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# Sphingosine 1-phosphate (S1P) signalling: role in bone biology and potential therapeutic target for bone repair

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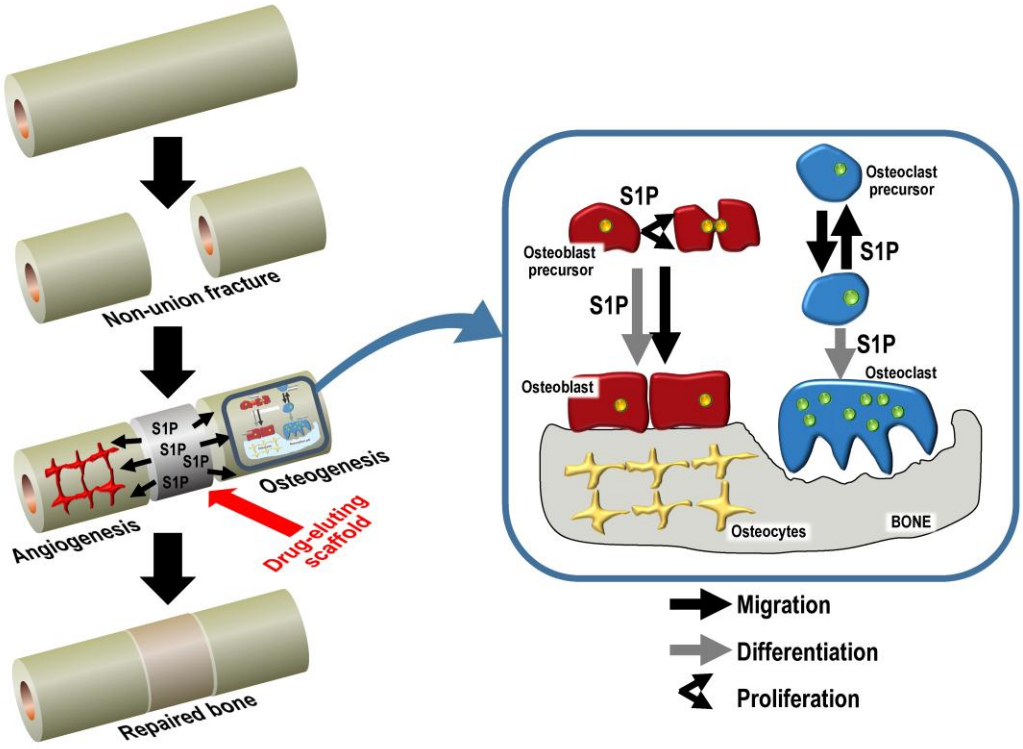
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Graphical abstract

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Abstract

The lipid mediator sphingosine 1-phosphate (S1P) affects cellular functions in most systems. Interest in its therapeutic potential has increased following the discovery of its G protein-coupled receptors and the recent availability of agents that can be safely administered in humans. Although the role of S1P in bone biology has been the focus of much less research than its role in the nervous, cardiovascular and immune systems, it is becoming clear that this lipid influences many of the functions, pathways and cell types that play a key role in bone maintenance and repair. Indeed, S1P is implicated in many osteogenesis-related processes including stem cell recruitment and subsequent differentiation, differentiation and survival of osteoblasts, and coupling of the latter cell type with osteoclasts. In addition, S1P's role in promoting angiogenesis is well-established. The pleiotropic effects of S1P on bone and blood vessels have significant potential therapeutic implications, as current therapeutic approaches for critical bone defects show significant limitations. Because of the complex effects of S1P on bone, the pharmacology of S1P-like agents and their physico-chemical properties, it is likely that therapeutic delivery of S1P agents will offer significant advantages compared to larger molecular weight

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factors. Hence, it is important to explore novel methods of utilizing S1P agents therapeutically, and improve our understanding of how S1P and its receptors modulate bone physiology and repair.

Keywords: Bone regeneration, bone defect, osteoblasts, osteoclasts, sphingosine 1-phosphate

## 1 Introduction

The incidence of non-union fractures is relatively low (20 per 100000 cases) (1). However, in severe fractures or in limb salvage following bone cancer, the incidence can be many fold higher (2). Current therapeutic options for non-union and other critical bone defects, mainly autologous grafts and allografts, suffer from drawbacks of both medical and logistical natures (3). There has been much hope that novel treatments based on the use of peptide or protein growth factors, mainly in combination with bone grafts or scaffolds, would show clinical benefit. Despite showing positive results, these strategies are limited by the need for high doses, as well as related ectopic growth (4-6). A potential promising alternative is the manipulation of lower molecular weight, non peptidic mediators, such as the bioactive lipid sphingosine 1-phosphate (S1P) (7).

S1P is the product of sphingosine kinase (SK)-mediated phosphorylation of sphingosine, itself derived from cell membrane sphingolipids (8, 9). S1P is an important player in cell death (10) and proliferation (11), with evidence that the balance between S1P and its pro-apoptotic precursors (sphingosine and ceramide) critically controls cell fate (12). Furthermore, S1P signalling is involved in cell adhesion and motility, smooth muscle contraction, and platelet aggregation (13).

S1P and its 5 known receptors ($S1P_{1\text{-}5}$) are expressed in several systems, including the vascular, immune, nervous, and reproductive systems (14). $S1P_1$ receptors have been detected in blood vessels and mesenchymal cells around day 12 of embryonic development (15). Their genetic deletion leads to defective limb chondrocyte development, and embryonic lethality from defective vasculature. Limb defects occur both following non-specific deletion and in mice specifically lacking endothelial $S1P_1$ receptors, and there is evidence that $S1P_1$ receptors may play a role in chondrocyte organization. Indeed, by day 16 of murine embryogenesis, $S1P_1$ receptor mRNA expression is abundant in bones undergoing ossification (16). As will be seen throughout this review, S1P receptors have also been identified in the key cells involved in bone remodelling and repair, including $S1P_{1\text{-}3}$ receptors expressed in osteoblasts, and $S1P_1$ and $S1P_2$ receptors in osteoclast precursor cells. Under basal conditions, the expression of $S1P_4$ and $S1P_5$ receptors seems to be limited to hematopoietic and lymphatic tissues ($S1P_4$) and the central nervous system ($S1P_5$) (17) and there is currently little evidence that either subtype plays a direct role in bone remodelling or repair. Semi-quantitative RT-PCR

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studies failed to detect mRNA for these two subtypes in primary rat osteoblasts (18), while they detected mRNA for all known S1P receptors except $S1P_5$ in bone marrow-derived macrophages and differentiating osteoclasts (19). However, a more recent quantitative RT-PCR study found mainly $S1P_1$, $S1P_2$, and $S1P_3$ receptor mRNA, with much lower levels of $S1P_4$ receptor mRNA, and no detectable $S1P_5$ receptor mRNA in primary osteoclasts or osteoblasts (20). Current pharmacological evidence for a lack of $S1P_{4/5}$ receptor involvement should be interpreted with caution due to the poor characterization and/or selectivity of available drugs (see Table 1). Studies using novel agents specific for $S1P_4$ and $S1P_5$ receptors (21) are needed to rule out, or possibly uncover, a role of these subtype in bone (patho)physiology.

The therapeutic potential of interfering with S1P signalling has mostly been explored in the immune (22), nervous (23), and cardiovascular systems (24). The function of S1P receptors in the immune system especially is increasingly better understood, with apparent roles in cell trafficking (25), allergic responses (26), and coagulation secondary to inflammatory conditions (27). The role of S1P in maintaining vascular integrity is also linked to inflammatory cell trafficking (28), suggesting that the effect of S1P on the immune and vascularization responses could contribute to bone repair, and could be exploited for therapeutic purposes in this context.

This review will focus on the role of S1P in bone regeneration, teasing out its interaction with the various cellular components of bone repair. It will evaluate whether the manipulation of S1P signalling has been effective in cases of critical bone defects, bearing in mind the complexity of S1P signalling, and the uncertainty regarding the specificity of the pharmacological tools used in the studies in question (29). Table 1 lists the S1P receptor agonists and antagonists frequently mentioned in this review, with their presumed subtype selectivity/specificity.

Other agents activating or blocking S1P receptors, or interfering with S1P metabolism have been described (30, 31). To the best of our knowledge, they have not yet been used to characterize the role of S1P signalling in bone biology and are therefore not listed here.

## 2 Bone repair

Bone is exceptionally proficient at self-repair, often able to avoid the formation of fibrous scar tissue in favour of complete regeneration (40). The cells responsible for bone development and repair are the same. Stem cells of mesenchymal origin are the source of bone forming osteoblasts and cartilage forming chondrocytes (41)

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whereas haematopoietic stem cells are the source of the monocytes and macrophages that differentiate into multinucleated osteoclasts, responsible for bone resorption (42). These cells collaborate in the formation of functional bone through intramembranous and endochondral ossification (43). Intramembranous ossification (IO) involves the direct differentiation of mesenchymal stem cells into osteoblasts and the deposition of bone, as occurs during the formation of bones of the skull. Endochondral ossification (EO), typical of long bone formation, involves an intermediary step, the formation of chondrocytes, and the deposition of cartilage, which acts as a template for osteoblasts as cartilage is systematically replaced by bone (44).

The process of bone repair echoes osteogenesis and resembles either EO or IO, depending on the size and location of the defect encountered. When the defect is sufficiently small and rigid, and adjacent bone cortices are in contact, deposition of bone may take place directly via IO, without intermediate cartilage formation. This direct, or primary, repair process requires the recruitment of osteoprogenitor cells, osteoclasts and undifferentiated mesenchymal stem cells to the fracture site. In contrast, indirect repair is similar to EO and involves the formation of a cartilaginous template (soft callus) that undergoes calcification into a hard callus and is eventually replaced by new woven bone (44). This process typically involves an acute inflammatory phase, which includes haematoma formation at the defect site, an early response by platelets, and neutrophils, followed soon after by monocytes and macrophages, resulting in thrombus formation, debris removal and the eventual formation of granulation tissue. Inflammation is continuously supported by positive feedback from the release of interleukins (primarily IL-1, and -6, along with -11, and -18) and tumor necrosis factor α (TNF-α) mainly in the first 24 hours after injury (45). Other important factors include platelet derived growth factor (PDGF) and macrophage colony-stimulating factor (M-CSF), which, together with stromal cell-derived factor 1 (SDF1, CXCL12) contribute to the recruitment of stem cells from the immediate bone environment and from the circulation (44, 45). These stem cells are essential for the next stage of regeneration, the formation of the soft callus. Hypoxic conditions in the haematoma may contribute to the promotion of chondrocyte differentiation from progenitor stem cells, and subsequent cartilage deposition (46, 47). Angiogenesis and blood vessel infiltration controlled by angiopoetin-1 and -2 and by vascular endothelial growth factor (VEGF) increase until hypoxic conditions begin to resolve (45). Improved circulation, as well as the activation of M-CSF, receptor activator of nuclear factor kappa B ligand (RANKL) and TNF-α, stimulate chondroclastogenesis and cartilage mineralization (48). The resolution of hypoxic conditions is followed by osteoblast proliferation and differentiation, leading to the deposition of woven bone. Cytokines such as transforming growth factors β2 and 3 (TGF-β) and bone morphogenetic proteins (BMP) -2, -5, and -6 exert control over the healing process by

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supporting continued proliferation, differentiation, and activity of osteoblasts, as well as the long term remodelling and restoration of woven bone into lamellar, functional bone (45, 49). The cell types and processes involved in bone repair are shown in Figure 1.

The role of several mediators and signalling pathways in bone repair (e.g., BMPs, VEGF, Wnt and Notch pathways) and therapeutic attempts at harnessing them to improve bone repair have been the subject of various reviews (4, 41, 50-52). Less attention has been paid to the role of S1P signalling in bone disorders and repair (53). This review will therefore summarise the key findings in this field, with emphasis on the effects of S1P on the migration, differentiation and survival of the cellular components of bone repair and their respective precursors. In addition to the well-known role of S1P in vascularization and immune cell trafficking, these effects are likely to underlie any observed improvement in repair of bone defects following pharmacological intervention targeting S1P signalling.

## 3 S1P effect on progenitor stem cells

After injury, bone healing relies not only on differentiated bone cells but also on the recruitment of undifferentiated cells from bone and adjacent tissues. S1P regulates cell trafficking through surface receptors that respond to the S1P gradient between tissues (where S1P is found in nanomolar concentrations) and the blood (where it is found at micromolar concentrations), a gradient which may arise due to high levels of S1P degrading enzymes in the tissue compared to the blood (54). In general S1P functions as a chemoattractant for quiescent stem cell populations (55), and also participates in their differentiation into specialist bone forming and bone resorbing cells, as will be explored in more detail in the forthcoming sections.

### 3.1 *S1P and stem cell migration*

The balance between the major chemo-attractants CXCL12 (also known as SDF-1), predominantly found in bone marrow, and S1P, mainly found in the blood, dynamically regulates haematopoietic stem cell recruitment to the circulation versus their retention in the bone marrow. The principal chemoattractant retaining progenitor stem cells in a quiescent state in the bone marrow is CXCL12. Dissipating the S1P gradient between the blood and bone marrow by inhibiting S1P degradation in tissues or downregulating stem cell $S1P_1$ receptors using fingolimod both reduce the number of circulating progenitor stem cells (56). The $S1P_3$ receptor has been shown to have the reverse effect, whereby $S1P_3$ agonism stimulates CXCL12-based retention of haematopoietic stem

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cells within the bone marrow, and $S1P_3$ antagonism contributes to increased stem cell egress (57). Stress, such as that occurring in a fractured bone, induces the downregulation of CXCL12 in the bone marrow and an increase in circulating S1P levels, leading to stem cell mobilization and migration into the blood stream (58). These observations support a role for S1P in the exit of cells from the bone marrow, a finding reminiscent of S1P-mediated lymphocyte egress from lymph nodes (22). Therefore, by manipulating S1P levels in the local environment of a tissue injury site, it may be possible to draw more of the local progenitor resources into the repair process.

S1P-treated stromal cells show increased expression of extracellular matrix protease (e.g., MMP1) (59), which are important in breaking down collagen during the cell migration process (60). S1P also induces stromal cell migration and formation of capillary-like structures (59) and Rho-dependent formation of stress fibres, followed by lamellipodia and filopodia, in bone marrow derived cells. MMP or MEK1-ERK1/2 inhibition reduces S1P-induced actin stress fibre formation, with no impact on lamellipodia or filopodia. MMP inhibition also interferes with S1P activation of RhoA and ERK, while Rho kinase blockage produces sustained S1P activation of ERK. This shows the intricate interplay downstream of S1P stimulation in the pathways involved in cell migration (61).

Medium conditioned by RANKL-differentiated bone marrow cells contains S1P that stimulates chemotaxis of mesenchymal stem cells (MSC) (62). Two parallel signalling pathways seem to be involved in this MSC migratory response: $S1P_1$ receptors activating the JAK/STAT pathway and $S1P_2$ receptors activating the FAK/PI3K/AKT pathway (62). Contrasting with these findings, a recent study showed that $S1P_2$ receptors played a critical role in the inhibition of MSC migration through ERK phosphorylation (63), an effect more in line with the more commonly observed inhibition of migration by $S1P_2$ receptors (64). Confirming the effects of S1P signalling on the recruitment of endogenous stem cells, exposure of bone marrow derived MSCs to the S1P agonist fingolimod released from biodegradable polymer scaffolds enhanced MSC migration toward CXCL12 (65), but the pharmacological profile of this response was not assessed. In these experiments fingolimod also led to cellular mineralization, an indicator of differentiation into the osteoblast lineage, and promoted vascularization (65).

### 3.2 *S1P and stem cell differentiation*

MSCs can differentiate into osteoblasts and adipocytes; commitment to one lineage inhibits commitment to the other due to the existence of negative feedback loops. S1P reduced adipogenic differentiation in MSCs (66) and

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increased their differentiation into osteoblasts as shown by increases in alkaline phosphatase and osteocalcin mRNAs, and the appearance of calcified deposits (66). While the MSC cell line expressed both $S1P_1$ and $S1P_2$ receptors, the inhibition of C/EBPβ expression by S1P was sensitive to pertussis toxin, suggesting that $S1P_1$ receptors played a key role (66). A recent study further defined the nature of the Wnt pathway involved in S1P-induced osteogenic differentiation of MSCs, implicating the Wnt5a ligand and LRP5/6 receptor (67). In another study, S1P-functionalized titanium oxide coated stainless steel used as a growth substrate for human adipose derived stem cells also fostered their osteogenic differentiation (68). Both the $S1P_{1/3}$ receptor antagonist VPC23019 and blocking of BMP6 with a neutralising antibody, polyclonal IgG reduced the mineralization response of human MSC to osteoclast-conditioned media, and similarly interferes with MSC migration. Indicating that osteoclasts and associated S1P release (among other osteoblast-osteoclast coupling factors) stimulate MSC differentiation and migration (69).

## 4 S1P and osteoblasts

### 4.1 *Proliferative effect*

Short (10-45 min) but not protracted (24 hr) treatment with S1P induces ERK-dependent proliferation of both rat and human osteoblasts (70, 71). This time dependence has been tentatively explained by the possibility that S1P might first induce an early phase of cell growth, but, upon longer stimulation, lead to a phase of differentiation in which proliferation stops. Alternatively, the differential increase in the PKCα isoform following short- vs. long-term exposure to S1P might also have played a role (71). This possibility is supported by the observation that, in response to a 10-minute S1P stimulation, PKCα immunoreactivity was redistributed from the cytosol to the nucleus (72). Osteoblasts are known to express $S1P_1$, $S1P_2$ and $S1P_3$ receptors (18-20), but none of the studies mentioned above addressed the identity of the receptor involved in the proliferation response; while pertussis toxin sensitivity pointed to an $S1P_1$-mediated effect (71), the S1P concentration used (10 μM) was higher than usually needed to activate S1P receptors. A more recent study reported increased DNA synthesis at S1P concentrations of 1 μM (18); S1P induced activation of p42/44 MAP kinases, in a Gi- and calcium-dependent manner, but independently of PKC, and proliferation was observed in response to 24-hour S1P treatment. When the effects of S1P were studied in human primary osteoblastic cells and the human osteosarcomal cell lines, G292 and MG-63, 10 minute incubations with 10 nM S1P increased proliferation in a pertussis toxin-sensitive manner, while the effect of 24-hr incubation were less consistent. In G292 cells, this

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longer exposure produced significant increases only with subnanomolar S1P, while higher doses had no effects; no proliferation was observed at any concentration in the other cell types (73). Both proliferation and apoptosis control the number of osteoblasts, and Gi proteins are not only involved in S1P-induced osteoblast proliferation but also in their survival. However, the role of PI3K appears to be restricted to the latter effect, since PI3K inhibition does not prevent the proliferative actions of S1P in osteoblastic cells (74).

### 4.2 *Osteoblast differentiation*

Differentiation of osteoblast precursors into mature osteoblasts is accompanied by an increase in SK 1 expression and enzyme activity, decreased levels of $S1P_1$ and $S1P_2$ receptor proteins, and increased levels of $S1P_3$ receptor proteins (75). Sphingosine kinase inhibitor (SKI-II), an anti-S1P antibody and the $S1P_{1/3}$ receptor antagonist VPC23019 all reduce alkaline phosphatase activity, while blocking $S1P_1$ receptors with W146, or $S1P_2$ receptors with JTE013, has no effect (75). A similar pharmacological profile was observed with RUNX2 expression (a key transcription factor associated with osteoblast differentiation), suggesting the existence of an autocrine SK1/S1P/$S1P_3$ signalling pathway during osteoblastic differentiation (75).

Other S1P receptors and signalling pathways may also mediate osteoblastogenesis. Activation of S1P receptors in C2C12 myoblasts enhanced BMP-2-induced expression markers of osteoblast differentiation (76). The expression of RUNX2 was likewise increased in the presence of S1P or fingolimod, as were Smad transcription factors and ERK1/2 (76). S1P and fingolimod also enhanced BMP-2-stimulated Smad1/5/8 phosphorylation in C2C12 cells, and cell differentiation was sensitive to Pertussis toxin, to a MEK1/2 inhibitor, to the $S1P_1$ receptor antagonist W146, and, to a smaller extent, to the $S1P_2$ antagonist JTE013, whereas an $S1P_3$ antagonist (CAY10444) had no effect. A similar pharmacological profile was observed for the effects of S1P on other osteoblast-like cell lines (human SaOS-2 and murine MC3T3-E1). In these cells, S1P activated PI3K/Akt signalling, inhibiting GSK-3β, promoting nuclear translocation of β-catenin and expression of osteoprotegerin (that inhibits osteoclastogenesis by acting as a soluble decoy receptor for RANKL), and enhancing ALP activity (77). In a more recent study by the same group, S1P stimulation of Smad1/5/8 phosphorylation was attributed to $S1P_2$-G12/13-RhoA activity, leading to the nuclear translocation of the Smad complex, up-regulation of RUNX2 leading to increased ALP (78). Of note, this (77) and another study (19) found that S1P also increased RANKL mRNA in osteoblasts, but the OPG/RANKL ratio was higher after S1P treatment, which should lead to an overall inhibition of osteoclast maturation (77). Increased SK activity indeed reduces osteoclastogenesis in a

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monoculture of osteoclast precursors; however, in an osteoblast/osteoclast co-culture system, which better reflects the reality of a healing bone, S1P stimulated osteoclastogenesis (19).

As mentioned above, S1P seems to act as a coupling factor between osteoclasts and osteoblasts, and is referred to as a clastokine (79). Osteoclasts lacking the bone degrading enzyme cathepsin K show increased SK 1 expression and culture media conditioned by these cells were shown to induce a larger increase in ALP and mineralized nodules in osteoblast cultures, due to their higher S1P content. This response was blocked by the $S1P_{1/3}$ antagonist VPC23019, in agreement with the studies described above (80).

### 4.3 *Osteoblast precursor migration*

Together with its activity on their proliferation and differentiation (18, 70-78, 80), S1P also affects the migration of osteoblast precursors (81). Treatment of mouse primary pre-osteoblasts with S1P drives cells toward the bone surface environment (81). However, when precursors differentiate into mature osteoblasts, they become insensitive to S1P, although they retain their chemotaxis to PDGF (81). The response to S1P is not sensitive to pertussis toxin, suggesting that a subtype other than $S1P_1$ is involved in the chemorepellent response to S1P. Indeed, expression studies and experiments with JTE-013 or with anti $S1P_2$ siRNA point to a developmental stage specific role of $S1P_2$ receptors. The chemorepellent effect of $S1P_2$ receptors is typical of this subtype in various cell types, whereas $S1P_1$ receptors are associated with chemotaxis to S1P in other cells important for bone repair: MSCs that give rise to cells of the osteoblast lineage (see (62) above), endothelial cells (82) or osteoclasts (see below). The lack of $S1P_1$-mediated positive chemotactic response in osteoblasts, despite high $S1P_1$ expression levels in these cells, is therefore unusual.

### 4.4 *Other effect of S1P signalling in osteoblasts*

S1P has long been known to release calcium from intracellular stores in pre-osteoblasts (83, 84). Because of calcium's central role in cell signalling, it is therefore not surprising that S1P is implicated in many osteoblast functions. Indeed, S1P stimulates IL-6 synthesis in these cells in a p42/p44 MAPK dependent manner (85), induces the synthesis of heat-shock protein 27 (HSP27) via p38 activation (86), and enhances PGF2α-induced phosphoinositide hydrolysis by phospholipase C through p38 MAPK (87, 88).

Administration of epidermal growth factor, a known mitogenic factor for osteoblasts, increased S1P levels which coincided with increased cell proliferation (89). There is also evidence for the involvement of S1P signalling in calcitonin activity (90). Calcitonin is an anti-resorptive hormone previously indicated in

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osteoporosis, however it may also influence bone formation through its interactions in S1P signalling. By decreasing the expression of the S1P transporter Spns2 in osteoclasts (20), limiting the cross-talk between osteoclasts and osteoblasts, and so also limiting S1P- or fingolimod-induced bone formation by osteoblasts which was found to be mediated by $S1P_3$ receptors (20).

S1P may also influence mature osteoblasts following their entombing as osteocytes in the bone matrix, as S1P signalling via the $S1P_2$ receptor has been shown to affect mechanotransduction in an osteocyte-like cell line (MLOY4) (91).

## 5 S1P and osteoclasts

Osteoclasts are multinucleated, resorptive cells whose development is influenced by osteoblast lineage cells (92). Osteoclasts are responsible for the continuous remodelling of bone, working in tandem with bone forming osteoblasts (93). The coupling between osteoclasts and osteoblasts in osteoclastogenesis is a clear example of the functional relationship between the two cell populations, and S1P seems to play a role in the crosstalk between these two cell populations and their differentiation, as represented in Figure 2.

### 5.1 *S1P and osteoclast recruitment*

S1P can regulate the migration of osteoclast precursors both in vitro and in vivo. Bone marrow derived monocytes (an in vitro model of osteoclast precursors) express both $S1P_1$ and $S1P_2$ receptors. Upon exposure to RANKL, these cells differentiate into osteoclast-like cells and show decreased $S1P_1$ expression, with concomitant loss of chemotactic response to S1P (94). Knockout mice with specific $S1P_1$ deletion in the monocyte lineage are osteoporotic, a phenotype that has been attributed to the loss of $S1P_1$ control of osteoclast precursor migration and increased residency time at the bone surface (94). The potential therapeutic significance of these findings was confirmed in an ovariectomy-induced osteoporosis model: fingolimod prevented bone loss in ovariectomized mice, but had no effects in sham-operated mice. This effect was due to a reduction of osteoclast deposition onto bone surfaces (94). In a rat model of periodontitis, fingolimod was found to reduce the number of osteoclast precursors and mature osteoclasts at the defect site, and increase the number of precursors in blood, an effect attributed to $S1P_1$-induced positive chemotaxis (95).

$S1P_2$ receptor deficient mice show higher bone density than control mice (96), and $S1P_2$ receptors seem to antagonize the effect of $S1P_1$ receptors on osteoclast precursor migration. Positive and negative chemotaxis are attributed to $S1P_1$-mediated activation of Rac via Gi, and $S1P_2$-mediated activation of Rho via $G_{12/13}$,

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respectively (96). An *in vitro* migration assay of osteoclast precursors expressing both receptors subtypes showed that lower S1P concentrations stimulate positive chemotaxis, while higher concentrations stimulate negative chemotaxis, or chemorepulsion, suggesting that $S1P_2$ receptors may only be active at high S1P concentrations. $S1P_1$-deficient osteoclast precursor cells show very little motility, while $S1P_2$-deficient cells showed positive chemotaxis, even at high S1P concentration (96). Intravital imaging confirmed the chemotactic effect of $S1P_2$ by showing that the antagonist JTE013 mobilised a small subset of monocytic lineage cells from the calvarium and led them to enter the blood circulation (96).

### 5.2 *Therapeutic manipulation of osteoclast trafficking*

While approved or investigational anti-resorptive agents (e.g., bisphosphonate or cathepsin K inhibitors) target mature osteoclasts, manipulating osteoclast precursors would provide a novel therapeutic modality for bone loss. Indeed, the opposing roles of $S1P_1$ and $S1P_2$ receptors on precursor recruitment might underlie therapeutic interventions (i.e., activation of $S1P_1$ or blockade of $S1P_2$ receptors) that could prevent bone loss in conditions associated with inflammation and/or remodelling imbalance. This potential was ascertained using murine models of rheumatoid arthritis (in which fingolimod was as effective as prednisolone) and osteoporosis (fingolimod improved bone loss, but prednisolone had no effect) (97). In a model of periodontitis, a bacteria-driven inflammatory bone loss disease, fingolimod inhibited osteoclastogenesis and pro-inflammatory cytokines involved in osteoclast precursor recruitment (98).

Vitamin D analogues are used for the treatment of osteoporosis, but their mechanism of action is not completely clear. For instance, *in vitro* calcitriol increased RANKL expression in bone marrow stromal cells, thereby activating osteoclasts and bone resorption (99). A recent study showed that vitamin D's effect on osteoclast precursor migration might underlie its anti-resorptive activity. Indeed, calcitriol and its analogue eldecalcitol were found to uniquely reduce $S1P_2$ receptor expression in monocytic osteoclast precursors (99), while circulating monocytes expressed fewer $S1P_2$ receptors in mice treated with calcitriol or eldecalcitol, and monocyte mobility was observed to increase in eldecalcitol-treated mice after treatment with JTE013 (99).

Whereas vitamin D analogues reduce $S1P_2$ receptor expression, a recent study showed that the inflammatory cytokine IL-6 induced $S1P_2$ mRNA, but not $S1P_1$ mRNA expression in osteoclast precursor cells (100). This effect was associated with a decrease in S1P-induced chemotaxis and an increased number of precursors in tibial bone marrow. Systemic treatment with an anti-IL-6 receptor antibody prevented bone loss

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and decreased the number of precursors in tibial bone marrow via $S1P_2$ receptor down-regulation (100), further validating the potential therapeutic value of $S1P_2$ antagonists.

The following table summarises some of the effects of S1P receptors on the cellular components of bone repair.

## 6 S1P in the vasculature and the role of angiogenesis

The repair of cranial bone defects by scaffold-mediated delivery of S1P agents involves not only the recruitment of bone cell progenitors, but also production of new vessels in the defect space (105, 106). Hence, while the previous sections focused on bone cells and their interactions, it is important to remember that bones are highly vascularized, perfused by up to 20ml of blood/100g of bone every minute (107). Blood vessels are not only an essential conduit for blood, providing minerals, nutrients, growth factors and osteoprogenitors, but the endothelium also acts as a paracrine and endocrine organ involved in growth factor production, coagulation, inflammation and the immune response (108). Fracture disrupts the bone's vasculature, leading to hypoxia and necrosis of adjacent tissue. Reestablishment of the circulation and neovascularization in the tissue formed in response to injury are critical for successful fracture healing (109). Unfortunately, bone repair strategies based on bone grafts or scaffolds have so far shown limited success due in part to the lack sufficient blood vessel supply during the early stages of the repair process (20, 21).

There are three main mechanisms for producing new vessels (110). Vasculogenesis refers to the *de novo* generation of blood vessels that occurs for instance during embryogenesis. It differs from angiogenesis, which is the generation of new vessels *from pre-existing ones*. Angiogenesis occurs during physiological (e.g., wound healing or menstrual cycle) or pathological processes (e.g., neovascular disorders, rheumatoid arthritis and cancer). It can result from the formation of a new vessel branching off an existing vessel (sprouting angiogenesis) or from the splitting of a blood vessel into two or more vessels (intussusceptive angiogenesis). Finally, arteriogenesis is the remodelling of an existing artery to increase its luminal diameter. While arteriogenesis, and possibly angiogenesis (111-113), occurs in response to physical forces such as increased blood flow, angiogenesis is initiated in poorly perfused tissues when low oxygen levels lead to increased levels of the transcription factor Hypoxia-Inducible Factor (HIF)-1α in parenchymal cells.

VEGF is the main HIF-1α–dependent pro-angiogenic factor, and inhibiting VEGF signalling impairs healing of femoral fractures and cortical bone defects in mice (114). Although VEGF is the archetypical pro-angiogenic factor, it promotes by itself the formation of immature and leaky vessels (115). In contrast, angiopoetin-1

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produces vessels that are resistant to leak (116), suggesting that different vascular growth factors play complementary and coordinated roles in new vessel formation, and that therapeutic strategies aimed at promoting angiogenesis should target more than one mediator. Indeed, when surgically implanted in the ear of mice, chemically modified hyaluronan hydrogels pre-loaded with both VEGF and angiopoetin-1 promote a larger angiogenic response than delivery of single growth factors (117). More recently, sequential delivery of VEGF and S1P using a porous hollow fibre in a skin Matrigel plug assay was shown to lead to more endothelial cell recruitment and a higher maturation index than single factor delivery, reverse sequential delivery or even co-delivery (118). The concept that temporal control of growth factor release produces more mature new vessels, able to integrate with the existing vasculature, was validated in similar experiments using Basic Fibroblast Growth Factor and Platelet-Derived Growth Factor (119).

These sequential release experiments were conducted over the course of a week, but the bone repair process takes months. Scaffold-mediated delivery of a low molecular weight, more lipophilic factor such as an S1P agent might be preferable to the delivery of recombinant proteins. The role of S1P in the vasculature and new vessel formation is well documented and has been the subject of numerous reviews (120-122). Endothelial cells express the same S1P receptor subtypes as intrinsic bone cells ($S1P_1$>$S1P_2$≈$S1P_3$); these receptors mediate generally similar cellular responses (proliferation, differentiation and migration), in addition to effects more specific to endothelial cells (modulation of cell adhesion and of the inflammatory/immune response).

S1P seems to play a key role in both vasculogenesis and angiogenesis. In a mouse hind limb ischemia model, S1P stimulates angiogenesis (123), while postischemic blood flow recovery and angiogenesis are accelerated in transgenic mice overexpressing SK1 (124). At variance with the effects of VEGF however, the angiogenic response to S1P is not associated with increased vascular permeability in the ischemic limb, and many studies have shown that S1P actually enhances endothelial barrier integrity (120). In fact, in this model, S1P-containing Poly(lactic-co-glycolic-acid) (PLGA) microparticles not only stimulated post-ischemic angiogenesis at 28 days but also blocked edema induced when VEGF was co-administered (125). The effects of $S1P_1$ and $S1P_3$ receptors on adherens junctions in endothelial cells were documented soon after the identification of these receptors (126). While $S1P_1$ and $S1P_3$ receptors strengthen the formation of endothelial cell junctions (28, 127-129), $S1P_2$ receptors increase vascular permeability *in vitro* via disruption of adherens junctions (130, 131). In vivo, $S1P_1$ receptor activation inhibit VEGF-induced vascular leakage in skin capillaries (132), whereas $S1P_1$ receptor antagonists have shown that they induce capillary leakage in the lung, kidney, skin, and intestine (133-135).

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$S1P_1$ receptors promote vascular stabilization by regulating the interactions between endothelial and mural cells during the maturation process (136, 137), and, in apparent contradiction with their pro-angiogenic effects mentioned above, $S1P_1$ receptors were recently shown to inhibit sprouting angiogenesis during vascular development (138), by stabilizing VE-cadherin at endothelial junctions and inhibiting VEGFR2 (111, 112), suggesting the existence of an alternative mechanism that helps stabilize the newly formed vascular network and improves its barrier function.

These data showing that S1P plays a role both at the early stages of angiogenesis and at the stage of new vessel stabilization, taken together with the effects of this lipid on bone cells, suggest that scaffold-mediated delivery of S1P (most likely $S1P_1$) agonists might promote bone repair via pleiotropic and possible synergistic mechanisms.

## 7 Current efforts in S1P delivery

The importance of S1P as a chemoattractant, and in coupling the activity of osteoblasts and osteoclasts suggests it could be utilized systemically in bone repair, and in disorders such as osteoporosis (53). However, a study of daily subcutaneous fingolimod (6mg/kg) did not lead to any improvement in fracture healing of a murine femoral defect (139), indicating that a more localised approach of delivering S1P and related analogue, may lead to more promising results.

Local administration of S1P has typically involved the use of scaffolds, which often have the dual role of acting as drug delivery device, and mimicking native tissue to elicit functional tissue development. Hence a range of biocompatible materials, including natural polymers (collagen, chitosan, silk), synthetic organic polymers PLGA and poly-ε-caprolactone [PCL]) and inorganic materials (ceramics and glasses) have been investigated to fabricate scaffolds that are conducive to tissue regeneration, and allow temporal control over the release of therapeutic cargoes (140). Biodegradable PLGA is among the commonest copolymers investigated (141) and has been used to control the release of S1P (105) and fingolimod (142), resulting in increased new bone formation post-implantation in a rat cranial defect model, an effect that was attributed to increased development of vasculature and the possible dose-dependent initiation of bone progenitor cell migration towards the defect site (142). The underlying mechanism was probed in a similar study investigating the delivery of S1P agonists and antagonists (S1P, fingolimod or VPC01091) from PLGA scaffold implants in a rat cranial defect model (106). Although S1P is subject to much more rapid *in vivo* degradation than fingolimod, scaffolds loaded with either agonist were equally effective in generating new bone over 6 weeks, while VPC01091-loaded scaffolds

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did not differ from unloaded controls (106). This study suggests that sustained release from scaffolds may offset the challenges of employing therapeutic cargoes (e.g. S1P) with short half-lives, and that $S1P_3$ receptors synergize with $S1P_1$ receptors to influence the various processes underlying repair (i.e., vascular remodelling, cell proliferation and migration, inflammation), albeit to differing extents. fingolimod has been incorporated into electrospun nanofibers composed of PLGA and biodegradable PCL and showed significant improvement in defect healing and vascularization in a rat critical mandibular defect (143). These fingolimod-loaded nanofibers increased neovascularization and enhanced the proportion of macrophages with an anti-inflammatory phenotype (M2) (143), a cell population that is also known to play an important role in tissue repair (144), and had been previously shown to be selectively attracted by fingolimod (145). A similar result of anti-inflammatory macrophage stimulation was found in another study using a PLGA coated allograft (146), and whilst SEW2871 was also observed to stimulate macrophage recruitment, details regarding phenotype were not reported (147). An electrospun amphiphilic copolymer was developed to act as a carrier for S1P to promote vascularization in tissue repair applications, the amphiphilic nature of the copolymer was anticipated to mimic the binding of S1P to apolipoprotein M. S1P was first applied directly to endothelial cells (HUVEC), and showed pro-angiogenic effects in a tube formation assay. Tube length and uniformity were then improved when S1P was administered as part of the amphiphilic scaffold, additional evidence of new vessel formation was shown in a 3-day chorioallantoic membrane assay (148).

Whether small molecule delivery alone will achieve sufficient and effective bone repair remains to be established, but it is worth noting that fingolimod PLGA microspheres in a chitosan gel improved bone regeneration in a rat cranial defect study, with no substantial improvement upon addition of BMP-2 to fingolimod-loaded microspheres (65), despite fingolimod being known to enhance BMP-2 mediated osteoblast differentiation *in vitro* (76). Conversely, SEW2871 alone failed to improve bone regeneration, but co-administration with platelet rich plasma improved the latter's performance, by enhancing macrophage recruitment and cell debris clearance (149). Combining S1P with low-cost, biocompatible, biodegradable polymers represents an enticing alternative prospect for current bone graft treatments. Unfortunately, results to date still show most polymeric biomaterials cannot match the efficacy of bone grafts, because they lack both the osteogenic and osteoinductive properties that make grafts so successful. Consequently, bioactive polymer-graft composites are a potential solution to recapitulate mechanical and biological properties of host tissue in an effort to repair critical-sized defects. In one case, fingolimod elution from a PLGA-coated devitalized-bone allograft in a critical rat tibial defect improved elastic modulus and ultimate compressive strength of the bone, outcomes

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attributed to evidence of enhanced active remodelling at the defect site (150). The same procedure was investigated further, and similarly attributed tissue regeneration to improved vascularization, while also presenting a more detailed discussion of the role of bone marrow derived cells in immune modulation (146). Another PLGA coated allograft delivery system for fingolimod showed a dose-dependent increase in bone volume in a cranial defect model at 2 and 4 weeks. Although differences in bone volumes were no longer significant at 8 weeks, fingolimod still enhanced host-graft integration at this time point (151). Notably, direct adsorption of fingolimod onto implanted allograft improved bone deposition and vascularisation (152). Predictably, this method produced higher local concentrations of fingolimod, but lower increases in bone density compared to polymer based delivery discussed above (151, 152).

## 8 Conclusion

Although the role of S1P in bone biology has been the focus of much less research than its role in the cardiovascular and immune systems, it is becoming clear that this lipid influences many of the functions, pathways and cell types that play a key role in bone repair. Indeed, S1P has a well-established role in promoting angiogenesis (14, 105, 148, 153, 154), but is also implicated in many other bone related processes including stem cell recruitment (59, 62, 155) and subsequent differentiation (66). S1P stimulates the differentiation and survival of osteoblasts (76, 77), and contributes to their intricate coupling with osteoclasts (19). S1P is not only a key factor in its own right, it also seems to mediate the functions of critical bone growth factors, such as BMPs (69, 76). Although the use of growth factors for bone repair has been widely explored, some issues remain, such as those related to supra-physiologic doses (156), short half-lives (157), an inability to maintain osteogenicity due to slow vascular integration of grafts (2), not to mention high costs (158). As summarized in earlier sections, various groups have therefore begun to explore the use of non peptidic agents, such as S1P and analogues, to promote bone repair in vivo, with generally promising results. Remaining issues regarding pleiotropic activity (159), solubility (147) and the need to maintain local concentrations over a number of weeks (159) may be addressed by using more specific agents and/or novel delivery options. A number of such delivery methods have been studied in the field of bone repair to enhance delivery of growth factors (158, 160, 161), small molecule drugs, and stem cell therapies (48, 162, 163). They have generally involved biomaterials for controlled release of drugs including biocompatible, biodegradable polymers, and bio-ceramics (4, 163) and the use of high affinity delivery systems, which have led to reductions in required doses (5).

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The use of S1P agents for bone repair is likely to be greatly accelerated by the much more active translational and clinical research of the role of S1P signalling in other fields, such as inflammation or cancer. The number of active clinical trials involving S1P receptor ligands in inflammatory conditions ranges from 2 and 3% of trials for inflammatory bowel disease and psoriasis, up to 32% of all trials for new multiple sclerosis therapies (164). $S1P_1$ receptors have been the focus of most research in this field, as evidenced by the great emphasis placed on the development of agents such as ponesimod, siponimod, and ozanimod, with improved specificity compared to fingolimod. Whilst other possible targets, such as S1P lyase inhibition have been less well investigated (165). In the field of bone repair, further basic and translational research will be needed to better define which S1P metabolic enzymes or receptors should be targeted, when and for what duration, and whether an agonist or an antagonist would be preferable. The latter issue is particularly critical considering that $S1P_1$ receptor agonists seem to exert their action as functional antagonists, with $S1P_1$ agonists and antagonists showing similar therapeutic effects (166). Furthermore, some of the work quoted in this review has been based on qualitative or semi-quantitative data, and the pharmacological profile of the response was sometimes unclear, either due to incomplete dose response studies, or the use of agents with questionable specificity (29, 35) .

To conclude, the manipulation of S1P signalling using systemic administration of therapeutic agents seems promising for the management of inflammatory or hormonally-related bone loss, as S1P agents can be used to affect osteoblast/osteoclast coupling, the unbalancing of which manifests as conditions such as osteoporosis. In contrast, local administration of S1P agents has shown more compelling results in bone defect studies, and so improving local delivery of these agents will be key to optimising their regenerative potential. Critically, this may be achieved by not only increasing the recruitment of osteogenic cell precursors but also by inducing and supporting vascularization and modulating the immune response; S1P agents may be unique in that they are known to possess all three activities (106, 142, 143, 146, 149-152).

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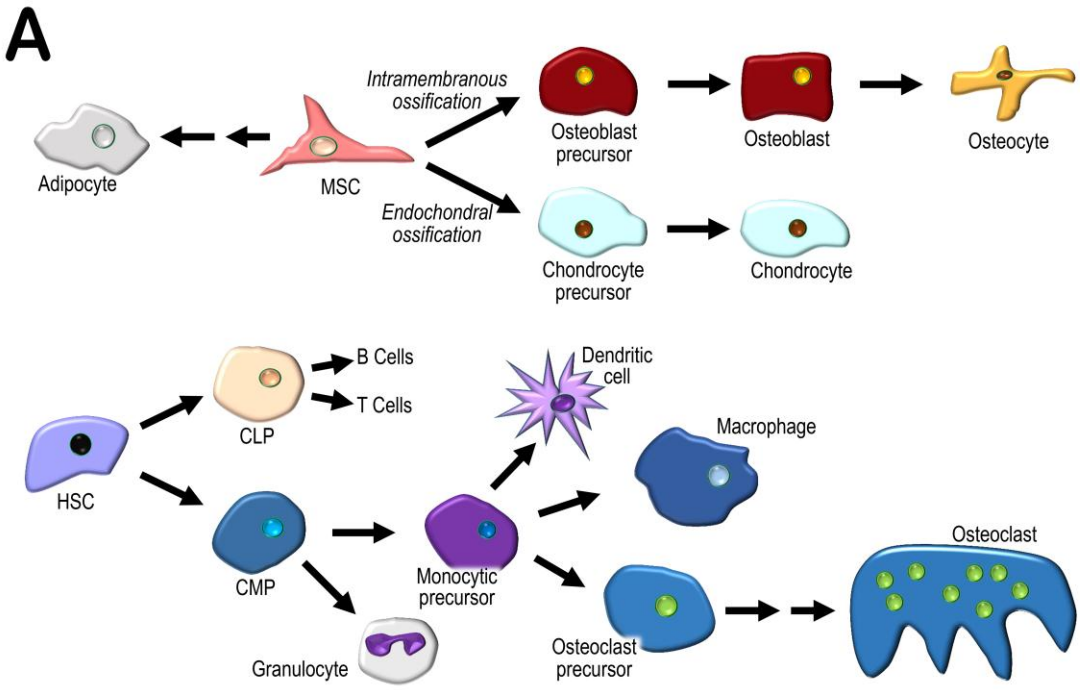
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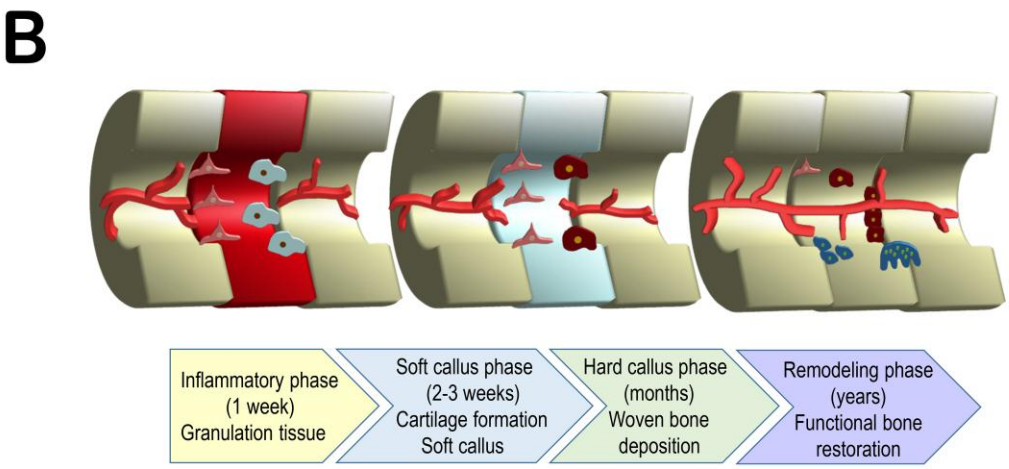
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***Fig. 1*** *(a) A simplified representation of the lineages of the cells involved in bone repair. Mesenchymal stem cells (MSC) differentiate into the major bone and cartilage forming cells, osteoblasts and chondrocytes (later replaced by osteoblasts), depending on whether ossification occurs through the intramembranous or endochondral pathways. Haematopoietic stem cells (HSC) differentiate into bone resorbing osteoclasts through the myeloid pathway. (b) Process of bone repair divided into 4 phases: inflammatory, soft callus, hard callus, and remodelling. Briefly, an early inflammatory response results in the removal of debris and the eventual recruitment of mesenchymal stem cells, initiating the soft callus phase and cartilage deposition. Improving vascularization leads to cartilage mineralization and deposition of bone, which is then slowly remodelled, restoring function.*

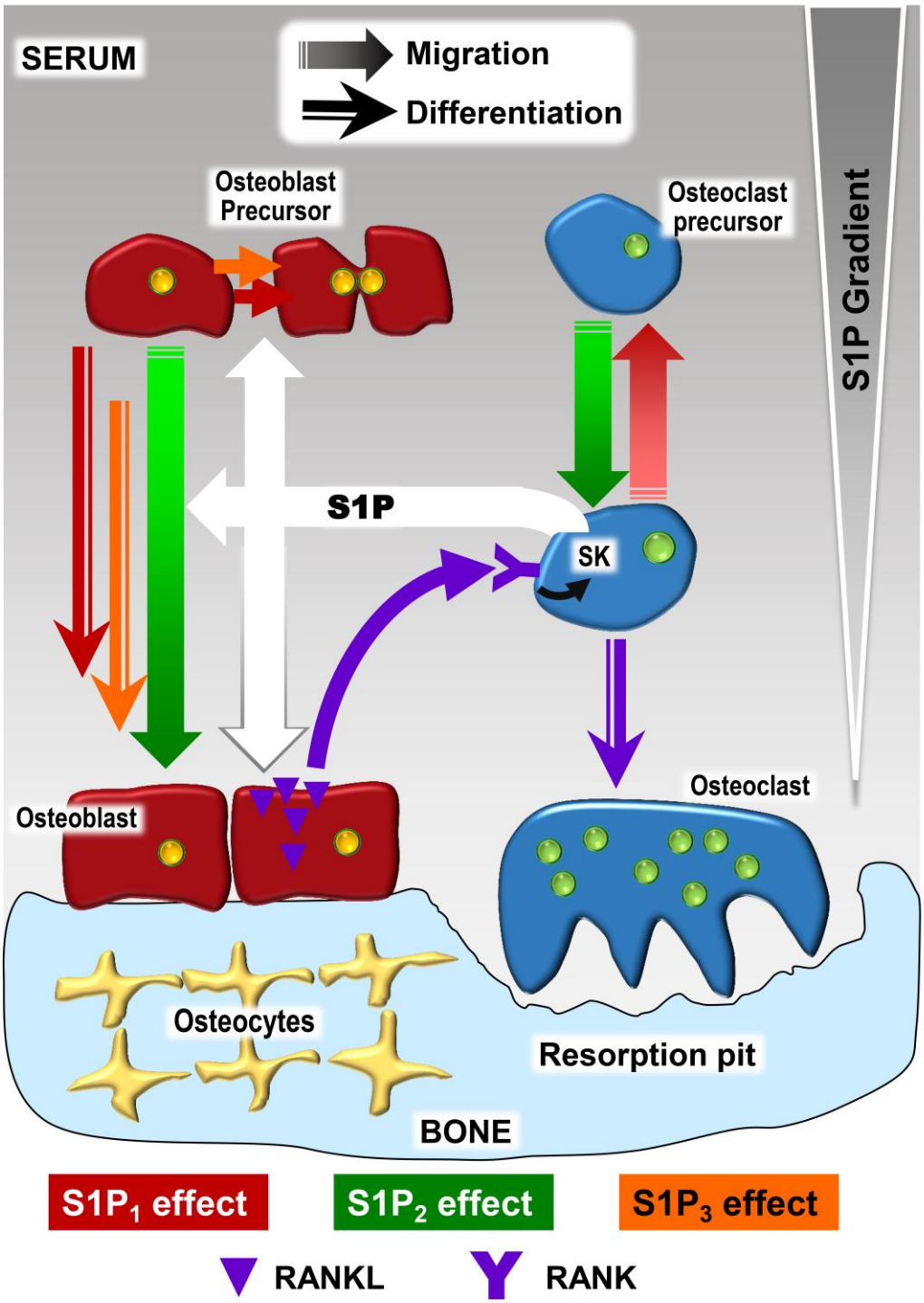
***Fig. 2*** *Simplified illustration of the effects of S1P and its receptors on osteoblasts, osteoclasts, their respective precursors, and the role of S1P in osteoblast-osteoclast coupling. The involvement of the 3 major S1P receptor subtypes (red: $S1P_1$, green: $S1P_2$, orange: $S1P_3$) in particular responses is indicated by different arrow shapes. Briefly, osteoclast and osteoblast precursor migration is influenced by $S1P_1$-mediated chemoattraction and $S1P_2$-mediated chemorepulsion in response to the the S1P concentration gradient (larger quantities of S1P are generated in serum mainly by red blood cells and endothelial cells, while lower S1P concentrations predominate in tissue compartments, such as bone). S1P, produced locally by osteoclasts or osteoclast precursors (20, 69, 80), directly stimulates the proliferation of osteoblast precursors and their differentiation into mature osteoblasts, while increasing RANKL mRNA in osteoblasts, indirectly stimulating osteoclast precursor differentiation via RANK. The RANKL/RANK signalling pathway also upregulates SK in osteoclast precursors.*

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*Table 1. List of S1P associated agents mentioned in the review. Of note, many of these agents only show subtype selectivity with a narrow range of concentrations, and have known non S1P receptor targets (for review see ((29))).*

| AGENT | SELECTIVITY/SPECIFICITY | NOTES |
|---|---|---|
| **S1P** | $S1P_{1-5}$ Agonist | Endogenous agonist |
| **FINGOLIMOD** | Activates all S1P subtypes except $S1P_2$, although recent evidence suggests $S1P_2$ might also be a target (32). | Fingolimod is a prodrug (activated by sphingosine kinase 2). Phosphorylated fingolimod is likely to act as a functional antagonist of $S1P_1$ in its approved therapeutic role, as it rapidly downregulates $S1P_1$ receptors. The extent and the kinetics of fingolimod-induced receptor internalization and of their recycling to the cell membrane seem to differ between various S1P receptor subtypes. Furthermore, the extent of receptor downregulation may also depend on fingolimod concentration, the concentration of endogenous S1P and the level of S1P receptor expression, possibly explaining why the functional effects of fingolimod in various systems can either resemble the effects of agonists or of antagonists (30).<br>It is also a potent protein phosphatase 2A (PP2A)–activating drug. Effects of sphingosine kinases and S1P lyase have also been shown. |
| **SEW2871** | $S1P_1$ Agonist | First described $S1P_1$-selective agonist. At variance with fingolimod, it demonstrates $S1P_1$ agonist activity without long-term decrease in surface receptor expression (33). It is 10 to 50 times less potent than CYM5442 and poorly water-soluble (34). |
| **JTE013** | $S1P_2$ Antagonist | Most commonly used $S1P_2$ receptor antagonist, but its selectivity is questionable (35). |

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| | | |
|---|---|---|
| **VPC23019** | $S1P_1$, $S1P_3$ Antagonist | pKB values of 7.5 and 6.0 for $S1P_1$ and $S1P_3$ receptors, respectively (36). |
| **VPC01091** | $S1P_1$ partial agonist, $S1P_3$ antagonist | The 1R,3S diastereomer is a conformationally constrained fingolimod analogue activated by sphingosine kinase 2 (37). |
| **W146** | $S1P_1$ Antagonist | W146 is an antagonist, but its *in vivo* effect often mimic those of S1P receptor agonists (38). |
| **CAY10444** | $S1P_3$ Antagonist | Also known as BML-241. Low potency and aqueous solubility agent. May also non-selectively inhibit increases in intracellular $[Ca^{2+}]$ (39). |

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*Table 2. Cell types involved in bone regeneration and some S1P receptor related effects.*

| CELL TYPE | AGENT | RECEPTOR | STUDY | EFFECT | REF. |
|---|---|---|---|---|---|
| **OSTEOBLAST CELL MODELS** | S1P as part of osteoclast conditioned medium | $S1P_{1/3}$ involvement determined using VPC23019 (2µM and 10µM) | Murine long bone osteoblasts cultured in osteoclast conditioned medium | ↑ALP<br>↑Mineralization | (80) |
| | S1P (1-30µM) | No S1P receptors were investigated | MC3T3-E1 cell line, treated with 1-30µM S1P, media contained 0.01% bovine serum albumin | ↑IL-6 | (85) |
| | S1P, various doses ranging from 1nM to 10µM | $S1P_1$ as determined by pertussis toxin (73). The remaining articles do not identify individual receptors. | Human osteoblast explant (71-73), Foetal rat osteoblasts (18, 70, 74), SaOS2 cell line (18), thymidine incorporation proliferation assays | ↑Proliferation | (18, 70-74) |
| | S1P (100nM) added to top and/or bottom compartments | $S1P_2$ as determined using pertussis toxin (200ng/mL), | MC3T3-E1 cell line, migration assay for PDGF and S1P pre-and post-differentiation | Negative chemotaxis | (81) |

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| | | | | | |
|---|---|---|---|---|---|
| | of migration chamber | JTE013 ($10^{-8}$-$10^{-5}$M), and RNA interference | | | |
| | Endogenous S1P | $S1P_3$ as determined using W146, JTE013, and VPC23019 (All 2µM) | MC3T3-E1 cell line cultured in osteoblast differentiation media, contained 10% serum | ↑Maturation | (75) |
| | S1P (0.01-0.1µM) or fingolimod* (0.01-0.1µM) | $S1P_1$ as determined by the pertussis toxin (100ng/mL), W146, JTE013, and CAY10444 (All 10µM) | C2C12 murine osteoblast precursor cultured in media containing 10% serum. S1P and fingolimod used supplementary to BMP-2 | ↑ALP (↑↑*)<br>↑Osteocalcin (↑↑*)<br>↑RUNX2(↑*) | (76) |
| | S1P (0.1-2µM) | $S1P_1$ as determined using W146, JTE013, and CAY10444 (BML-241) | Human SaOS2 and murine MC3T3-E1 cell lines, cultured in media containing 10% serum | ↑ALP<br>↑Mineralization<br>↑Osteoprotegerin<br>↑RANKL mRNA<br>Nuclear localization of β-catenin | (77) |
| **OSTEOCLAST CELL MODELS** | S1P (0.1µM and 1µM) | No receptors were investigated | Osteoclast from minced rabbit bones incubated on dentine slices. Treated for 16 hours with S1P in media containing 10% serum | ↓ Resorption | (101) |

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| S1P ($10^{-10}$-$10^{-7}$) | $S1P_1$ as determined by osteoclast lineage specific conditional $S1P_1$ knockout | Murine monocyte cell line migration assay<br>Cells cultured in media containing 10% serum | Positive chemotaxis | (94, 96) |
| fingolimod (3mg/Kg) intraperitoneal injection | $S1P_1$ determined from $S1P_1$ knockout osteoclasts collected from transgenic mice | Murine model of osteoporosis | ↓Bone density loss<br>Positive chemotaxis | (94, 97) |
| $S1P_2$ receptor deficiency or blockade | $S1P_2$ as determined *in vitro* by targeting with RNA interference.<br>And *in vivo* by use of JTE013 3mg/Kg | *In vitro* and *in vivo* investigation of the role of $S1P_2$ in the migration of osteoclast precursors | Osteopetrosis<br>↑Bone density<br>↓Negative chemotaxis (osteoclast precursors remain in circulation) | (96) |
| fingolimod (3mg/Kg/Day) intraperitoneal injections | $S1P_1$ as determined using immunohistochemistry and an anti-$S1P_1$ receptor antibody | Rat model of periodontitis | Positive chemotaxis | (95) |

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| | Calcitriol and eldecalcitol (*In vitro*: $10^{-9}$-$10^{-8}$M<br>*In vivo*: 50ng/Kg) effect on S1P ($10^{-6}$M) chemotaxis | $S1P_2$ receptor expression as determined by PCR | Monocytoid cell line migration assay<br>*In vivo* osteoporosis model | ↓$S1P_2$ receptor expression<br>Positive chemotaxis<br>↑Bone mineral density | (99) |
| | IL-6 (1-10ng/mL) effect on S1P ($10^{-7}$M) chemotaxis | $S1P_2$ receptor expression as determined by PCR | Murine osteoclast precursors cultured in media containing fatty-acid free bovine serum albumin, migration assay.<br>*In vivo* arthritis model | ↑$S1P_2$ Receptor expression<br>Negative chemotaxis<br>↓Bone volume | (100) |
| **MESENCHYMAL STEM CELLS** | S1P (1µM) | No receptors were investigated | Murine bone marrow stromal cells cultured in 10% inactivated serum | ↑Stress fibre formation<br>↑Migration | (61) |
| | S1P as part of murine osteoclast conditioned medium | $S1P_1$ as determined using VPC23019 (1µM), without any discussion of $S1P_3$ antagonism | Human mesenchymal stem cells cultured in media containing 10% serum and 10-fold concentrated conditioned media | ↑Mineralization<br>↑Migration | (69) |
| | S1P as part of osteoclast conditioned | $S1P_{1/2}$ as determined using | Human bone marrow derived MSCs, cultured in | ↑Migration (Both $S1P_{1/2}$ led to increased migration | (62) |

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| | medium, and $S1P_1$ agonist VPC24191 (5μM) | VPC23019 (100nM), and JTE013 (20nM), and $S1P_1$ antagonist W143 (1μM) | media containing 10% serum | although through different pathways) | |
| | S1P (1μM) | $S1P_1$ as determined by pertussis toxin (100ng/mL), and W146 (10μM) receptor blockade | C3H10T1/2 murine MSCs incubated with S1P for 15 minutes to 24 hours. Media contained 10% serum | ↑ALP<br>↑Osteocalcin<br>↑Mineralization<br>↓Adipogenic differentiation<br><br>No effect on proliferation | (102) |
| | S1P (40mg/mL and 80mg/mL) | $S1P_{1/2}$ as determined by changes in gene expression | Human adipose derived stem cells cultured on titanium oxide coated stainless steel doped in S1P, cells were exposed to S1P for 120 hours | ↑Proliferation<br>↑Mineralization<br>↑Expression of $S1P_1$ and $S1P_2$ at 80mg/mL<br>↑Expression of $S1P_2$ only at 40mg/mL | (68) |
| **CHONDROCYTES** | S1P (0.1-3μM) | $S1P_{1-3}$ receptors exhibit increased expression as determined by PCR | Bovine and human cartilage explants (monolayer culture), proliferation assay | ↑Proliferation | (103) |
| | S1P (0.1-10μM) | Broad S1P receptor expression, though no | Human articular chondrocytes from osteoarthritis patients. | ↑PGE2 release<br><br>↑Cartilage degradation | (104) |

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| | | specific receptor roles were identified, although Gi protein blockade with pertussis toxin reduced PGE2 induction by S1P | Treated following serum starving (0.5% serum) | No effect on proliferation and viability | |
| **OSTEOCYTES** | Mechanical stimulation-S1P (100nM) | $S1P_2$ as determined by pre-treatment with JTE013 (10μM) | MLO-Y4 cell line, oscillatory fluid flow, JTE013 | ↑PGE2 release<br>↓RANKL/OPG | (91) |