruction (ACLR) model. High expression of PDGF-BB might promote the migration of BMSCs to the implantation site and promote good osseointegration [179]. Liu et al. found that Mg-containing medium significantly promoted the expression of PDGF-BB in MC3T3-E1 cells. Downregulation of PDGF-BB expression resulted in decreased osteogenic differentiation and extracellular matrix mineralization of MC3T3-E1 cells [95]. Notably, the interactions between ECs and osteogenesis-related cells are complex [180]. The role of Mg^{2+} in these interactions remains to be further explored.

Interestingly, neuropeptides have also been shown to involve in Mg-induced bone regeneration. Mg^{2+} released at the implant site can diffuse through the bone to the periosteum. Through the Mg^{2+} transporters MagT1 and TRPM7, Mg^{2+} enters the dorsal root ganglia (DRG) and promote CGRP-vesicles accumulation and exocytosis. The released CGRP will activate the phosphorylation of cAMP-responsive element binding protein 1(CREB1) in periosteum-derived stem cells (PDSCs) via cAMP and finally activate the expression of SP7 (Osx) (Fig. 5) [36].

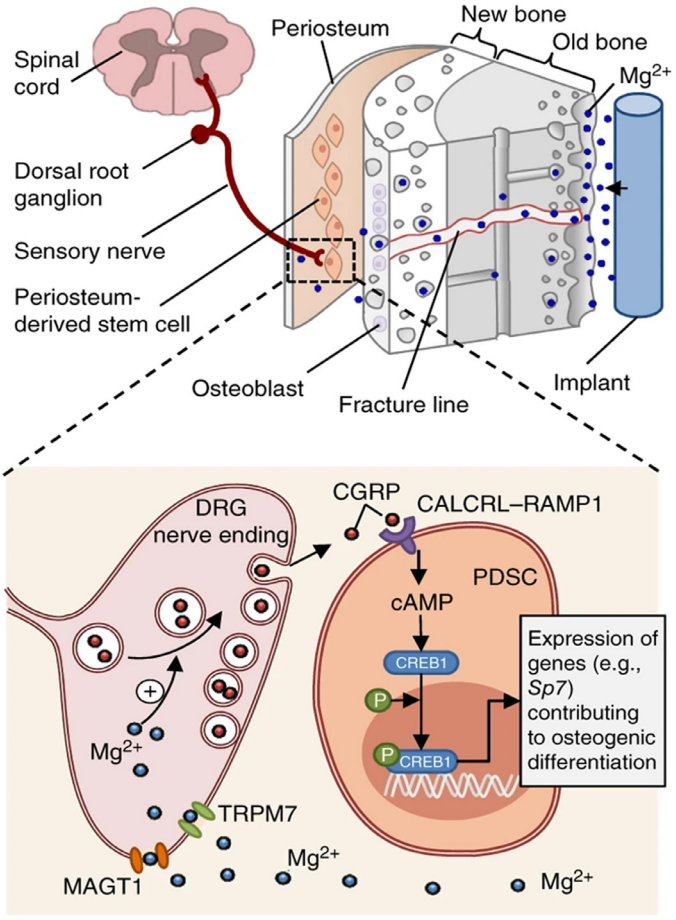

Fig. 5. The schematic diagram shows that implant-derived Mg promotes bone healing by inducing local neuronal production of CGRP. Mg^{2+} released at the implant site can diffuse through the bone to the periosteum. Through the Mg^{2+} transporters MagT1 and TRPM7, Mg^{2+} enters the DRG and promotes CGRP-vesicles accumulation and exocytosis. The released CGRP will activate the phosphorylation of CREB1 in PDSCs via cAMP and finally activate the expression of SP7 [36].

5.2.6. The weak alkaline environment-mediated signaling pathway activation

Although the weak alkaline environment produced by Mg alloy degradation has been proven to be beneficial to the osteogenic differentiation of cells, the exploration of its potential mechanism is still rare. The mechanism of pH regulating osteoblast behavior in other environments may provide some clues to us. In the study of Tao et al., G-protein-coupled receptor 4 (GPCR 4) of BMSCs was proven to inhibit YAP activation and subsequent osteogenic differentiation in response to proton induction. And cAMP/PKA seems to be the downstream pathway of GPCR 4, leading to the degradation of YAP [181]. On the contrary, Wang et al. confirmed that the alkaline environment formed by sodium bicarbonate on the Ti surface can activate the integrin receptor, which stimulated the phosphorylation of FAK and then led to the upregulation of ALP and Runx2 expression [151]. Galow et al. cultured MC3T3-E1 osteoblast-like cells at pH 7.4, 7.8, and 8.4 for 14 days and identified the differential gene expression. A functional gene network reflecting different pH-dependent cellular responses was made combined with biological information analysis. Results found the common feature of these networks is the core function of p38 MAPK [182]. Besides, in the study of M.W. Chen et al., the pH value of 8.5 will significantly promote the expression of apoptosis-related factor LC3 of MSCs and the level of osteogenic differentiation, suggesting that alkaline environment can improve osteogenic activity by promoting autophagy of cells. However, the specific molecular mechanism of alkaline microenvironment and autophagy remains to be further explored [154].

In conclusion, many intracellular signaling pathways including integrin-related signaling pathway, MAPK signaling pathway, Wnt signaling pathway, Smad signaling pathway, hedgehog pathway, PI3K/AKT signaling pathway, CaM/CaMKIV/CREB1 signaling pathway have been confirmed to play an important role in the process of Mg alloys or Mg^{2+} promoting osteogenic differentiation. And Mg^{2+} transport receptors TRPM7 and MagT1 play important roles in Mg-mediated bone regeneration (Table 4 and Fig. 6). In ad-

Table 4
The signaling pathway involved in the osteogenic effect of Mg alloys.

	Cell line/ animal models	Signaling pathway	Effects	Refs.
Mg-0.2Si-1.0Ca alloy	hMSCs	Fak/AKT/ GSK3β signaling; Fak/ERK/p38MAPKs signaling	Promotes osteogenic differentiation.	[12]
Mg-1Ca-2.0Sr alloy	MC3T3-E1	ERK1/2 MAPK signaling	Promotes osteogenic differentiation.	[130]
B-TCP/Mg-Zn composite	hBMSCs	ERK1/2, p38 and JNK MAPK signaling	Up-regulates the expression of BMPs, Col-I, RUNX2 and OSX. Elevates the level of ALP and the formation of calcium nodules.	[27]
$MgCl_2$	rBMSCs	ERK1/2 MAPK signaling	Promotes osteogenic differentiation.	[146]
Mg-1Y alloy	hBMSCs	BMP2/Smad signaling; TGF-β/Smad signaling	Promotes osteogenic differentiation.	[173]
Mg-3.5Li-0.5Ca alloy	hBMSCs	Wnt/β-catenin signaling	Promotes osteogenic differentiation.	[131]
Mg-Nd-Zn-Zr (JDBM) alloy	The rat osteochondral defect model	Wnt/β-catenin signaling	Increases the relative expression of Runx2, BMP2, OPN, OCN. Promotes new bone regeneration.	[169]
High purity Mg	The rat femoral DO model	Hedgehog-alternative Wnt signaling	Enhances bone consolidation in the DO application.	[37]
High purity Mg	The rat femoral fracture model	CGRP /cAMP/ CREB1 signaling	Up-regulates the expression of osteogenic genes such as SP7. Promotes fracture healing.	[36]
High purity Mg	MC3T3-E1; MG63 The rat femoral implantation model	CaM/CaMKIV/CREB1 signaling	Enhances the expression of OPN. Enhances the expression of OPN and promotes new bone regeneration.	[177]
$MgSO_4$	hFOB1.19	TRPM7/PI3K signaling	Increases the expression of Runx2 and ALP. Up-regulates the expression of MMP2, MMP9 and VEGF to promote cell migration.	[147]
$MgCl_2$	rBMSCs	Notch signaling	Promotes cell proliferation and osteogenic differentiation.	[176]

dition, PGDF-BB, an important factor of angio-osteogenesis coupling, neurons and CGRP they secreted are also involved in osteogenesis under the induction of Mg^{2+}. The summary of these pathways and mechanisms provides a theoretical basis for Mg alloy design and orthopedic application. However, the osteogenic mechanism of the weak alkaline environment caused by Mg alloy degradation is still unknown, which should also be paid attention to and further studied. Finally, most of the current studies choose to directly verify the signaling pathways that have been confirmed to be involved in osteogenic differentiation, lacking a unique and comprehensive understanding of the osteogenic mechanism of Mg alloys. And most of the validations are still focused on *in vitro* and simple magnesium salt simulation experiments. In view of the differences in surface morphology, degradation behavior and bioactive components of Mg alloys with different compositions, it is necessary to study the osteogenesis mechanism of different Mg alloys and verify them *in vivo*. A comprehensive understanding of the osteogenesis mechanism of Mg alloys will provide more molecular biology theories and foundations for the development and improvement of Mg alloy orthopedic implant materials.

6. Mg alloys play a role in the formation and function of osteoclasts to avoid osteolysis and regulate bone remodeling

Numbers of researches has been focused on the regulation of osteoblast-related cells function and underling mechanism by implants. The osteolytic effect of osteoclasts, the interaction between osteoblasts and osteoclasts is neglected. In fact, the successful implants and bone remodeling are inseparable from the regulation of osteoclasts [183]. Janning et al. implanted pure $Mg(OH)_2$ in rabbit femur condyles. It was found that the number of osteoclasts around the implant was temporarily reduced during the first four weeks [184]. Similarly, Jahn et al. found that the number of osteoclasts and bone resorption at the implanted site decreased within 3 weeks of Mg-2Ag alloy implantation in a mouse femoral fracture model [184]. In addition, several *in vitro* experiments verified the inhibitory effect of Mg and Mg alloy extracts on osteoclast differentiation and function [57],[185–187]. These evidences suggest the effect of Mg alloy corrosion products on osteoclast differentiation and function. Mg^{2+}, alkaline environment, and H_2 release have all been confirmed to play a role in this phenomenon [57],[186],[188],[189].

6.1. Magnesium ion, pH value and hydrogen release regulate osteoclast differentiation and function

The study by Wu et al. confirmed that $MgCl_2$ promoted the formation and activation of osteoclasts when reaching a certain concentration (5 mM), and this phenomenon was reversed by further increasing the concentration (25 mM) [186]. However, this dual effect was not found in Mg extracts. Conversely, osteoclast differentiation continued to decline with increasing Mg or Mg alloy extract concentration (increased Mg^{2+} concentration, osmolarity, and pH) [186],[187]. More-

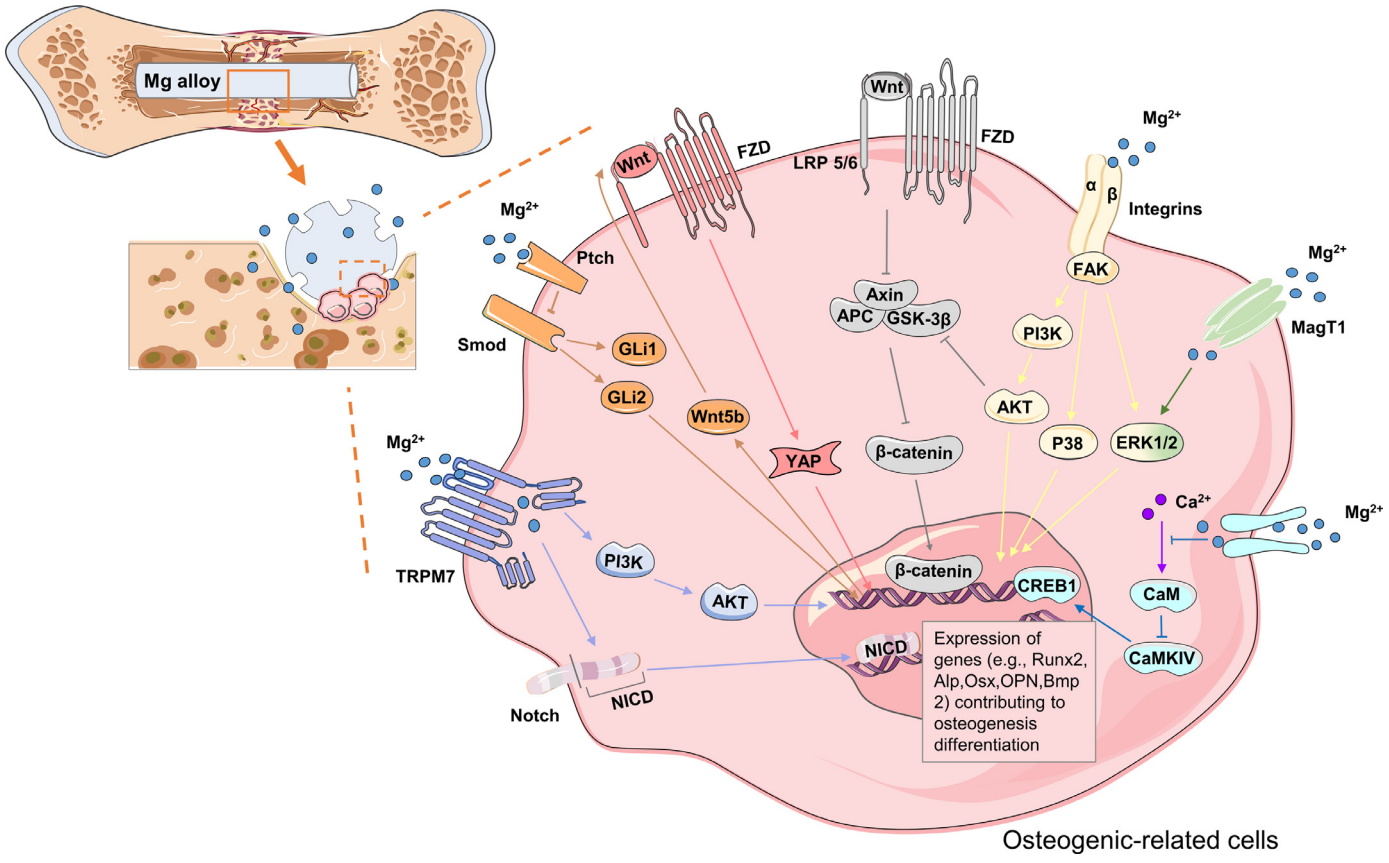

Fig. 6. The signaling pathway involved in the osteogenic effect of Mg alloys. Mg^{2+} upregulates integrin expression and activates FAK signaling pathway via an integrin-dependent manner. Then, the downstream signaling pathways included MAPK (ERK1/2 and p38) and PI3K/AKT are activated. PI3K/AKT signaling pathway increases the levels of GSK3-β phosphorylation, thereby activating canonical Wnt pathway. Moreover, Mg^{2+} transported into the cytoplasm through MagT1 promotes the activation of ERK1/2 signaling pathway. Mg^{2+} acting on TRPM7 with kinase activity activates PI3K/AKT signaling pathway. TRPM7 can also transport Mg^{2+} into the cytoplasm for intracellular effect. The intracellular Mg^{2+} activates Notch signaling pathway. Furthermore, intracellular Mg^{2+} competes binding sites on CaM with Ca^{2+}, promotes osteogenesis through CaM/CaMKIV/CREB1 signaling pathway. Moreover, under the mediation of Mg^{2+}, both the canonical Wnt pathway and the non-canonical Wnt pathway are activated. In particular, the Hedgehog signaling pathway may be an upstream pathway of the non-canonical Wnt pathway, which can promote the expression of Wnt5b under the action of Mg^{2+}.

over, high Mg extract concentration can significantly inhibit osteoclast differentiation and expression of osteoclast markers (tartrate-resistant acid phosphatase, TRAP, cathepsin K, CTSK, receptor activator of nuclear factor kappa-B, RANK, and nuclear factor-activated T cells c1, NFATc1) [140],[186]. This suggests that in addition to Mg^{2+} concentration, the effects of pH and osmolarity changes on osteoclasts cannot be ignored. At the same time, Mg and Mg alloy extracts are the better choice for the *in vitro* study of the effect of Mg and its alloys on osteoclasts. Kim et al. and Zhai et al. both found that the inhibitory effect of Mg or Mg alloy extract on osteoclast differentiation and activation was partially reversed after neutralization of pH, further confirming the important role of pH [57],[188]. Indeed, it has long been demonstrated that the activity of osteoclasts is pH-dependent. The differentiation and proliferation of osteoclasts are optimal at pH 7.0–7.5 [190], while pH 6.8 is considered to be the optimum acidic environment to stimulate osteoclast bone resorption activity [191]. This is the exact opposite to the effect of pH on osteoblasts mentioned above, suggesting that alkaline environment has dual regulatory effects on osteogenesis and osteoclastogenesis. This effect is also confirmed in the research of other materials. In the study of Liu et al., when the surface pH of the biomaterial was higher than 7.8, the differentiation and absorption ability of osteoclasts were almost completely inhibited, while the function of BMSCs remained unaffected [192]. Chen et al. demonstrated that the alkali-heat-treated material could generate an alkaline microenvironment (about pH 8.5) for a long time, which significantly promoted osteogenesis while inhibited the expression of osteoclast markers [154]. Surprisingly, in the study of Liu et al., they found that the higher initial microenvironment pH value after material implantation would lead to the low reactivity of osteoclasts, as evidenced by almost no TRAP osteoclasts around the implant in the first week. Then, with the gradual stabilization of implant degradation, the environmental pH decreased. TRAP osteoclast-like cells appeared in the 4th week and persisted until the 9th week [193]. This will be more consistent with the bone healing process in which bone remodeling involving osteoclasts is a crucial part. Whether the degradation of Mg alloy *in vivo* will produce this long-term bidirectional effect remains to be further explored.

Notably, the expression of osteoclast-specific genes (TRAP, NFATc1) was not significantly changed by the presence of Mg corrosion products in the study of Maradze et al. They suggested that the low number of multinucleated TRAP positive osteoclast-like cells was due to the inhibition of fusion at the pre-osteoclast cell surface by Mg corrosion products [185]. It should also be emphasized that Wu et al. used a co-culture system of osteoblasts and osteoclasts to better mimic the *in vivo* bone healing environment. They confirmed that higher concentrations of Mg extract increased the activity of osteoblasts, and the pro-osteoclast factors (RANKL and M-CSF) released by osteoblasts were also enhanced. At the same time, the extract was acidified to neutralize the pH due to the increase in cell density. These are favorable for both differentiation and activity of osteoclasts. However, the expression of osteoclast markers was still inhibited by high Mg extract concentration. This may be due to the fact that neither pro-osteoclast factors nor osteoblast-induced pH neutralization is sufficient to counteract the negative effects of high pH and osmolarity [140]. Moreover, the difference of pH environment between the implantation site and the extracts cannot be ignored [194]. In the process of bone healing *in vivo*, the host response to the material, the adhesion of various cells on the material surface, tissue fluid, and blood will all affect the local pH value [193],[195]. Therefore, it is necessary to directly detect the pH environment around materials *in vivo* and explore its regulation on osteoclastogenesis and osteogenesis.

In addition, H_2 release was confirmed to have inhibitory effects on the osteoclast formation and function of bone marrow mononuclear cells (BMMCs). Liu et al. simulated exposure scenarios of different H_2 levels in tissues or organs by changing the H_2 concentration in the incubator. They found that when exposed to H_2, especially 50% or 75% H_2, the osteoclastogenesis of osteoclast-induced BMMCs was significantly inhibited. H_2 exposure inhibited the proliferation of BMMCs and promoted their apoptosis. At the same time, the expression of osteoclast-related genes and proteins in BMMCs was significantly inhibited [189].

6.2. Underlying mechanisms of Mg alloys regulating osteoclast differentiation and function

As for the regulation mechanism of Mg or Mg alloy extract on osteoclast differentiation and function, there are still few studies. Zhai et al. demonstrated that Mg extract significantly inhibited the activation of NF-κB by inhibiting the degradation of the inhibitory subunit of NF-κB and the subsequent nuclear translocation of NF-κB. At the same time, Mg extract attenuated the expression of NFATc1. The expression of downstream genes TRAP, calcitonin receptor (CTR), and CTSK related to osteoclast differentiation and function was inhibited. Therefore, the high Mg extract concentration significantly inhibited the differentiation and activation of osteoclasts by blocking NF-κB signaling pathway. Besides, since Mg is a natural calcium antagonist and has a strong inhibitory effect on L-type calcium channels, they also speculated that Mg leach liquor (MLL) might inhibit the auto-amplification of NFATc1 by blocking Ca^{2+}-dependent calcineurin signaling (Fig. 7) [188]. Recently, Zheng et al. demonstrated the inhibitory effect of Mg on intracellular Ca^{2+} response. The addition of Mg^{2+} significantly inhibited the influx of Ca^{2+} induced by RANKL or LPS, thereby inhibiting osteoclast differentiation by blunting NF-κB and NFATc1 signaling pathway (Fig. 7) [196]. RANK/RANKL signaling pathway, as the core signaling pathway of osteoclast activation, has been paid attention to for a long time for its negative effects in osteoporosis, bone defects and other bone diseases [197]. Therapeutic methods targeting the RANK/RANKL signaling pathway, including RANK antagonists, have been developed [197],[198]. The regulating effect of Mg alloys on RANK/RANKL related pathway has further laid a foundation for its application in orthopedics.

Based on these evidences, we speculate the ideal cellular response after Mg alloy implantation. Mg alloys undergo rapid corrosion in the initial stage, resulting in a high concentration of degradation products. On the one hand, high-concentration degradation products promote early inflammatory response and help debridement of the implanted site to provide a favorable environment for subsequent bone regeneration. On the other hand, the formation and activation of osteoclasts are inhibited to avoid early excessive osteolysis. At this stage, caution is required for the degree and duration of early rapid degradation to avoid unnecessary cytotoxicity and excessive inflammatory reaction. Certainly, the early high-concentration degradation products may not be suitable for the effective differentiation of angioblast and osteoblast-related cells. After a short period of rapid degradation, the release of Mg^{2+} and hydroxyl turned to a stable level. Stimulated by the appropriate concentration of Mg^{2+} and weak alkaline pH, the activity of precursor cells increased. New blood vessels grow in the implantation site. Osteoblast-related cells proliferate and differentiate into osteocytes. The excessive inhibitory effect of high pH value and Mg^{2+} concentration on osteoclasts gradually weakened. At the same time, osteoblasts secrete pro-osteoclast cytokines during differentiation, which promotes the formation and activation of osteoclasts. Under the interaction of osteoblasts and osteoclasts, bone reconstruction is carried out at the implanted site, and bone healing achieves eventually. In the process, controlling the concentration of degradation products, including Mg^{2+}, hydroxide, and H_2, is crucial for tissue reaction. The results of cell effect and mechanism *in vitro* can serve as tentative references. The important to note is, however, that considering the three-dimensional space of the implant site different from the culture medium *in vitro*, the complex cell interaction, and the influence of body fluids, the situation after Mg alloy implantation is complicated. Numerous studies have shown that the corrosion rate of Mg alloys *in vivo* is usually significantly lower than that *in vitro* [199–201]. In the study of Sanchez et al., a systematic literature survey speculates that corrosion rate of Mg alloys *in vivo* is 1–5 times lower than that obtained *in vitro* [199]. More research is needed on the tissue reaction and mechanism of Mg alloy *in vivo*. Of course, all

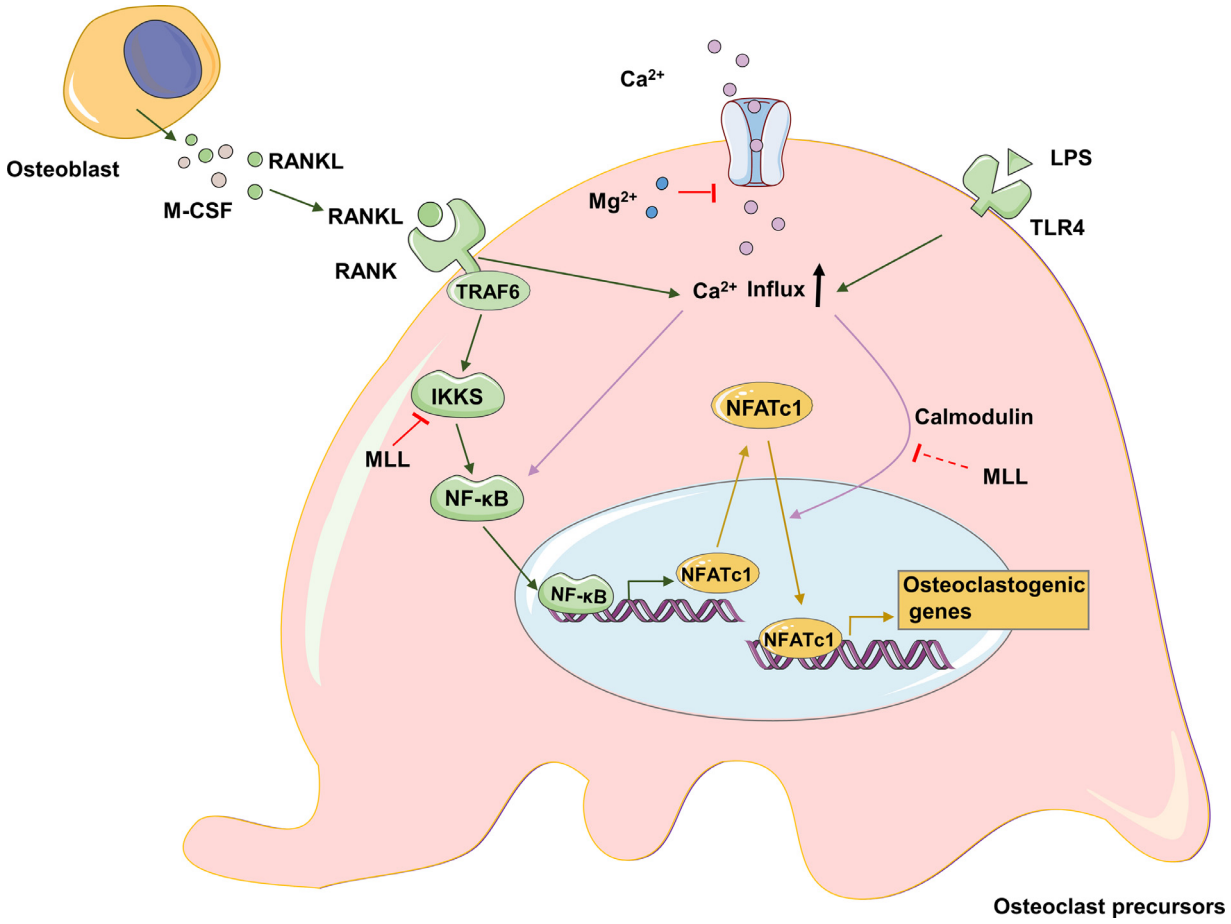

Fig. 7. The schematic diagram shows the molecular mechanism of Mg that inhibits osteoclast differentiation and function. Pro-osteoclast cytokines such as RANKL and M-CSF secreted by osteoblasts promote the differentiation and function of osteoclasts. However, MLL inhibits the NF-κB signaling pathway and probably also blocks Ca^{2+}-dependent calcineurin signaling, thereby suppressing NFATc1 expression, activation and nuclear translocation. Mg^{2+} significantly inhibits the influx of Ca^{2+} induced by RANKL or LPS, thereby blunting NF-κB and NFATc1 signaling. The inhibition of osteoclast marker gene expression under the action of MLL/Mg^{2+} ultimately leads to the inhibition of osteoclastogenesis and function.

this should be aimed at explaining different bone regeneration effects of different Mg alloys *in vivo* and helping to obtain the best implant response.

7. The cellular effects and underlying mechanisms of bioactive coatings promoting bone healing of Mg alloys

It is well known that the excessive release of Mg^{2+}, the accumulation of a large amount of H_2 and the production of local alkalinity caused by the rapid degradation of Mg alloys result in cytotoxicity. At the same time, the rapid degradation brings about the premature disappearance of the mechanical support of the implants which is not conducive to the application of Mg alloys in orthopedics [17],[202]. To overcome these difficulties, a large number of researchers have focused on the development of coatings for Mg alloys [22]. Considering the possible additional biological effects of coatings, bioactive coatings are increasingly being studied [203–206]. In addition to effectively controlling the degradation of Mg alloys to bring about the moderate release of Mg^{2+}, H_2 and hydroxide, which are beneficial to cellular responses, these coatings also have unique effects on osteogenesis, angiogenesis, immunomodulation and even osteoclast function regulation [206–209]. This section summarizes the cellular effects and underlying mechanisms of bioactive coatings including polymers, calcium phosphate (CaP), etc. promoting bone healing of Mg alloys.

7.1. Inorganic coating

Ca-P coatings are widely studied due to its good biocompatibility and osteoconductivity [210]. The promotion effect of Ca-P coatings on the bioactivity of Mg alloys has been confirmed by some reports [203],[204],[211]. Xu et al. covered Ca-P coatings on Mg-Mn-Zn alloys, which provided Ca^{2+} led to more fibronectin and vimentin attachment, then promoted L929 cell attachment and diffusion [211]. Similarly, in the study of Liu et al., calcium metaphosphate (CMP) coatings lead to faster adhesion of BMSCs in flat morphology. The high expression of vinculin and F-actin was detected, indicating the focal adhesion formation. Additionally, higher expression of Ki-67, a nuclear protein associated with cellu-

lar proliferation, reflected CMP coatings facilitated BMSCs proliferation. More importantly, the expression of osteogenic genes, including ALP, COL1 and RUNX2, in BMSCs on the surface of CMP coatings were upregulated, which confirmed osteogenic activity of the coatings [203]. Kim et al. manufactured amorphous Ca-P composite layer coated Mg-3Al-1Zn-1.5Ca alloy. Interestingly, Ca-P compound reduced production of cellular stress indicator hydrogen peroxide. Moreover, Ca-P compound increased osteoblast differentiation while decreasing osteoclast differentiation and activity significantly in osteoblasts and RAW 264.7 cells co-culture system with the coated Mg alloy extract [204].

Wang et al. discovered the improvement of dicalcium phosphate dihydrate (DCPD) coatings to the bioactivity of porous JDBM scaffold. The results demonstrated that compared to MgF_2 coatings, rBMSCs cultured on the DCPD coatings possessed better adhesion and spreading morphology. Meanwhile, DCPD-coated scaffold extracts significantly boosted the expression of osteogenic-related genes (ALP and OCN) and angiogenesis-related genes (VEGF, HIF-1α) [212]. It is inseparable from the vigorous cellular bioactivity of DCPD. DCPD shares a similar composition to bone minerals and is more conducive to cell adhesion [213]. Besides, Ca and P ions released by DCPD can stimulate cell proliferation and differentiation [214]. However, the role and mechanism of DCPD in angiogenesis-related gene expression remain to be explored. A β-tricalcium phosphate (β-TCP) coating on the Mg scaffold was proven to promote the polarization of macrophages to the M2 phenotype. The expression of the anti-inflammatory cytokine IL-1ra was thus significantly upregulated. At the same time, macrophages expressed abundant VEGF and BMP-2 in response to the coating, which stimulated the osteogenic differentiation of BMSCs. In contrast, the differentiation of macrophages into osteoclasts was inhibited by the coating, as shown by the downregulation of macrophage colony-stimulating factor (MCSF) and TRAP. The anti-inflammatory rather than osteoclastic immune microenvironment brought by β-TCP coating may be accomplished by its inhibition of the TLR signaling pathway and RANKL/RANK system [206].

Hydroxyapatite (HA), as the main mineralized matrix component of bone, especially HA with three-dimensional nanomorphology, has been shown to have strong bone regeneration ability and was widely explored in the field of Mg alloy coatings [215–217]. Li et al. encapsulated the MgO coated Mg alloy using HA nanorods, and it testified the nano-HA coating promoted the early secretion of BSP and OPN by osteoblasts and BMSCs, which further induced mineralization to form a bone cement line matrix, enabling tight integration of the implant and new bone. Osteogenic related genes ALP and OCN in osteoblasts was also significantly expressed under the action of the nano-HA coating [207]. Besides the osteogenic effect of HA itself, this result may also be closely related to the surface nanostructures that have been reported to synergistically affect integrin, BMP-2 signaling pathway and Cx43-related intercellular communication [217],[218]. More interestingly, the HA coating endowed the Mg implants with stronger immunomodulatory properties. HA coating significantly down-regulated the expression of pro-inflammatory cytokines TNF-α and IL-1β secreted by macrophages and upregulated the secretion of anti-inflammatory cytokine IL-10 [207]. Furthermore, under the effect of HA coating, the differentiation of macrophages into osteoclasts was blocked, implying the inhibition of bone resorption [207]. In addition to the regulatory effects on osteogenesis, osteoclastogenesis and immunomodulation, the role of HA coating in angiogenesis was also confirmed. In the study of Cheng et al., the HA-coated Mg extracts showed enhanced expression of angiogenic-related genes including KDR, VEGF, and HIF-α in HUVECs. In the tube formation assay, the HA coating showed certain angiogenic activity and promoted the formation of a tubular network structure [208].

Furthermore, some studies have been devoted to adding active metal elements to Ca-P coatings to further improve the coating bioreactivity [219–221]. As a natural bone trace element, strontium (Sr) can promote the binding of cells to adhesion proteins to create an active environment conducive to cell behavior and support the differentiation of osteoblasts [222]. Makkar et al. demonstrated that calcium phosphate (Ca-Sr-P) coatings on ZK60 alloy significantly promoted the adhesion, proliferation and osteogenic differentiation of MC3T3-E1 cells [219]. The addition of Sr into the nano-HA coating to partially replace Ca^{2+} will further enhance the promoting effect on the expression of osteogenic-related genes, including Runx2, ALP, OSX, Col-I, OCN, OPN in BMSCs. It is partly attributed to the addition of Sr, leading to the formation of a nano-network with a larger contact area on the surface of the HA coating, and the other part is attributed to the promoting effect of Sr on osteogenic differentiation with unknown mechanism [220]. Interestingly, a $SrHPO_4$ coating suitable for JDBM was developed. $SrHPO_4$-coated JDBM was confirmed to promote the proliferation and growth of MC3T3E1 cells by upregulating TLR4 and activating the downstream PI3K/AKT signaling pathway. And in a rat femoral fracture model implanted with SrHPO4-coated JDBM, the application of TLR4 inhibitors attenuated new bone formation [221].

The improvement of Mg-Al layered double hydroxide (LDH) coating on the bioactivity of Mg implants has been investigated. It is reported that Mg-Al LDH-coated Mg alloys supported the activity, adhesion and proliferation of rBMSCs and MC3T3-E1 cells [223],[224]. Furthermore, in the study of Cheng et al., the multiple regulatory effects of Mg-Al LDH coating on osteogenesis, angiogenesis and immunomodulation were confirmed. First, MC3T3-E1 cells culture showed that Mg-Al LDH coating was more conducive to the expression of osteogenic-related genes such as ALP, BMP-2, Col-I and OCN. Second, Mg-Al LDH coating upregulated the expression of angiogenesis-related genes KdR, VEGF and HIF-α in HUVECs and promoted their interaction to form vascular structure. Additionally, Mg-Al LDH coating induced the polarization of macrophages to the M2 phenotype, and the secreted cytokines were shown to improve the osteogenic differentiation of rBMSCs. The significant down-regulation of p65 expression suggested that Mg-Al LDH may relieve its

negative effect on the osteogenic differentiation of rBMSCs by inhibiting the activation of NF-κB signaling pathway. Finally, the excellent cell behavior regulation ability mentioned above maked the Mg-Al LDH-coated Mg alloy exhibit excellent osseointegration and regeneration ability in femoral implantation model [225].

7.2. Organic coating

PCL is a widely used biocompatible polymer coating material. Smooth PCL coating on Mg alloy surface can guide cells for uniform adhesion and spreading, avoiding cell tearing caused by localized corrosion cracks of Mg alloys [226]. However, the bioactivity of PCL is low, and other components need to be added to enhance its regulatory effect on cell behavior. Zheng et al. put copper-based metal-organic framework (MOF) to PCL matrix to prepare PCL coatings capable of releasing low concentrations of Cu^{2+}. Coated Mg alloy significantly increased the ALP activity of cells grown on the surface due to the positive effect of low concentrations of Cu^{2+} on osteogenic differentiation [226]. In the study of Kim et al., electrospun PCL coating containing ZnO nanoparticles exhibited better promotion of MC3T3-E1 osteoblasts activity and proliferation than bare Mg due to rough surface nanostructure and good hydrophilicity [227]. Negrescu et al. explored the effect of ZnO/PCL coating on osteoimmunoregulation by regulating the behavior of macrophages. In their study, ZnO/PCL coating appeared supportive for macrophage survival, proliferation, and early inflammatory responses. The expression of IL-1β and TNF-α was significantly increased within 48 h when macrophages were cultured with the coated Mg alloy extracts. Meantime, the coating did not appear to perpetuate the early inflammatory response and convert it into an undesirable chronic inflammation, as indicated by the low degree of macrophage fusion under the LPS-induced inflammatory response [209].

Peng et al. coated polydopamine (PDA) film on the surface of hydrothermally treated AZ31 alloy. Since abundant functional group surface and osteogenic activity of PDA [228], the coating exhibited strong adhesion to MC3T3-E1 cells and promotion of osteogenic differentiation [229]. Zhou et al. utilized PDA intermediate layer to induce HA coating on the Mg alloy surface, which exhibited superior proliferation, adhesion and spreading of osteoblasts compared to the pure HA coating due to the presence of the inner layer of PDA [230]. These results also confirmed the regulation of PDA to the behavior of osteoblasts. However, the specific mechanism that PDA-containing coatings on Mg alloys promote bone regeneration remains to be explored. To further enhance the bioactivity of the alloy, Peng et al. added Zn to the PDA coating, which then increased the expression of MC3T3-E1 osteogenic-related genes ALP and OCN. More importantly, Zn–containing PDA film sample extracts activated RAW264.7 to polarize to an anti-inflammatory phenotype. The secretions of inflammatory cytokines CCL-3, TNF-a, IL-1b, and IL-6 were significantly down-regulated [229]. This effect may come from the sustained release of Zn^{2+}, which has been shown to have anti-inflammatory effects [231],[232].

The natural protein silk fibroin (SF), which has been proven to have osteogenic activity, is favored for the fabrication of coating for Mg alloys. Xiong et al. prepared a multilayer coating system on Mg alloy using SF as the outermost layer. On SF-coated surfaces, MC3T3-E1 cells showed good adhesion and spreading along the SF fibers. In the meantime, SF endowed the coating with impressive osteogenic activity, which was manifested by a significant increase in ALP activity, collagen secretion and mineral deposition [233]. SF has previously been shown to enhance the expression of ALP and Runx2 by significantly downregulating Notch signaling [234]. Furthermore, cellulose nanocrystals were composited with SF to fabricate a composite coating with a nano-surface structure, which significantly promoted the adhesion and spreading of human fetal osteoblasts [235]. Besides, SF films containing Ca, Sr, and P on Mg alloys were developed. The coating exhibited a biomimetic structure similar to that of the extracellular matrix, with the support of the osteogenic activity of SF, Ca and Sr, the MC3T3-E1 cells on the coating responded strongly to proliferation, spreading and differentiation [236].

It has been reported that Mg guided bone regeneration membrane (GBRM) coated with chitosan showed similar bone regeneration effect as commercial GBRM [135]. Jia et al. applied a chitosan coating loaded with the corrosion inhibitor trivalent cerium on the surface of Mg alloy after MAO pretreatment. The coated alloy showed significant promotion of proliferation and adhesion of MC3T3-E1 cells. In addition to the effective control of the release of Mg^{2+} and hydroxide by the coating, this positive effect seems to be related to the promotion of cell adhesion and proliferation by chitosan and the micro/nanotopologies obtained by coating treatment [237]. Furthermore, Liu et al. demonstrated that CaP/chitosan-coated Mg alloys significantly promoted the osteogenic differentiation of MSCs, as shown by the elevation of early ALP expression and late expression of BSP. And this effect inhibited by Wnt inhibitor, suggesting the role of Wnt/β-catenin pathway [238].

7.3. Drug-loaded coating

To achieve osteogenic-osteoclastic regulation, zoledronic acid (ZA)-loaded coatings for Mg alloys were developed [239]. Li et al. loaded ZA on CaP-coated JDBM alloy. It was found that ZA/CaP bilayer-coated Mg alloys achieved the stable release of ZA for at least several weeks *in vitro*. The alloy extract showed obvious promotion of osteogenic differentiation of rBMSCs while inhibiting the differentiation of osteoclasts. Implantation of ZA/CaP double-coated Mg alloy in femur fracture in ovariectomy-induced osteoporotic rats also confirmed the inhibitory effect of ZA-loaded coating on osteoclast formation, as demonstrated by TRAP-positive multinucleate osteoclasts around the implant significantly decreased [239]. Similarly, in the study of Li et al., ZA-CaP double-coated Mg-Sr alloy significantly promoted the proliferation, osteogenic differentiation and extracellular matrix mineraliza-

tion of pre-osteoblasts compared with CaP single-layer coated Mg-Sr alloy. Conversely, ZA-CAP double-coated Mg-Sr alloy selectively induced pro-osteoclast apoptosis and inhibited osteoclast differentiation and cytoskeleton organization [240]. To the underlying mechanism, the ZA-CaP bilayer coating upregulated the expression of ERα on the membrane of pre-osteoclasts to block the continuous formation of osteoclasts. Meanwhile, the coating inhibited the activation of the NF-κB signaling pathway and down-regulated osteoclastogenesis-specific biomarkers, including TRAP, c-Src, central transcriptional factor in osteoclastogenesis (c-fos), CTSK and MMP-9, most of which are targets of NFATc1. More importantly, in the osteoclast-osteoblast co-culture system, ZA-CaP-coated Mg-Sr alloy was confirmed to regulate the osteoblast-osteoclast interaction through the OPG/RANKL/RANK signaling pathway, which is critical for the balance of the bone remodeling process [240]. Besides, Qi et al. added simvastatin (SIM) to gelatin nanospheres/chitosan (GNS/CTS) coating for WE43 alloy, realizing the regulation of angio-osteogenesis coupling. The co-culture system of BMSCs and HUVECs confirmed that SIM released from the coating increased the chemokine SDF-1 and angiogenic factors VEGF, bFGF secreted by BMSCs, which promoted the migration and tube formation of HUVECs, respectively. At the same time, the chemokines SDF-1 and BMP-2 secreted by HUVECs, which enhanced the migration and osteogenic differentiation of BMSCs, were up-regulated by SIM [241].

8. Conclusion

This review summarizes current research going on the mechanism of Mg alloys promoting bone healing. Mg alloys play an essential role across the stages in bone healing by regulating the functional status and interactions between numerous cells that are involved in bone healing, including immune cells, endothelial cells, osteogenic-related cells, osteoclasts, nerve cells, etc. At the early stage of implantation, Mg alloys participate in activating acute inflammatory response, then regulate macrophage polarization, enabling the transformation of implantation sites into an anti-inflammatory state that benefits tissue regeneration and repair. At bone repair phase, Mg alloys promote new bone formation by mediating the adhesion, proliferation, differentiation, and extracellular matrix secretion of osteogenic-related cells. More importantly, Mg alloys show unique properties in angiogenesis by regulating behavior of endothelial cells and modulating the interactions between immune cells, osteogenic-related cells, nerve cells and endothelial cells. Additionally, Mg alloys have an important regulatory effect on osteoclasts, avoiding abnormal osteoclast activation and bone resorption at bone repair phase. Finally, Mg alloys achieve good bone remodeling by regulating osteoclast and osteoblast functions. As for the coatings for Mg alloys, the moderate release of Mg^{2+} and hydroxide is beneficial to cell reaction achieved by controlling alloy degradation. In addition, due to the introduction of bioactive coating materials, Mg alloys are endowed with unique immunomodulation, angiogenesis, osteogenesis, and osteoclastogenesis functions. Current knowledge about the mechanisms of Mg alloys on promoting bone healing provides the theoretical basis and insights for the application of Mg alloys in orthopedic fields. Nevertheless, according to the literature reviewed here, the mechanisms of Mg alloys on promoting bone healing are extremely complicated, involving in different cell types, numerous regulators, interwoven signaling networks as well as multi-step regulation and balance, to achieve optimal bone healing. There is still a lot of knowledge that is understudied. In particular, the role of alkaline environment and H_2 on different cells remain unknown, and more exploration is needed in the future. Besides, current studies mainly choose to directly verify whether the factors and signaling pathways that have been proven to play a role in bone regeneration are involved in the promotion of bone healing by Mg alloys. A comprehensive and pioneering knowledge of the mechanism of Mg alloys promoting bone healing is lacking. Another fact, which cannot be ignored, is that most of studies are performed *in vitro* with one kind of cell as the object. To better simulate the cellular effect *in vivo*, the crosstalk between two or more kinds of cells is worth studying. Biological effects and underlying mechanisms caused by *in vivo* implantation of Mg alloys need more exploration and validation. Finally, the ultimate goal of a large number of studies on cell effects and mechanisms is to serve Mg alloy design and *in vivo* experiments. More verification of the implantation effect *in vivo* is necessary. According to the existing and future knowledge about mechanism and effect, it is possible to prepare the optimal alloy system by speculating the causes of different bone healing outcomes *in vivo* and adjusting the alloy design. Incidentally, the development of coating, especially multilayer or hybrid coating for Mg alloys, has potential, as it not only controls the degradation of Mg alloys to bring appropriate cellular reaction but also has special immune, angiogenesis or osteogenic-osteoclastogenic regulation mechanism. Reasonable selection of coating composition and related performance verification are issues worthy of attention in the future.

Declaration of Competing Interest

The authors have declared that no competing interest exists.

Acknowledgments

The work was supported by grants from The National Key Research and Development Program of China (No. 2020YFC1107501), National Natural Science Foundation of China (No. 51971222), STS program (No. 20201600200042), DongGuan Innovative Research Team Program, the Natural Science Foundation of Liaoning Province (2021-BS-103) and the China Postdoctoral Science Foundation Grant (2020M681020).

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