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Adjuvant drug-assisted bone healing: Part II – Modulation of angiogenesis

Rebecca Rothe^a, Sabine Schulze^{b,c}, Christin Neuber^a, Sandra Hauser^a, Stefan Rammelt^{b,c,d,1,*} and Jens Pietzsch^{a,e,1,*}

^a*Department of Radiopharmaceutical and Chemical Biology, Helmholtz-Zentrum Dresden-Rossendorf (HZDR), Institute of Radiopharmaceutical Cancer Research, Dresden, Germany*

^b*University Center of Orthopaedics and Traumatology (OUC), University Hospital Carl Gustav Carus, Dresden, Germany*

^c*Center for Translational Bone, Joint and Soft Tissue Research, University Hospital Carl Gustav Carus and Faculty of Medicine, Technische Universität Dresden, Dresden, Germany*

^d*Center for Regenerative Therapies Dresden (CRTD), Tatzberg 4, Dresden, Germany*

^e*Technische Universität Dresden, School of Science, Faculty of Chemistry and Food Chemistry, Dresden, Germany*

Abstract. The treatment of critical-size bone defects following complicated fractures, infections or tumor resections is a major challenge. The same applies to fractures in patients with impaired bone healing due to systemic inflammatory and metabolic diseases. Despite considerable progress in development and establishment of new surgical techniques, design of bone graft substitutes and imaging techniques, these scenarios still represent unresolved clinical problems. However, development of new active substances gives cause for hope. This work discusses therapeutic approaches that influence angiogenesis or hypoxic situations in healing bone and surrounding tissue. In particular, literature on sphingosine-1-phosphate receptor modulators and nitric oxide (NO^{*}) donors, including bi-functional (hybrid) compounds like NO^{*}-releasing cyclooxygenase-2 inhibitors, was critically reviewed with regard to their local and systemic mode of action.

Keywords: Critical-size bone defects, neovascularization, nitric oxide donors, signaling, small molecules, sphingosine-1-phosphate receptor

List of abbreviations

2-OG	2-oxoglutarate
AC	adenylate cyclase
Akt	protein kinase B
Asn	asparagine
ATP/GTP	adenosine/guanosine triphosphate
BMP	bone morphogenetic protein

¹SR and JP share senior authorship.

*Corresponding author: Prof. Dr. Stefan Rammelt, University Center for Orthopaedics and Traumatology, University Hospital Carl Gustav Carus, Fetscherstrasse 74, 01307, Dresden, Germany. Tel.: +49 351 4583211; Fax: +49 351 4584307; E-mail: stefan.rammelt@uniklinikum-dresden.de and Prof. Dr. Jens Pietzsch, Department of Radiopharmaceutical and Chemical Biology, Helmholtz-Zentrum Dresden-Rossendorf, Institute of Radiopharmaceutical Cancer Research, Bautzner Landstraße 400, 01328, Dresden, Germany. Tel.: +49 351 2602622; Fax: +49 351 26012622; E-mail: j.pietzsch@hzdr.de.

cAMP/cGMP	cyclic adenosine/guanosine monophosphate
CINOD	COX-inhibiting nitric oxide donator
COX	cyclooxygenase
CREB	cAMP response element-binding protein
DFO	deferoxamine
DMOG	dimethylloxaloylglycine
EGFR	epidermal growth factor receptor
ELK	ETS domain-containing protein Elk
eNOS/iNOS/nNOS	endothelial/inducible/neuronal nitric oxide synthase
ERK	extracellular signal-regulated kinase
FDA	Food and Drug Administration
GC	guanylate cyclase
GPCR	G-protein coupled receptor (G _i , G _q , G _s , G _{12/13} subunits)
GSK-3 β	glycogen synthase kinase-3 β
HIF	hypoxia-inducible factor
HRE	hypoxia response element
I κ B	inhibitor of kappa B
IKK	inhibitor of kappa B kinase
L-NAME	N(G)-nitro-L-arginine methyl ester
L-NMMA	N(G)-monomethyl-L-arginine
LPA	lysophosphatidic acid
LPP1	lipid phosphate phosphohydrolase type 1
LRP	low-density lipoprotein receptor-related protein
MAPK	mitogen-activated protein kinase
MEK	mitogen-activated protein kinase kinase
BMSCs	bone marrow-derived mesenchymal stem cells
NFAT	nuclear factor of activated T-cells
NF κ B	nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NO \bullet	nitric oxide (radical)
NOG	N-oxaloylglycine
OPG	osteoprotegerin
PCL	poly-caprolactone
PDE	phosphodiesterase
PEG	polyethylenglycol
PG	prostaglandin
PHD	prolyl hydroxylase domain enzyme
PI3K	phosphatidylinositol-3-kinase
PKA	protein kinase A
PKC	protein kinase C
PKG	protein kinase G
PLGA	poly-lactic-co-glycolic acid
PLC	phospholipase C
Pro	proline
PRP	platelet-rich plasma
Rac	Ras homolog (GTPase)
RAF	rapidly accelerated fibrosarcoma
RANKL	receptor activator of nuclear factor κ B ligand

Ras	rat sarcoma
RGD	Arginine – Glycine – Aspartate motif
rh	recombinant human
Rho	Ras homolog (GTPase)
ROCK	Rho-associated, coiled-coil-containing protein kinase
ROS	reactive oxygen species
S1P	sphingosine-1-phosphate
SDF-1	stromal cell-derived factor 1
Smad	contraction of Sma and Mad (small mothers against decapentaplegic homolog)
SPHK	sphingosine kinase
Spns2	spinster homolog 2
Src	sarcoma oncogene cellular homolog/proto-oncogene tyrosine-protein kinase
TNF- α	tumor necrosis factor alpha
Ub	ubiquitin
VEGF	vascular endothelial growth factor
VHL	Von Hippel-Lindau protein
Wnt	Wingless-related integration site

1. Modulation of angiogenesis¹

Besides inflammation [1], angiogenesis is the second important process during bone repair and it is necessary to initiate osteogenesis by supply of oxygen (O₂), nutrients, and cell recruitment [2–8]. These two key processes of healing are closely interlinked and successively take over determining role in the course of repair [9]. However, angiogenesis plays an essential role during all bone fracture healing phases. At the beginning of bone healing, pro-angiogenic mediators are primarily involved in cell recruitment and migration, while subsequently promoting formation and networking of blood vessels [10]. Endothelial cells are the cells dominating during angiogenesis and vascularization [11, 12]. In process of bone remodeling, matrix metalloproteases play an important role, especially by enabling invasion of formed blood vessels [13, 14]. A delay in angiogenic progression may lead to increased chondrocyte settlement, which occupy a predetermined role in structure of new tissue [15]. Angiogenic processes have to be highly regulated and, if necessary, controlled through clinical intervention in order to guarantee a stable vasculature and to prevent pathological states. This requires a controlled, temporally defined expression and function of pro-angiogenic factors and signals [16–19]. Angiogenesis is mainly regulated via VEGF (vascular endothelial growth factor) in combination with PGE₂ (prostaglandin E₂) or angiopoietin to promote bone formation [5, 20, 21]. Besides, signaling via ephrin receptors and ephrins is essential for vascularization and angiogenesis and thereby may support fracture healing [22]. For example, an increased expression of ephrin receptor B4 in a mouse model leads to an increase in bone strength in the course of fracture healing [23]. Moreover, ephrin B2 and corresponding above mentioned ephrin receptor B4 maintain bone homeostasis due to bi-directional regulation of osteoblastic and osteoclastic differentiation resulting in enhanced bone formation and decreased bone resorption “via ephrin receptor forward signaling” [24–26]. In comparison to VEGF, other growth factors such as transforming growth factor-beta, platelet-derived growth factor, fibroblast growth factor as well as the interaction of all of these growth factors contribute to angiogenesis to a

¹ A PubMed database search was performed in November 2018 using key words and phrases ‘agonists’, ‘anabolic’, ‘anti-resorptive’ ‘angiogenesis’, ‘antagonists’, ‘drugs’, ‘inflammation’, ‘inhibitors’, ‘local’, ‘small molecule compounds’, ‘systemic’ linked to the key words ‘critical bone defect’, ‘fracture’, and ‘healing’ by AND/OR as Boolean function.

lesser extent [27–29]. A “major driving force” for angiogenesis is hypoxia, being required for initiating the healing process [30–34].

1.1. Role of hypoxia-inducible factors in bone healing

Hypoxia-inducible factor (HIFs) are heterodimeric proteins, more specifically transcription factors, with a basic helix-loop-helix motif acting as cellular adaptors to prevalent oxygen tension [21, 35, 36]. HIF-1 β subunit is constitutively expressed and permanently functional whereas posttranslational function of HIF- α subunits depend on oxygen availability [37, 38]. There are three isoforms of HIF- α subunit having different transcription profiles and consequent functions in various tissues, for example upregulation of hypoxia-induced endothelial nitric oxide synthase (eNOS) [39]. HIF-1 α is present in almost all tissues, whereas HIF-2 α is predominantly expressed by endothelial cells during embryogenesis. Moreover, both HIFs are key players in adaptation of tumor cells to hypoxia. Comparatively, function of HIF-3 α subunit is currently poorly understood, but there are some indications that transcription of this subunit is intensified upon hypoxia. However, HIF-3 α is probably not involved in pro-angiogenic processes and may function as a negative regulator [40–44]. As depicted in Fig. 1, under “normoxic conditions”, distinct proline residues (Pro402, Pro567) of HIF-1 α are hydroxylated by prolyl hydroxylases further recruiting E3 ubiquitin ligase-Von Hippel-Lindau (VHL) complex. Afterwards, HIF-1 α becomes degraded by 26 S proteasome. However, hydroxylation of asparagine (Asn803) by asparaginyl hydroxylase probably leads to inhibition of interactions with nuclear auxiliary proteins being essential to trigger downstream transcription [40, 45, 46]. During hypoxia, defined by an oxygen level lower than 5 %, HIF-1 α subunit is stabilized by several kinases and thereby accumulates in cytosol due to prevented hydroxylation and ubiquitin (Ub)-mediated proteasomal degradation. Further, HIF-1 α translocates into the nucleus. After heterodimerization with HIF-1 β as well as binding of additional assistant proteins, HIF complex induces downstream transcription of about 100 target genes regulating several cellular processes like glucose metabolism, proliferation, cell survival, motility, extracellular matrix metabolism, and angiogenesis, the last due to regulation of VEGF (Fig. 1) [15, 30, 31, 36, 44, 47, 48]. Depending on the particular fracture repair stage either hypoxic or normoxic conditions are required to ensure optimal wound healing. After an initial hypoxic phase lasting only few days, recovery of normal oxygen tension within tissue is essential since chronic hypoxia is detrimental for bone repair. Hyperoxia, on the other hand, can positively influence angiogenesis, but it can also impair bone healing due to occurrence of superoxide radicals. Therefore, a controlled oxygen level is needed throughout the healing process because oxygen is required to maintain normal cellular metabolism as well as to fulfill optimal function of various enzymes [1, 32, 49, 50]. Moreover, HIF-1 α prevents its own accumulation based on upregulated expression of prolyl hydroxylase domain enzyme (PHDs) under hypoxic conditions via a negative feedback loop. This control mechanism probably is necessary to reestablish normal tissue conditions concerning oxygen level [40, 51, 52]. Lechler and coworkers as well as Utting and coworkers showed *in vitro* that oxygen availability is essential for viability, proliferation and differentiation of osteoblasts as well as for exerting their functions [53, 54]. Hypoxia attenuates these important processes due to delayed gene expression until these processes arrest resulting in impaired bone formation. Influence of different oxygen levels from hypoxia to hyperoxia has been investigated *in vivo* by Lu and coworkers using a murine tibia fracture model [49]. The authors detected significant differences with regard to bone formation between fractures under normoxic and hypoxic conditions only after ten days, suggesting that hypoxia delays fracture repair in later healing phases. Even hyperoxic conditions do not significantly affect callus and bone volume within the first ten days. In this context, Komatsu and coworkers postulated post-fracture day ten as the “key angiogenic time point” in fracture repair using a rodent femoral fracture model [55]. At this time point, highest HIF expression associated with activation of downstream transcription targets VEGF

and inducible nitric oxide synthase (iNOS) was recognized. Low oxygen tension increasing HIF-1 α downstream transcription characterized the early stages of fracture healing. According to Tazzyman and coworkers, acute hypoxia due to lowest oxygen level after an injury with about 0.6–0.9 kPa oxygen partial pressure, has been detected after three to five days [56]. Thereby, transcription of several pro-angiogenic as well as pro-inflammatory and proliferation-enhancing mediators like VEGF, TNF- α (tumor necrosis factor alpha), or iNOS is triggered emphasizing the essential role of HIF during bone healing [2, 30, 40, 57]. VEGF further promotes osteogenesis and bone formation by initializing vasculogenesis providing recruitment of osteoprogenitor cells, nutrients, and oxygen to fracture site. It becomes obvious that HIF signaling pathway is a key link between osteogenesis and angiogenesis during bone repair [35, 38, 58–60]. Promoted proliferation and differentiation of osteoblasts under hypoxic conditions are important downstream signaling results. Also RANKL (receptor activator of nuclear factor κ B ligand)-based activation of osteoclast-mediated bone resorption as a consequence of hypoxia has been demonstrated to be essential during early stages of fracture repair to provide basis for formation of new bone substance [38, 61–63]. Wang and coworkers as well as Wan and coworkers investigated effects of genetically inactivated HIF-1 α in osteoblasts utilizing a murine model [64–66]. Loss of HIF-1 α resulted in skeletal defects, impaired bone formation as well as reduced bone volume and mineralization together with decreased vascularization and impaired angiogenesis. The question arose whether HIF-1 α overexpression or constitutive activation probably enhances fracture healing in critical-size bone defects to gain a strategy for transferring the beneficial HIF impact in therapeutic use. Zou and coworkers examined the mentioned issue by transducing lentiviral constructs with HIF-1 α or stable constitutive HIF-1 α to bone marrow-derived mesenchymal stem cells (BMSCs) and by further implantation of these BMSCs into rats [67]. Thereby, authors observed an increase in bone volume and mineral density as well as an enhancement in blood vessel number and area. Results have been confirmed by Wang and coworkers as well as Wan and coworkers [64–66]. Here, the authors used genetically manipulated mice lacking VHL suppressor, thus preventing degradation of HIF-1 α . They demonstrated that resultant HIF-1 α overexpression along with VEGF overproduction in mature osteoblasts induced angiogenesis and vascularization as well as osteogenesis, finally leading to an increased bone volume and enhanced bone regeneration. This highlights the great importance of HIF signaling with its key mediator VEGF for accelerated angiogenesis and osteogenesis in treatment of critical-size bone defects. However, cell therapy is limited in clinical use due to time expenditure, efficacy, costs, and practicability [38].

1.2. Angiogenesis-modulatory agents

1.2.1. Prolyl hydroxylase domain protein inhibitors

PHDs are dioxygenases requiring molecular oxygen as well as 2-oxoglutarate (2-OG) and iron (Fe^{2+}) as cofactors for proline hydroxylation on target proteins [68, 69]. There are three different PHD isoforms. PHD1 and PHD3 presumably prefer HIF-2 α as target substrate, whereas PHD2 seems to be predominant in HIF-1 α regulation under normoxic conditions [30, 38, 40, 43, 70]. PHD enzymes are decisively involved in adaptation to prevalent oxygen availability due to hydroxylation of HIF- α subunit under normoxic conditions and further initiation of proteasomal HIF degradation. Under hypoxia, enzyme activity is suppressed, associated with absent hydroxylation and degradation of HIF, leading to HIF-triggered transcription of certain target genes [57]. Since there is an essential role of HIF in promoting angiogenesis and osteogenesis, especially during early bone formation, some scientific efforts have focused on targeting HIF pathway in order to promote fracture healing (Table 1). To achieve pro-angiogenic effects in therapeutic use, a promising approach is to stabilize HIF by interfering PHD function with the aid of small inhibitory molecules competing with above-mentioned cofactors [3]. For instance, non-selective inhibitors such as competitive 2-OG analogs like

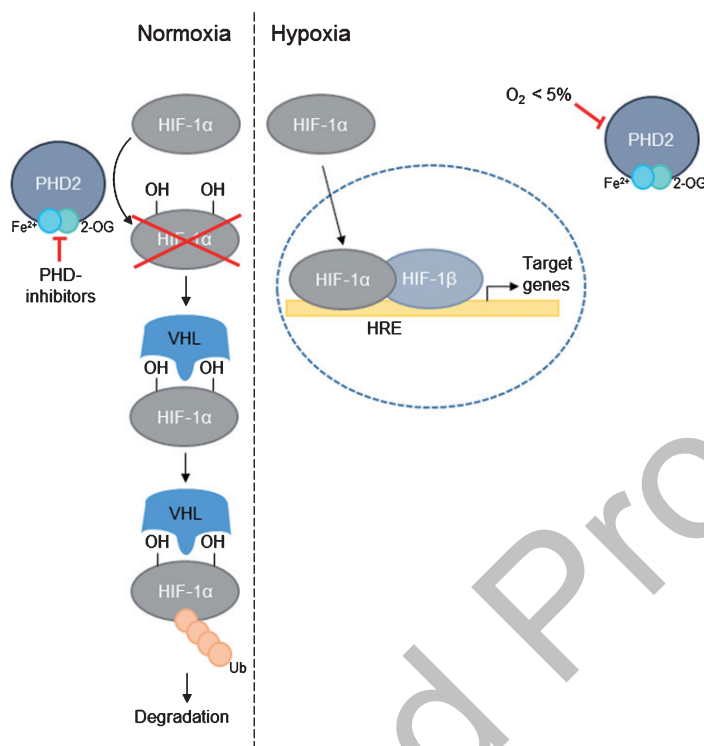


Fig. 1. HIF-1 α signaling pathway depends on oxygen availability. Under normoxia, HIF-1 α gets hydroxylated by PHD enzymes which PHD-inhibitors can prevent. Further ligase and ubiquitin-mediated (Ub) degradation takes place. On the contrary, during hypoxia PHD enzymes are blocked and HIF-1 α is able to stimulate transcription of several target genes in cooperation with HIF-1 β (modified according to Maes and coworkers [35]).

dimethylaloxaloylglycine (DMOG) or N-oxaloylglycine (NOG) and iron chelating agents such as cobalt chloride or the FDA (Food and Drug Administration)-approved deferoxamine (DFO) in common with competitive iron inhibitors such as cobalt (Co²⁺), copper (Cu²⁺), zinc (Zn²⁺) and manganese (Mn²⁺) were under investigation to elicit HIF/VEGF-dependent pro-angiogenic effects [35, 57, 71]. Shen and coworkers investigated angiogenic and osteogenic impact of cell penetrating small molecule DMOG when locally applied to a murine femur fracture model [72]. Treatment with this PHD inhibitor resulted in an increased bone formation due to raised callus size and vascularity already after 14 days. Another approach utilizing DMOG to improve fracture repair has been shown by Ding and coworkers [73]. Therein, adipose-derived stem cells treated with DMOG exhibited dose-dependently enhanced VEGF expression *in vitro* due to raised HIF-1 α activation leading to an increased angiogenic and osteogenic potential. Cell-loaded hydrogels served as carrier matrix for transfer of these cells into critical-size calvarial defects in rats. DMOG treatment resulted in accelerated bone regeneration and vascularization. In addition, Wu and coworkers loaded a bioactive glass scaffold with DMOG [74]. *In vitro*, the authors demonstrated an enhanced angiogenesis and osteogenesis due to stabilization of HIF-1 α and further stimulated expression of VEGF and other related targets. Furthermore, iron chelating agent DFO has been examined by Donneys and coworkers, Drager and coworkers, and Wan and coworkers [65, 75, 76]. The authors determined accelerated angiogenesis and bone formation after locally injected DFO in either rats, rabbits, or mice. Also Stewart and coworkers indicated enhanced fracture healing based on increased angiogenesis and mechanical bone stiffness using scaffold-delivered DFO in a rodent critical-size femoral defect model [77]. These studies demonstrated effective enhancement of fracture healing by applying small molecules interfering with HIF – VEGF signaling pathway. Mentioned results have

been confirmed by Shen and coworkers although possible side effects should be considered since the unspecific iron chelator also interferes with non-HIF-related signaling pathways [72]. Cho and coworkers revealed that iron chelators exhibit different effects relative to their iron specificity and membrane permeability [78]. Furthermore, Wu and coworkers formed a bioactive glass scaffold with incorporated Co^{2+} being able to mimic hypoxic environment *in vitro* [79]. Co^{2+} in low concentration, i.e. less than 5% of the scaffold, was able to increase HIF-1 α and VEGF expression. This shows another possibility of increasing angiogenesis concomitant with bone regeneration by specifically loaded biomaterials. Indeed, systemic application of cobalt chloride (15 mg/kg/day via intraperitoneal injection) mimicking hypoxia resulted in an enhanced mechanical strength and fracture repair due to stabilized HIF-1 α and, consequently, promoted downstream transcription of HIF-1 α target genes [80]. However, clinical application of the above-mentioned non-selective and non-specific inhibitors is limited due to their (adverse) side effects on other important signaling pathways. Therefore, new innovative approaches came into focus of research using specific PHD inhibitors, utilizing the three-dimensional enzyme conformation, such as FG-2216 (glycine derivative [46]), FG-4592 (2-OG analog [46]), and GSK360A (glycine derivative [46]) to trigger pharmacologically enhanced bone formation [30, 38, 46]. Since PHDs have a conserved active site and variable N- or C-termini, these domains are potential targets for selective inhibitors. For instance, novel PHD inhibitors TM6008 and TM6089 (pyrimidine derivatives [81]) bind selectively to the catalytic center of PHD2 [30]. Still, safety and selectivity of these inhibitors are considered as critical, so that only a short-term, local treatment would be possible in clinics [71]. In addition to controlled local administration, changed and optimized dose can also reduce adverse side effects such as osteosclerosis, bone marrow fibrosis, or incurrence of osteosarcoma progression as possible consequences of an excessive HIF-1 α activation [38]. While hypoxia is essential during bone healing at an early stage, chronically excessive course is detrimental for fracture healing. After early hypoxic phase, rapid restoration of cellular oxygen level is necessary. If this does not happen by itself, therapeutic intervention is needed. Therefore, either increased levels of cofactors 2-OG and Fe^{2+} or small molecules like lipid second messengers, which activate PHDs, can be promising in driving HIF degradation, as summarized by Nagel and coworkers [69].

1.2.2. Nitric oxide donors

Free radical nitric oxide (NO^\bullet) is a paracrine and autocrine bioactive messenger molecule and is produced by nitric oxide synthases (NOS) [82, 83]. However, NO^\bullet itself can regulate its production via a feedback mechanism. When NO^\bullet is present in high concentrations, it binds to heme center of NOS, thus decreasing NOS activity [84]. Generally, NO^\bullet exhibits many physiologically important functions like modulating immune response, angiogenesis, platelet function, vascular regulation or wound healing [85–88]. NO^\bullet activates soluble guanylate cyclase (GC) producing the intracellular second messenger cyclic guanosine monophosphate (cGMP) (Fig. 2). cGMP further activates target proteins like soluble or membrane-bound PKG (protein kinase G), in turn regulating cation channels, or recruits adapter proteins such as Src (sarcoma oncogene cellular homolog) and, finally, drives physiological processes, for instance proliferation, via activated downstream pathways such as the MAPK (mitogen-activated protein kinase) signaling cascade [89–91]. Moreover, a direct activation of transcription factors β -catenin and CREB (cAMP response element-binding protein) has also been demonstrated (Fig. 2) [92]. In addition, there is a cross-talk between NO^\bullet and COX (cyclooxygenase, impact of COX activity on bone healing was discussed in Part I – Modulation of inflammation [1]), whereby produced mediators NO^\bullet and PGs, respectively, seem to affect the determining enzyme of the other signaling pathway in a way yet to be clarified. Probably, NO^\bullet affects COX function through interplay with heme center, since NO^\bullet activates soluble GC also due to heme interactions [90, 93]. However, cross-talk between NOS and COX may also happen via NO^\bullet -induced posttranslational protein modification, since it is able to activate COX via S-nitrosylation of various cysteine residues

Table 1
Summary of *in vitro* and predominantly *in vivo* studies regarding the effect of promising angiogenesis-modulatory drugs on bone metabolism

Compound	Model	Dose	Application	Effect	Reference
lipid mediators					
LPA and LPA receptors					
LPA	osteocyte-like cells	0.01–10 μ M	–	↑	Karagiosis <i>et al.</i> , 2007 [202]
LPA ₁ receptor	LPA ₁ ^{-/-} mice	–	–	↓	David <i>et al.</i> , 2014 [192]
	LPA ₁ ^{-/-} mice	–	–	↓	Gennero <i>et al.</i> , 2011 [207]
LPA ₄ receptor	LPA ₄ ^{-/-} mice	–	–	↑	Liu <i>et al.</i> , 2010 [193]
S1P and S1P analogs					
	human umbilical vein endothelial cells	5 nmol	PEG hydrogel	↑	Wacker <i>et al.</i> , 2006 [163]
S1P	mice	1:400 S1P:PLGA	PLGA-coated implant	↑	Sefcik <i>et al.</i> , 2011 [160]
	mice	1800 μ M	subcutaneous injected matrigel	↑	Tengood <i>et al.</i> , 2010 [165]
	rat	1:400 S1P:PLGA	PLGA scaffold	↑	Petrie Aronin <i>et al.</i> , 2010 [136]
	rat	1 mg/ml	PLGA 3D scaffold	↑	Sefcik <i>et al.</i> , 2008 [164]
FTY720 (fingolimod)	mice	6 mg/kg	subcutaneous injection	→	Heilmann <i>et al.</i> , 2013 [158]
	mice	3 mg/kg/day	intraperitoneal injection	↑	Ishii <i>et al.</i> , 2009 [147]
	mice	1:200 FTY720:PLGA	PLGA-coated implant	↑	Sefcik <i>et al.</i> , 2011 [160]
	mice	1–10 nM	local injected matrigel	↑	Wang <i>et al.</i> , 2016 [174]
	rat	1:200 FTY720:polymer	PCL/PLGA nanofiber implant	↑	Das <i>et al.</i> , 2013 [175]
	rat	1:200 FTY720:PLGA	PLGA-coated allograft	↑	Das <i>et al.</i> , 2014 [176]/2015 [173]
FTY720 (fingolimod)	rat	1:40 or 1:200 FTY720:PLGA	PLGA-coated allograft	↑	Huang <i>et al.</i> , 2012 [172]
	rat	1:200 FTY720:PLGA	PLGA scaffold	↑	Petrie Aronin <i>et al.</i> , 2010 [136, 151]
	rat	1.5 mg/0.6 ml	coated allograft	↑	Wang <i>et al.</i> , 2016 [174]
JTE-013 (pyrazolopyridine derivative)	mice	3 mg/kg	intraperitoneal injection	↑	Ishii <i>et al.</i> , 2010 [208]
SEW2871 (oxadiazole derivative)	mice	0.4–1.6 mg SEW2871 micelles	SDF-1 incorporated gelatin hydrogel	↑	Kim <i>et al.</i> , 2016 [209]
	rat	7.5–15 μ g SEW2871 micelles	PRP incorporated gelatin hydrogel	↑	Kim <i>et al.</i> , 2014 [210]
VPC01091	mice	1:200 VPC01091:PLGA	PLGA-coated implant	↑	Sefcik <i>et al.</i> , 2011 [160]
(octylphenyl-substituted cyclophenyl derivative)	rat	1:200 VPC01091:PLGA	PLGA scaffold	↑	Petrie Aronin <i>et al.</i> , 2010 [136]
	rat	1–5 mg/kg	intraperitoneal injection	↑	Selma <i>et al.</i> , 2018 [211]

Table 1
(Continued)

Compound	Model	Dose	Application	Effect	Reference
nitric acid					
NO• donors					
carboxybutyl chitosan NONOate	rat	200 mg (250 nmol NO release per 5 mg of chitosan-NO over 185 minutes)	local	↑	Diwan <i>et al.</i> , 2000 [85]
isosorbide mononitrate	human	5–20 mg/day	oral	↑	Jamal <i>et al.</i> , 2004 [212]
	human	20 mg/day	oral	↑	Nabhan <i>et al.</i> , 2008 [213]
L-arginine	guinea pig	100 mg/kg	oral	↑	Kdolsky <i>et al.</i> , 2005 [214]
L-arginine	human	18 g L-arginine hydrochloride (14.8 g free L-arginine)	oral	→	Baecker <i>et al.</i> , 2005 [215]
nitroglycerin	rat	0.2 mg, 0.4 mg, or 2.0 mg 2% nitroglycerin	dermal (ointment)	↑ (low and middle dose) → (high dose)	Hao <i>et al.</i> , 2005 [95]
	rat	0.2 mg 2% nitroglycerin	dermal (ointment)	↑	Wimalawansa <i>et al.</i> , 2000 [216]
	rat	0.2 mg 2% nitroglycerin once, twice or three times a day	dermal (ointment)	↑ (once daily) ↓ (higher frequency)	Wimalawansa <i>et al.</i> , 2000 [217]
	human	15 mg/day	percutaneous (ointment)	↑	Wimalawansa <i>et al.</i> , 2000 [218]
	rat	0.3 mmol/l	local	↑	Baldik <i>et al.</i> , 2002 [219]
nitrosobovine serum albumin	rat	0.3 mmol/l	local	↑	Baldik <i>et al.</i> , 2002 [219]
nitrosyl-cobinamide	mice	10 mg/kg/day	intraperitoneal injection	↑	Kalyanaraman <i>et al.</i> , 2017 [102]
NOC-18/DETA-NONOate (slower release/mimicking eNOS)	calvarial osteoblasts	1–10 μM	–	↑	Lin <i>et al.</i> , 2008 [220]
	primary rat osteoblast-enriched Cultures	10–100 μM	–	↑	Mancini <i>et al.</i> , 2000 [82]
S-nitrosoglutathione	rat	100 μmol/l in hydrogel 8 nmol/application	local hydrogel	↑	Amadeu <i>et al.</i> , 2008 [109]
sodium nitroprusside (rapid release/mimicking iNOS)	primary rat osteoblast-enriched cultures	10–100 μM	–	↓	Mancini <i>et al.</i> , 2000 [82]
NOS and NOS inhibitors					
eNOS	eNOS ^{-/-} mice	–	–	↓	Aguirre <i>et al.</i> , 2001 [221]
	eNOS ^{-/-} mice	–	–	↓	Armour <i>et al.</i> , 2001 [222]
	eNOS ^{-/-} mice	–	–	↓	Meesters <i>et al.</i> , 2016 [223]

(Continued next page)

Table 1
(Continued)

Compound	Model	Dose	Application	Effect	Reference
iNOS	iNOS ^{-/-} mice	-	-	↓	Baldik <i>et al.</i> , 2005 [224]
	iNOS ^{-/-} mice	-	-	↓	Meesters <i>et al.</i> , 2016 [223]
nNOS	nNOS ^{-/-} mice	-	-	↑	Van't Hof <i>et al.</i> , 2004 [225]
aminoguanidine (iNOS inhibitor)	rat	400 mg/dl	oral	↑	Baldik <i>et al.</i> , 2002 [219]
L-NAME (non-selective NOS inhibitor)	rat	1 mg/ml	oral (drinking water)	↓	Diwan <i>et al.</i> , 2000 [85]
L-NMMA (non-selective NOS inhibitor)	calvarial osteoblasts	1 mM	-	↓	Lin <i>et al.</i> , 2008 [220]
PDE inhibitors					
rolipram (selective PDE4 inhibitor)	mice	10–20 mg/kg/day	subcutaneous injection	↑	Horiuchi <i>et al.</i> , 2002 [129]
	mice	1–30 mg/kg/day	subcutaneous injection	↑	Kinoshita <i>et al.</i> , 2000 [128]
	mice	50–5000 nmol (500 nmol equals 150 µg)	rhBMP-2-loaded PEG discs	↑	Tokuhara <i>et al.</i> , 2010 [125]
avanafil (selective PDE5 inhibitor)	rat	10 mg/kg	oral	↑	Huyut <i>et al.</i> , 2018 [226]
sildenafil (selective PDE5 inhibitor)	mice	5 mg/kg/day	oral	↑	Histing <i>et al.</i> , 2011 [124]
	rat	10 mg/kg/day	oral	↑	Dincel <i>et al.</i> , 2018 [227]
	rat	5 mg/kg/day	oral (stomach tube)	↑	Togral <i>et al.</i> , 2015 [121]
	rat	10 mg/kg/day	orogastric tube	↑	Yaman <i>et al.</i> , 2011 [123]
tadalafil (selective PDE5 inhibitor)	mice	45–75 mg/kg/day	oral	↓	Gong <i>et al.</i> , 2014 [119]
	rat	10 mg/kg/day	oral (tablet)	↑	Alp <i>et al.</i> , 2017 [228]
	rat	2 mg/kg/day	oral	→	Raifer <i>et al.</i> , 2017 [97]
	rat	1 mg/kg/day	oral (stomach tube)	↑	Togral <i>et al.</i> , 2015 [121]
	rat	2.5 – 10 mg/kg/day	oral	→	Wang <i>et al.</i> , 2018 [229]
udenafil (selective PDE5 inhibitor)	rat	10 mg/kg/day	oral (tablet)	↑	Alp <i>et al.</i> , 2017 [228]
vardenafil (selective PDE5 inhibitor)	rat	10 mg/kg/day	oral (tablet)	↑	Alp <i>et al.</i> , 2017 [228]
zaprinast (selective PDE5 inhibitor)	rat	10 mg/kg	oral	↑	Huyut <i>et al.</i> , 2018 [226]
pentoxifylline (non-selective PDE inhibitor)	mice	50 – 300 mg/kg/day	subcutaneous injection	↑	Kinoshita <i>et al.</i> , 2000 [128]
	mice	5 – 300 mg/kg/day	subcutaneous injection	↑ (higher dose)	Horiuchi <i>et al.</i> , 2001 [230]
	rat	50 mg/kg/day	intraperitoneal injection	↑	Atalay <i>et al.</i> , 2015 [231]
pentoxifylline (non-selective PDE inhibitor)	rat	50 mg/kg/day	intraperitoneal injection	↑	Aydin <i>et al.</i> , 2011 [122]
	rat	50 mg/kg/day	intraperitoneal injection	→	Dincel <i>et al.</i> , 2018 [227]
	rat	200 mg/kg/day	-	→	Vashghani Farahani <i>et al.</i> , 2017 [232]

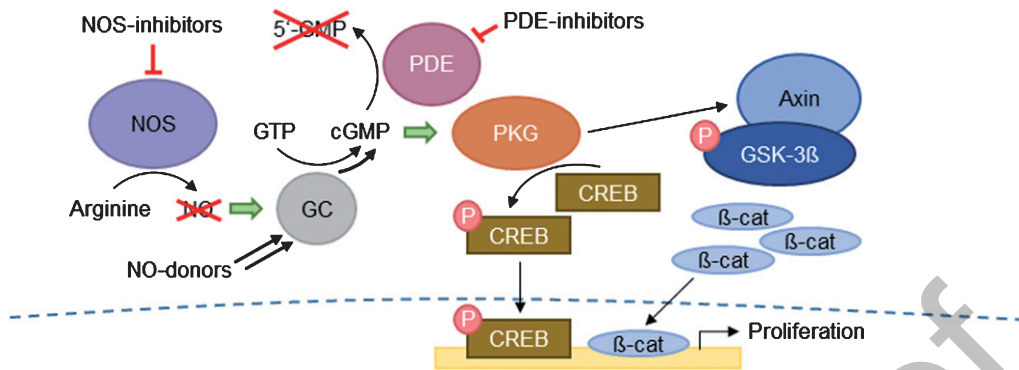


Fig. 2. Intracellular NO[•] pathway. NO[•] production by NOS enzymes is based on L-Arginine. NO[•], in line with the effect of NO-donors, activates further downstream signaling pathways. Activation of GC, PKG, and the main part of Wnt signaling cascade stimulates proliferation-enhanced transcription via CREB and β -catenin. Effects of NOS- and PDE inhibitors on their target molecules are highlighted in red (modified according to Gong and coworkers [119]).

and further stimulates PG synthesis [94]. NO[•] displays biphasic effects towards activity of osteoclasts and osteoblasts, with NO[•] concentration having to be balanced within a certain range. Low NO[•] concentrations (nanomolar range) released slowly appear to increase cellular activity, but when NO[•] level drops below physiological limit, bone fades. Also, high NO[•] concentrations (micromolar range) being quickly released inhibit cell proliferation and differentiation [82–84, 87, 90, 95]. If NO[•] is present in high concentrations, apoptosis of osteoblasts is stimulated via activation of caspase-3 or reduction of anti-apoptotic proteins [96]. NO[•] prevents differentiation of osteoclasts probably by increasing OPG (osteoprotegerin) production acting as a decoy receptor for RANKL [87]. Optimal dosage is probably in the middle, as this prevents osteoclast-mediated bone resorption as well as promotes growth of osteoblasts [83]. Three different dimeric NOS subtypes are known. They are differentiated as endothelial, neuronal (nNOS), or inducible according to calcium-dependency, site of main expression and function [85, 93, 97]. NO[•] production by NOS depends on availability of oxygen and source amino acid L-arginine. However, three NOS subtypes have different production rates. Whereas constitutively active eNOS and nNOS produce NO[•] at a basal physiological level, iNOS generates NO[•] to a greater extent under hypoxia or after an inflammatory stimulus such as certain inflammation-related cytokines [82, 83, 85, 86, 90, 96]. NOS subtypes not only differ in NO[•] production but also in terms of their major function and time-dependent expression during wound healing processes. For this reason, NO[•] is able to influence all stages of fracture healing from inflammation to remodeling. At the beginning of healing process, macrophages produce large quantities of NO[•] within the first five days in order to destroy possible pathogens due to formation of ROS (reactive oxygen species) including NO[•]. Later in healing process, when NO[•] level is lower, cell proliferation as well as angiogenesis is promoted [86, 87]. More precisely, iNOS activity was already detected 24 h after fracture with a steeply rising activity until 4 d or 15 d according to literature, presumably recruiting cells for bone healing to fracture site. In contrast, eNOS is significantly expressed later in healing process with a peak at day 14 or steady increase within one month being probably related to differentiation of osteoblasts and regulation of angiogenic processes [83, 85, 98]. Moreover, nNOS shows highest expression rate on day 21. For this reason, nNOS is probably linked to regulation of bone remodeling processes. In addition, there is not only a time-dependent NOS expression after a fracture incidence, but also a site-specific expression of certain NOS isoforms in fracture callus. While cells of intramembranous region at the edge of callus express all three NOS isoforms at beginning of callus formation, in the middle to late repair phases cells of chondral and fibrochondral regions only show enhanced expression of eNOS and nNOS [99].

When therapeutic intervention is desired, time-dependent expression of the three NOS enzymes together with appropriate NO• production capacity during fracture healing has to be considered [98]. It is known that knockout or inhibition of NOS and inhibited NO• production, has detrimental effects on bone healing, such as decreased mineral density, stiffness, and bone strength, due to increased bone resorption (Table 1) [83, 85, 100]. Administration of NOS inhibitor N(G)-nitro-L-arginine methyl ester (L-NAME), for instance, delays healing process [86]. Systemic application of 1 mg/ml L-NAME led to significantly reduced mechanical stiffness in rats with femoral fractures [85]. Moreover, inhibition of NOS had not only direct consequences due to prevented NO• synthesis, but also influenced other signaling pathways. For instance, selective iNOS inhibitor 1400 W (acetamidine derivative) prevents production of PGs shown in an *in vitro* cell experiment [94]. However, increasing NO• concentration, for example by locally adding NO• donors, can reverse these negative effects [83, 85]. Thereby, NO• donors exhibit different NO• release mechanisms depending on their structure [90, 101]. For instance, enzymatic cleavage is required for NO• release from nitrates, thus generating ROS and concomitant cell damage [102]. NO• delivery was not trivial due to short half-life and high reactivity of NO•, which is why a variety of options has been investigated. Focusing on cardiovascular diseases, Miller and Megson summarized development and clinical application of many NO• donors including nitroglycerine, isosorbide mononitrate, sodium nitroprusside, diazeniumdiolates, S-nitrosothiols as well as novel NO• hybrid drugs [103]. According to Nichols and coworkers, N-diazeniumdiolates and S-nitrosothiols are “the two most diverse NO• donor classes” being able to release NO• without any further enzymatic assistance [87]. Moreover, metal nitrosyl complexes like sodium nitroprusside, consisting of a transition metal (Fe²⁺) and NO• attached to it, are in the focus of research for regulated and specific NO• release and resulting vasoactive effects [104]. In principal, organic nitrates as nitroglycerin are in clinical use to treat osteoporosis depicting dose-related impacts on bone mineral density. Additionally, studies have shown that other organic nitrates such as isosorbide mononitrate and isosorbide dinitrate also reduce fracture risk. The FDA approved cost-effective organic nitrates for long-term treatment, but development of tolerances complicates clinical use. Hypotheses on nitrate tolerance formation are based on an increase in oxidative stress triggered by products of biotransformation, which further lead to uncoupling of NOS and production of additional ROS [83, 102, 105–107]. For fracture healing to be as optimal as possible, a slow release of small amounts of NO• or an intermittent administration of NO• donors releasing NO• rapidly is desirable [87]. Krausz and coworkers summarized a great number of NO• donor delivery methods such as gaseous nitrate, acidified nitrate creams, diazeniumdiolates, probiotic NO•-releasing patches, or topical delivery of NO• nanoparticles concerning improved wound healing [86]. Most promising approaches seem to be incorporation of NO• donors into locally applicable biomaterials like hydrogels reviewed by Frost and coworkers [108]. During preparation of such matrices, it is possible to covalently or non-covalently bind NO• donors to polymer side chains or backbone [108]. Amadeu and coworkers determined optimal NO• donor time during cutaneous wound healing of rats using an S-nitrosoglutathione-containing hydrogel [109]. Authors detected accelerated wound repair after topical application of the mentioned hydrogel during inflammatory and proliferative healing phases suggesting that NO• exerts positive effects during temporally and functionally different phases. Besides local administration, NO• donors are used in systemic or targeted approaches. In the latter strategy, bisphosphonates being functionalized with a NO• donor, such as nitrobisphosphonates, purposefully direct NO• to bone due to capacity of bisphosphonates for calcium ion chelation. Thus, differentiation of osteoclasts is prevented, in turn reducing bone resorption [87]. In addition, NO• donor nitroglycerin is mainly delivered systemically. Hao and coworkers showed that application of nitroglycerin in a low or middle dose range (0.2–0.4 mg 2% nitroglycerin ointment) prevents bone loss and promotes bone formation due to inhibited osteoclast or enhanced osteoblast proliferation in an ovariectomized rodent model [95]. Hence, the authors concluded that “optimal dose of NO• supplement should produce a NO• level similar or slightly greater than physiologic NO• concentration”.

Wimalawansa and coworkers confirmed this by determining an anabolic effect of nitroglycerin in a dose range of 0.2–0.5 mg/kg as well as increased bone resorption when applying higher dose in a rodent model [110]. Using a murine model, Kalyanaraman and coworkers investigated impact of the novel NO• donor nitrosyl-cobinamide (10 mg/kg/day intraperitoneal injection), which does not form ROS compared to nitrates [102]. The authors demonstrated an increased bone formation due to enhanced proliferation of osteoblasts and a reduced differentiation of osteoclasts. Anabolic impact on bone is probably due to signaling via cGMP – PKG and Wnt (Wingless-related integration site) – β -catenin pathways as well as decreased expression of RANKL and promoted OPG production.

In addition, several scientists including our own group aim to combine modulation of inflammation and angiogenesis by synthesizing a bi-functional (hybrid) molecule, for example based on a non-selective or selective COX-inhibitor lead structure and a NO•-releasing moiety [101, 111]. Thereby, linker or conjunction between the two important functional units, for instance enzyme-cleavable ester bonds, plays a major role. Non-selective COX-inhibiting nitric oxide donors (CINODs) display anti-inflammatory and protective effects regarding gastrointestinal tract due to released NO• in comparison to traditional non-steroidal anti-inflammatory drugs. If synthesis is based on a selective COX-2 inhibitor, inhibition of COX-2 should remain as selective and effective as possible [101, 112–116]. Naproxcinod (NO•-Naproxen; AZD3582) was the first representative of CINODs to be examined in preclinical and clinical phase III studies, where 375–750 mg naproxcinod were applied to patients with osteoarthritis twice daily demonstrating above-mentioned benefits of CINODs [117]. Investigation regarding impact of CINODs on bone metabolism and fracture healing still remains open.

1.2.3. Phosphodiesterase inhibitors

Phosphodiesterases (PDE) are able to cleave cyclic nucleotides and are clinically used to treat erectile dysfunction, chronic heart failure, or pulmonary hypertension [118]. Eleven PDE subtypes with tissue-specific distribution are described in literature, whereby PDE5 degrading 3'-5'- cGMP is the most investigated one [119]. PDE5 inhibitors such as the long-acting tadalafil (half-life of about 17.5 h) provide NO•-induced cGMP accumulation and further activation of PKG. Other PDE5 inhibitors like sildenafil and vardenafil have a biological half-life of only 4 h and seem to be less affine and effective in comparison to tadalafil [118, 120]. These inhibitors have been widely studied for their effect on bone healing (Table 1). For instance, Togral and coworkers treated rats with a femur fracture with 5 mg/kg/day and 1 mg/kg/day sildenafil and tadalafil, respectively [121]. Both PDE5 inhibitors accelerated bone repair similarly. In contrast, Raifer and coworkers did not observe any beneficial effect of tadalafil on fracture healing in a rodent model [97]. However, reason for that might be that treatment comprised only a small amount of the inhibitor (2 mg/kg), probably being not sufficient to trigger osteogenic effects due to cGMP accumulation. Moreover, Gong and coworkers demonstrated a reduction of bone mass and osteoblastogenesis due to inhibition of PDE5 and further impaired Wnt signaling by orally applying 45–75 mg/kg/day tadalafil over a period of two months to mice [119]. The authors proved *in vitro* that systemic PDE5 inhibition leads to an activated cGMP-dependent protein kinase. Subsequently, phosphorylation of the downstream target GSK 3 β (glycogen synthase kinase-3 β) results in phosphorylated cytosolic β -catenin that is further recognized by E3 ubiquitin ligase and degraded through the proteasome. Detrimental impact of tadalafil on bone metabolism in this study is probably a consequence of long-term treatment with the PDE5 inhibitor and administration of a very high dose. Long-term treatment with a PDE inhibitor can delay healing process, as has been proven by Aydın and coworkers [122]. The authors investigated the influence of the non-selective competitive PDE inhibitor pentoxifylline in a rodent model. An accelerated bone healing after intraperitoneal injection of 50 mg/kg/day pentoxifylline was determined only within the first post-operative week leading to detrimental characteristics of newly formed bone after three weeks of treatment. Furthermore, Yaman and coworkers analyzed the impact of sildenafil in a rodent model applying 10 mg/kg/day sildenafil

citrate via orogastric tube [123]. An improved bone healing, especially an enhanced inflammatory and repair phase, has been detected probably due to NO[•]-dependent raised blood flow. Histing and coworkers also examined the pro-angiogenic effect of sildenafil in a murine femur fracture model. Oral administration of 5 mg/kg PDE5 inhibitor promotes biomechanical stiffness and accelerates bone healing probably via upregulation of angiogenic and osteogenic growth factors [124]. Significantly improved fracture healing could be demonstrated already after two weeks of treatment. Based on a longer half-life, which facilitates oral intake, and a greater selectivity, resulting in less adverse side effects, treatment with tadalafil is more promising from a clinical perspective [121]. Further studies dealt with the influence of inhibitors on cAMP (cyclic adenosine monophosphate)-specific PDE. Thereby, PDE4 represents an important target being predominantly active in inflammatory cells. PDE4 inhibitors such as rolipram or roflumilast promote anti-inflammatory and osteogenic effects mainly for clinical treatment of dermal diseases [125–127]. Kinoshita and coworkers studied the effect of the selective PDE4 inhibitor rolipram on bone formation using a mouse model [128]. After subcutaneous injection of 1–30 mg/kg rolipram over the most used treatment period of five weeks they observed an increased bone mass. Furthermore, Horiuchi and coworkers as well as Tokuhara and coworkers confirmed positive impact on bone formation [125, 129]. In the first study, mice received rhBMP-2 (recombinant human bone morphogenetic protein; 5 µg) containing sponges, and in addition, subcutaneous injections of 10–20 mg/kg/day rolipram resulting in BMP-dependent accelerated bone repair. In the latter study, the authors used polyethyleneglycol (PEG) implants containing 150 µg (500 nmol) rolipram and 5 mg rhBMP-2 in a murine model. Local release of rolipram supports osteogenic effects of BMP-2. As result, a reduction of the applied dose in accordance with adverse side effects such as nausea and emesis and production costs as well as enhancement of efficacy and clinical practicability occurs [125, 126]. However, PDE4 inhibition by rolipram not only leads to increased proliferation and differentiation of osteoblasts, but also intensifies RANKL expression, which promotes osteoclast formation. Increased RANKL expression may be due to activation of PKA (protein kinase A) as a result of the accumulation of cAMP or via activation of MAPK signaling pathway [130].

1.2.4. Agonistic targeting of lipid mediators: sphingosine-1-phosphate and lysophosphatidic acid

Two prominent lipid mediators sphingosine-1-phosphate (S1P) and lysophosphatidic acid (LPA) are able to exert strong effects on vascular system and also contribute to inflammatory responses to a lesser extent [131–133]. S1P is a phospholipid produced by sphingosine kinase (SPHK)-mediated phosphorylation of the membranous precursor molecule sphingosine [131, 134, 135]. Two isozymes SPHK1 and 2 are described in literature being distinguished due to their kinetic as well as tissue-specific expression profile [136, 137]. Hypoxia, for instance, results in an increased SPHK expression. This is based on HIF-dependent transcriptional regulation, as hypoxia-stabilized HIFs can bind to the hypoxia-inducible factor-responsive elements of the SPHK promoter and thus drive its expression [138]. It turned out that SPHK1 is located in cytosol, more precisely in proximity of the membrane, whereas SPHK2 is thought to be localized not only in cytosol, but also in cell organelles, such as cell nucleus or endoplasmatic reticulum [139–142]. After the lipid mediator has been formed, S1P is transported out of the cell into the plasma by specific transporters such as Spns2 (spinster homolog 2) [131, 143–145]. This mechanism is called “inside-out” signaling [138]. In general, S1P acts as an intracellular messenger or extracellular ligand regulating vessel formation, inflammation, and bone regeneration as well as innate and adaptive immunity [136, 137, 146]. The small molecule regulates important cellular processes such as proliferation, differentiation, and migration of osteoblasts and osteoclasts. More precisely, the lipid mediator exerts chemoattractive effects on osteoprogenitor cells controlling migratory behavior of these cells in the context of bone repair [135, 147]. S1P can stimulate recruitment and proliferation of osteoblasts as well as osteoclasts via regulating RANKL expression, whereby S1P gradient between blood and bone microenvironment defines migratory and chemotactic effect determining

bone remodeling decisively. Osteoclasts are likely to recruit osteoblastic precursor cells via secretion of S1P, in turn activating Wnt – BMP signaling pathway. *Vice versa*, activated osteoblasts presumably stimulate osteoclastogenesis via secretion of RANKL [148]. However, chemotactic influence seems to depend on differentiation status of cells as well as on activation of S1P downstream signaling pathway [149]. S1P signaling occurs via G-protein coupled receptors (GPCRs), whereby five S1P receptor types (S1P_{1–5}) are described [150]. S1P signaling activates downstream targets like COX-2, PI3K (phosphatidylinositol-3-kinase), PLC (phospholipase C), and eNOS (Fig. 3), or further cross-activates receptor tyrosine kinases such as VEGF receptor [134, 138, 143, 151–154]. In addition, an increased differentiation of osteoblasts via MAPK cascade or Smad (small mothers against decapentaplegic homolog)-dependent signaling is described in literature [155]. S1P_{1–3} receptors are predominantly expressed by endothelial cells, whereas S1P₄ and S1P₅ are expressed in immune and neuronal cells [156]. Osteoblasts express the receptor subtypes S1P_{1–3}, while expression of S1P_{1–2} is seen in osteoclasts [134, 157, 158]. S1P₁ receptor just couples to an inhibitory G-protein promoting activation of ERK (extracellular signal-regulated kinase) and Rac protein (Ras homolog/GTPase), further inducing cell migration. On the contrary, S1P₂ inhibits Rac and its mediated migratory effects. Instead, S1P₂ receptor signals also via other G protein subtypes like G_{12/13} activating Rho (Ras homolog/GTPase) and Rho-associated, coiled-coil-containing protein kinase (ROCK) [143, 157, 159]. Like S1P₂, S1P₃ participates in formation of integrin-mediated focal adhesion contacts via Rho and seems to play a greater regulatory role in vasculature than in bone remodeling. However, activation of Rho via activated G_{12/13} protein appears to be less efficient than activation of downstream signals by G_i, because migratory effect of S1P₃-mediated signals is comparable to those of S1P₁ [136, 143, 159]. Since both S1P₁ and S1P₃ have a positive influence on cell proliferation and migration as well as on vascularization, a synergistic interplay of both receptor subtypes is suspected to potentially enhance downstream effects [160]. There are two different ways to inactivate S1P. Degradation via lyases is irreversible, whereas dephosphorylation by phosphatases, especially phosphohydrolases, is a reversible process [131, 135, 137, 152, 161]. However, S1P receptor activation appears to be low during homeostatic conditions and becomes enhanced particularly in cases of inflammation due to higher ligand availability [162]. Wacker and coworkers prepared a RGD (Arginine – Glycine – Aspartate motif)-modified hydrogel releasing S1P specifically depending on concentration of lipid transporter albumin being incorporated into the hydrogel [163]. The authors found that at higher albumin concentration, S1P release is slower but still capable of promoting cell migration and angiogenesis.

Furthermore, Sefcik and coworkers demonstrated an enhanced bone formation and angiogenesis after scaffold-based S1P release in a rodent critical-size cranial defect model [164]. In addition, Tengood and coworkers investigated dual release of S1P together with the growth factor VEGF in terms of best timing [165]. It turned out that best cell recruitment and vascularization was seen due to sequential delivery with initial VEGF and following S1P release. Therefore, authors concluded that use of S1P in therapeutic intervention might be an option for stabilizing vascular structures only in later angiogenic processes. In order to investigate effects of S1P signaling pathways in more detail and to address them therapeutically, several small molecules have been investigated and summarized in terms of their vascular and bone effects by Segar and coworkers as well as Sartawi and coworkers (Table 1) [57, 166]. The authors have titled molecules such as FTY720 (fingolimod; S1P analog; S1P_{1,3–5} agonist), SEW2871 (oxadiazole derivative; S1P₁ agonist), VPC01091 (octylphenyl-substituted cyclophenyl derivative; S1P₁ agonist and S1P₃ antagonist), and JTE-013 (pyrazolopyridine derivative [167]; S1P₂ antagonist). All above-mentioned small molecules elicit positive impact on cellular migration, proliferation, angiogenesis, or bone healing. However, FTY720 is the only FDA-approved drug for treatment of multiple sclerosis and is the only one being able to address multiple cellular targets, wherefore the S1P_{1,3–5} receptor agonist is predominantly used to further potentiate anabolic effects of endogenous S1P [135, 151, 168]. FTY720 is a structural analog of sphingosine being phosphorylated by SPHK to generate

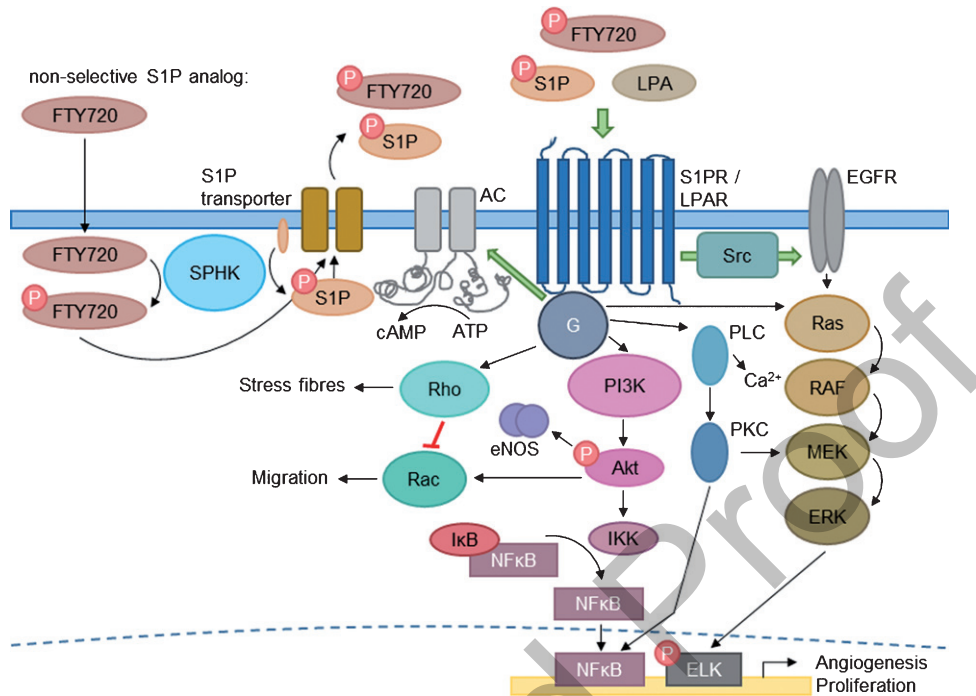


Fig. 3. Lipid-mediated signaling. Characteristic for G-protein coupled lipid receptor (S1PR or LPAR)-mediated downstream signaling pathways, initiated via S1P, its analog FTY720 or LPA, is the activation of certain cytosolic mediators like AC, PI3K – Akt – eNOS, IKK or Rac, Rho, PLC – PKC. Likewise, MAPK cascade including Ras, RAF, mitogen-activated protein kinase kinase (MEK) and ERK is stimulated in order to enhance proliferation and angiogenesis due to activated transcription factors. However, S1P and its analog first have to be phosphorylated intracellularly by SPHK and then have to be discharged via S1P transporter (modified according to Binder and coworkers [187]).

phospho-FTY720, in turn binding to S1P receptors in a highly affine manner [134]. However, phosphorylation of FTY720 by SPHK2 appears to be more efficient compared to SPHK1 [169]. Unlike S1P having a short biological half-life of about 15 min, FTY720 does not degrade quickly since the agonist avoids enzymatic degradation by lyases. Nevertheless, FTY720 undergoes conversion from a phosphorylated to a non-phosphorylated state due to activity of lipid phosphohydrolases [136, 161, 170, 171]. Heilmann and coworkers examined impact of systematically applied 6 mg/kg FTY720 three days post-operatively via subcutaneous injections in a murine femur fracture model [158]. The authors concluded that the agonist had no effect on bone regeneration since no significantly changed mechanical characteristics or osteoclast quantity could be observed. This assessment may result from a diminished local attraction of osteoprogenitor cells [158]. By contrast, Aronin and coworkers showed an improvement of mechanical stability by administration of FTY720 [151]. The authors used polymer-coated bone allografts for local release of the agonist FTY720 in a rodent tibia defect model. Likewise, Huang and coworkers employed allografts as basis of locally released FTY720 to treat critical-size calvarial bone defects [172]. Increased angiogenesis and osteogenesis resulted, based on enhanced recruitment of precursor cells. Additionally, an increase in bone regeneration was detectable, especially within the first four weeks, which could be due to an initial burst of FTY720. The positive effects of FTY720 reported above have been confirmed by Das and coworkers [173]. Moreover, they demonstrated an immunomodulatory function by switching macrophage from a pro- to an anti-inflammatory state measured two weeks after treatment. Wang and coworkers investigated two approaches regarding the delivery of FTY720 [174]. The authors examined both allografts with absorbed drug (193 μg FTY720/ mm^3 bone

graft) in a rodent critical-sized cranial defect model and local release of the S1P analog (1–10 nM) from an injectable extracellular matrix gel using a murine tibial fracture model. Treatment with FTY720 resulted in an increased bone volume, accelerated vascularization, and promoted recruitment of osteoprogenitor cells. Furthermore, Das and coworkers considered local delivery of FTY720 by use of polymer nanofiber scaffolds in a rodent mandibular critical-size defect model resulting in enhanced vascularization and bone repair based on recruitment of anti-inflammatory macrophages [175]. Release kinetic of S1P analog from degradable polymer scaffolds depends on chemical material-specific properties as well as on distribution of FTY720, wherein a near-surface binding of the agonist to the carrier matrix has led to a rapid accumulated release after application at the fracture site [176]. Hughes and coworkers as well as Awojodu and coworkers confirmed the observation that S1P molecule and S1P_{1/3} receptor downstream signaling pathways, result in prevalent anti-inflammatory macrophage phenotype during the acute inflammatory phase promoting further angiogenesis [177, 178]. Possible reasons for this could be a reduction of pro-inflammatory cytokines and transcription factors such as NF κ B (nuclear factor ‘kappa-light-chain-enhancer’ of activated B-cells), which leads to a reduction in the iNOS expression and NO[•] production, as well as a shift to cytokines predominantly present during regeneration processes. In contrast to S1P induced receptor recycling, after FTY720 triggered downstream signaling pathways as an agonist, the lipid mediator also exerts functional antagonistic effects due to ultimate desensitization and lysosomal degradation of the S1P₁ receptor based on polyubiquitinylation. Downregulation of S1P₁ receptor further results in reduced angiogenesis [135, 143, 179, 180]. In addition to the S1P analogues triggering or amplifying pro-osteogenic and pro-angiogenic downstream signaling pathways, key enzymes of S1P activity and function, SPHKs and lyases, might also be interesting therapeutic targets [181, 182]. Ji and coworkers already showed that the SPHK activator K6PC-5 (decanamide derivative) significantly increased survival of glucocorticoid-treated cells and relieved dexamethasone-induced apoptosis [181]. The authors suggested an underlying increase in S1P production and further enhanced Akt (protein kinase B)-mediated signaling. Investigation of whether activators in case of SPHKs or inhibitors against S1P degrading lyases positively influence healing process after a critical-size bone defect is still pending.

Another lysophospholipid is LPA being generated based on the conversion of precursor phospholipids such as lysophosphatidylcholine, -ethanolamine, or -serine via phospholipase A1/A2 and lysophospholipase D [131, 148, 183–185]. Similar to S1P, when LPA occurs in serum, it is immediately bound and stabilized by carrier protein albumin as it is not assailable for cleavage enzymes like phospholipase B in the carrier-bound state [183–186]. The lipid mediator elicits similar cellular responses regarding enhanced angiogenesis, chemotaxis, and proliferation like S1P, but relies on its own subset of six different G-protein coupled signaling receptors (LPA_{1–6}) evoking various downstream effects depending on the activated G-protein subtype (G_s, G_i, G_q or G_{12/13}) [150, 184, 187–189]. For example, LPA stimulates osteoblastogenesis, whereby a synergistic interaction with the vitamin D3 metabolite is supposed to support this process [186]. LPA receptors have a high sequence similarity differing mainly with respect to their C-termini. These C-terminal ends are particularly associated with phosphorylation reactions on distinct serine and threonine residues, which subsequently control recruitment of adapter proteins and formation of signal complexes via specific domain binding motifs [185]. Usually, activated downstream signal pathways mainly includes MAPK and PI3K signaling [190]. For example, ERK is activated by epidermal growth factor receptor (EGFR) as a result of LPA- and GPCR-mediated transactivation in wound healing processes [191]. However, Karagiosis and coworkers showed *in vitro* that activation and nuclear translocation of ERK can also take place independent of usual EGFR transactivation and further stimulation of adaptor protein Ras (rat sarcoma) by LPA ligand [188]. Still promoted chemotaxis of preosteoblasts is probably due to cross-activation of ERK by phosphorylation of phosphoinositide- or calcium-dependent kinases. This suggests that EGFR does not seem to be essential for triggering ERK stimulation [185]. It is known that receptors LPA_{1–3}, which are the only

LPA receptors with a phylogenetic similarity to S1P₁ receptors, are able to activate PI3K and MAPK. Further, these LPA receptors stimulate PLC and PKC (protein kinase C) via the active coupled G_i subunit and by acting via G_q. LPA₁ and LPA₂ can also control cell migration through regulation of Rho and ROCK via the coupled G_{12/13} protein. LPA₁ and LPA₃ might elicit pro-angiogenic effects based on LPA-induced COX-2 activation together with EGFR transactivation, and finally resulting in NFκB-dependent raised VEGF transcription. LPA₄ is also able to activate G_s, in addition to the previously mentioned G protein subtypes, in turn stimulating AC (adenylate cyclase). Presumably, LPA₁ and LPA₄ mainly determine bone metabolism, perhaps in opposite ways, as LPA₁ promotes osteoblast differentiation, whereas LPA₄ inhibits this [57, 150, 183, 184, 192–195]. LPA₅ transduces pleiotropic effects of the LPA ligand through G_{12/13} or G_q whereas LPA₆ may be coupled to G_i, G_{12/13} or G_s [57, 184, 192]. Analogous to S1P, in addition to the frequent GPCR-mediated signal pathways, a connection or cross-talk to inflammation-modulating PG synthesis pathway is also assumed in the case of LPA-induced downstream signaling. Preliminary experiments indicate an increase in PGE₂ production as a result of LPA administration and LPA₁-mediated signals *in vitro* [196]. Certainly, LPA can also be enzymatically degraded due to dephosphorylation by lipid phosphate phosphatases like LPP1 forming monoacylglycerol [131, 184, 185, 197]. However, signaling may also be terminated by ligand-induced desensitization of the receptor, being a typical mechanism of signal regulation for GPCRs [185]. Loss of LPA₁ activity leads to detrimental effects regarding bone metabolism because of reduced osteogenesis. On the contrary, LPA₄ knockout results in an opposite effect, more precisely in increased bone mass due to osteoblastic differentiation [184, 187]. LPA₁ is not only involved in osteoblastogenesis, but also in osteoclastogenesis, suggesting a balancing role of LPA₁-mediated signals in bone remodeling [57, 192]. LPA is believed to stimulate survival of osteoclasts directly via LPA₁-mediated and calcium-dependent activation of NFAT (nuclear factor of activated T-cells) or indirectly through an increase of osteoblast-driven RANKL secretion [198–201]. Certainly, signaling promoting bone formation appears to be stronger and superior compared to triggered bone resorption [194]. Apart from direct effect on osteoblasts, LPA₁ downstream signals show a positive influence on mature bone cells deriving from osteoblasts. Thereby, LPA stimulates formation of dendritic cell contacts to build a functional connective network consisting of established cell-cell-communication and mechanical load-bearing structures in the course of bone healing [202]. Moreover, LPA₃ downstream signaling seems to promote osteoblastogenesis of precursor cells. Certainly, the lipid regulates osteoclastogenesis probably via PI3K – Akt signaling as well [187]. In addition to osteogenic effects, LPA also elicits pro-angiogenic responses, as LPA_{1–3} downstream signals favor vessel vascularization and vessel growth [57]. For example, loss of LPA₁ leads to development of vascular defects. Indeed, signaling via several LPA receptors seems to be responsible for vessel stability [184]. Blackburn and coworkers outlined a variety of *in vitro* experiments investigating impact of LPA on various cell types, such as osteoblasts, osteoclasts, and chondrocytes of rodent or human origin [183]. These studies revealed an LPA-dependent stimulation of Rho and MAPK signaling pathway as well as a PI3K-dependent calcium increase favoring cellular migration and maturation. LPA has only a short half-life of less than one minute due to a rapid liver accumulation and metabolism. For this reason, lipid mediator in this form would not be practicable with respect to a possible use in the clinic. Therefore, investigation of new lipid mimetics as possible therapeutics that selectively and efficiently bind to receptors is essential [187, 203]. Recently, Yu and Coworkers showed that an LPA analogue more efficiently supports bone formation compared to LPA itself [204]. In addition, regulated local release systems should be established analogously to the already described S1P, for instance with the aid of suitable biomaterials [187, 203].

When considering regulation of angiogenic processes in the context of critical-size bone defect treatment, not only cell membrane-derived lipids could play an important role, but also receptors involved in membrane remodeling might be of particular interest. Low-density lipoprotein receptor-

related proteins (LRPs) act, among others, as co-receptors of Wnt signaling pathway (see section ‘Agonistic targeting of Wnt signaling pathway’ in Part III – Further strategies for local and systemic modulation [205]) and are of recent interest based on involvement in lipid homeostasis and growth factor-triggered signal transduction resulting in affected inflammation or angiogenesis. In addition to the typical downstream Wnt signaling pathway, possible activated LRP downstream target molecules are presumably PKA and eNOS. Hypoxia-induced activity of LRPs support the hypothesis regarding the participation of angiogenesis regulation [17]. Mao and coworkers summarized a series of publications dealing with the role of LRPs in angiogenesis [17]. It was shown that, for instance, loss of LRP function leads to disturbed blood vessel formation in zebrafish and mouse models probably due to an affected BMP, S1P, ERK or VEGF signaling [206]. As LRP receptors recently also came into focus of research regarding angiogenesis regulation in the context of bone repair, investigation of the precise signaling mechanisms and examination of whether LRP receptors are suitable for therapeutic intervention remains to be done. For development of critical-size bone defects treatment approaches, alongside with compounds regulating inflammatory and angiogenic processes (like COX-inhibitors, NO• donors or lipid mediators) being already discussed in detail, other substance classes (e.g. statins, strontium or bisphosphonates [205]) and target structures might play an important role. For providing the best strategy for fracture healing, drug concentration or combination as well as delivery method and, especially, treatment duration should be included in establishing new approaches.

2. Conclusion

One of the key processes in healing critical-size bone defects is formation of new blood vessels. New therapeutic strategies that stimulate and regulate blood vessel formation potentially contribute to improvement of bone regeneration. Very promising approaches are currently those involving local release of nitric NO• by small molecule bi-functional (hybrid) drugs like NO•-COXIBs. Furthermore, lipid analogues released via e.g. biomaterial-based drug release systems show promising results. However, as discussed elsewhere, each case has to be considered individually and the type of drugs, possible co-medications, dosage, beginning and duration of therapy has to be optimized accordingly.

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Author contributions

All authors have jointly conceived and written this review article. All authors read and approved the final manuscript.

Conflicts of interest

The authors declare that they have no conflict of interest. The founding sponsors had no influence on the conception of the article, the interpretation of literature data or the conclusions drawn, and in the decision to publish this review article.

References

- [1] Rothe R, Schulze S, Neuber C, Hauser S, Rammelt S, Pietzsch J. Adjuvant drug-assisted bone healing: Part I – Modulation of inflammation. *Clin Hemorheol Microcirc.* in press.
- [2] Xing Y, Wang R, Chen D, Mao J, Shi R, Wu Z, et al. COX2 is involved in hypoxia-induced TNF-alpha expression in osteoblast. *Sci Rep.* 2015;5:10020. doi: 10.1038/srep10020
- [3] Rahman SU, Lee MS, Baek JH, Ryoo HM, Woo KM. The prolyl hydroxylase inhibitor dimethylxalylglycine enhances dentin sialophosphoprotein expression through VEGF-induced Runx2 stabilization. *PLoS One.* 2014;9(11):e112078. doi: 10.1371/journal.pone.0112078
- [4] Chaparro O, Linero I. Regenerative Medicine: A New Paradigm in Bone Regeneration. *Advanced Techniques in Bone Regeneration: InTech;* 2016. doi: 10.5772/62523
- [5] Hankenson KD, Dishowitz M, Gray C, Schenker M. Angiogenesis in bone regeneration. *Injury.* 2011;42(6):556-61. doi: 10.1016/j.injury.2011.03.035
- [6] Kanczler JM, Oreffo ROC. Osteogenesis and angiogenesis: The potential for engineering bone. *Eur Cell Mater.* 2008;15:100-14. doi: 10.22203/eCM.v015a08
- [7] Dittmer KE, Firth EC. Mechanisms of bone response to injury. *J Vet Diagn Invest.* 2017;29(4):385-95. doi: 10.1177/1040638716679861
- [8] Grüneboom A, Hawwari I, Weidner D, Culemann S, Müller S, Henneberg S, et al. A network of trans-cortical capillaries as mainstay for blood circulation in long bones. *Nat Metab.* 2019;1(2):236-50. doi: 10.1038/s42255-018-0016-5
- [9] Schmidt-Bleek K, Schell H, Lienau J, Schulz N, Hoff P, Pfaff M, et al. Initial immune reaction and angiogenesis in bone healing. *J Tissue Eng Regen Med.* 2014;8(2):120-30. doi: 10.1002/term.1505
- [10] Beamer B, Hettrich C, Lane J. Vascular endothelial growth factor: An essential component of angiogenesis and fracture healing. *HSS J.* 2010;6(1):85-94. doi: 10.1007/s11420-009-9129-4
- [11] Hauser S, Jung F, Pietzsch J. Human Endothelial Cell Models in Biomaterial Research. *Trends Biotechnol.* 2017;35(3):265-77. doi: 10.1016/j.tibtech.2016.09.007
- [12] Beyer S, Koch M, Lee YH, Jung F, Blocki A. An In Vitro Model of Angiogenesis during Wound Healing Provides Insights into the Complex Role of Cells and Factors in the Inflammatory and Proliferation Phase. *Int J Mol Sci.* 2018;19(10). doi: 10.3390/ijms19102913
- [13] Ghanbari H, Vakili-Ghartavol R. Bone Regeneration: Current Status and Future Prospects. *Advanced Techniques in Bone Regeneration* 2016. doi: 10.5772/63912
- [14] Kosaki N, Takaishi H, Kamekura S, Kimura T, Okada Y, Minqi L, et al. Impaired bone fracture healing in matrix metalloproteinase-13 deficient mice. *Biochem Biophys Res Commun.* 2007;354(4):846-51. doi: 10.1016/j.bbrc.2006.12.234
- [15] Araldi E, Schipani E. Hypoxia, HIFs and bone development. *Bone.* 2010;47(2):190-6. doi: 10.1016/j.bone.2010.04.606
- [16] Chu H, Wang Y. Therapeutic angiogenesis: Controlled delivery of angiogenic factors. *Therapeutic delivery.* 2012;3(6):693-714. doi: 10.4155/tde.12.50
- [17] Mao H, Xie L, Pi X. Low-Density Lipoprotein Receptor-Related Protein-1 Signaling in Angiogenesis. *Front Cardiovasc Med.* 2017;4:34. doi: 10.3389/fcvm.2017.00034
- [18] Yoo SY, Kwon SM. Angiogenesis and its therapeutic opportunities. *Mediators Inflamm.* 2013;2013:127170. doi: 10.1155/2013/127170
- [19] Jiang B, Brey EM. Formation of stable vascular networks in engineered tissues. *Regenerative Medicine and Tissue Engineering-Cells and Biomaterials: InTech;* 2011. doi: 10.5772/23223
- [20] Ai-Aql Z, Alagl AS, Graves DT, Gerstenfeld LC, Einhorn TA. Molecular mechanisms controlling bone formation during fracture healing and distraction osteogenesis. *J Dent Res.* 2008;87(2):107-18. doi: 10.1177/154405910808700215
- [21] Hoeben A, Landuyt B, Highley MS, Wildiers H, Van Oosterom AT, De Bruijn EA. Vascular endothelial growth factor and angiogenesis. *Pharmacol Rev.* 2004;56(4):549-80. doi: 10.1124/pr.56.4.3

- [22] Mosch B, Reissenweber B, Neuber C, Pietzsch J. Eph receptors and ephrin ligands: Important players in angiogenesis and tumor angiogenesis. *J Oncol.* 2010;2010:135285. doi: 10.1155/2010/135285
- [23] Arthur A, Panagopoulos RA, Cooper L, Menicanin D, Parkinson IH, Codrington JD, et al. EphB4 enhances the process of endochondral ossification and inhibits remodeling during bone fracture repair. *J Bone Miner Res.* 2013;28(4):926-35. doi: 10.1002/jbmr.1821
- [24] Zhao C, Irie N, Takada Y, Shimoda K, Miyamoto T, Nishiwaki T, et al. Bidirectional ephrinB2-EphB4 signaling controls bone homeostasis. *Cell Metab.* 2006;4(2):111-21. doi: 10.1016/j.cmet.2006.05.012
- [25] Pasquale EB. Eph-ephrin bidirectional signaling in physiology and disease. *Cell.* 2008;133(1):38-52. doi: 10.1016/j.cell.2008.03.011
- [26] Allan EH, Hausler KD, Wei T, Gooi JH, Quinn JM, Crimeen-Irwin B, et al. EphrinB2 regulation by PTH and PTHrP revealed by molecular profiling in differentiating osteoblasts. *J Bone Miner Res.* 2008;23(8):1170-81. doi: 10.1359/jbmr.080324
- [27] Carano RA, Filvaroff EH. Angiogenesis and bone repair. *Drug Discov Today.* 2003;8(21):980-9. doi: 10.1016/S1359-6446(03)02866-6
- [28] Saran U, Piperni SG, Chatterjee S. Role of angiogenesis in bone repair. *Arch Biochem Biophys.* 2014;561:109-17. doi: 10.1016/j.abb.2014.07.006
- [29] Largo RA, Ramakrishnan VM, Ehrbar M, Ziogas A, Plock JA, Eberli D. Angiogenesis and Vascularity for Tissue Engineering Applications. *Regenerative Medicine and Tissue Engineering-Cells and Biomaterials: InTech;* 2011. doi: 10.5772/25141
- [30] Fan L, Li J, Yu Z, Dang X, Wang K. The hypoxia-inducible factor pathway, prolyl hydroxylase domain protein inhibitors, and their roles in bone repair and regeneration. *Biomed Res Int.* 2014;2014:239356. doi: 10.1155/2014/239356
- [31] Krock BL, Skuli N, Simon MC. Hypoxia-induced angiogenesis: Good and evil. *Genes Cancer.* 2011;2(12):1117-33. doi: 10.1177/1947601911423654
- [32] Kimmel HM, Grant A, Ditata J. The Presence of Oxygen in Wound Healing. *Wounds: A compendium of clinical research and practice.* 2016;28(8):264-70. issn: 1044-7946.
- [33] Stegen S, van Gastel N, Carmeliet G. Bringing new life to damaged bone: The importance of angiogenesis in bone repair and regeneration. *Bone.* 2015;70:19-27. doi: 10.1016/j.bone.2014.09.017
- [34] Jung C, Jung F, Kelm M. The microcirculation in hypoxia: The center of the battlefield for oxygen. *Clin Hemorheol Microcirc.* 2016;63(3):169-72. doi: 10.3233/CH-1663301
- [35] Maes C, Carmeliet G, Schipani E. Hypoxia-driven pathways in bone development, regeneration and disease. *Nat Rev Rheumatol.* 2012;8(6):358-66. doi: 10.1038/nrrheum.2012.36
- [36] Rankin EB, Giaccia AJ, Schipani E. A central role for hypoxic signaling in cartilage, bone, and hematopoiesis. *Curr Osteoporos Rep.* 2011;9(2):46-52. doi: 10.1007/s11914-011-0047-2
- [37] Burke B, Giannoudis A, Corke KP, Gill D, Wells M, Ziegler-Heitbrock L, et al. Hypoxia-induced gene expression in human macrophages: Implications for ischemic tissues and hypoxia-regulated gene therapy. *Am J Pathol.* 2003;163(4):1233-43. doi: 10.1016/S0002-9440(10)63483-9
- [38] Drager J, Harvey EJ, Barralet J. Hypoxia signalling manipulation for bone regeneration. *Expert Rev Mol Med.* 2015;17:e6. doi: 10.1017/erm.2015.4
- [39] Coulet F, Nadaud S, Agrapart M, Soubrier F. Identification of hypoxia-response element in the human endothelial nitric-oxide synthase gene promoter. *J Biol Chem.* 2003;278(47):46230-40. doi: 10.1074/jbc.M305420200
- [40] Fong GH. Regulation of angiogenesis by oxygen sensing mechanisms. *J Mol Med (Berl).* 2009;87(6):549-60. doi: 10.1007/s00109-009-0458-z
- [41] Hirota K, Semenza GL. Regulation of angiogenesis by hypoxia-inducible factor 1. *Crit Rev Oncol Hematol.* 2006;59(1):15-26. doi: 10.1016/j.critrevonc.2005.12.003
- [42] Pugh CW, Ratcliffe PJ. Regulation of angiogenesis by hypoxia: Role of the HIF system. *Nat Med.* 2003;9(6):677. issn: 1546-170X.
- [43] Aprelikova O, Chandramouli GV, Wood M, Vasselli JR, Riss J, Maranchie JK, et al. Regulation of HIF prolyl hydroxylases by hypoxia-inducible factors. *J Cell Biochem.* 2004;92(3):491-501. doi: 10.1002/jcb.20067
- [44] Semenza GL. Targeting HIF-1 for cancer therapy. *Nat Rev Cancer.* 2003;3(10):721-32. doi: 10.1038/nrc1187
- [45] Riddle RC, Khatri R, Schipani E, Clemens TL. Role of hypoxia-inducible factor-1 α in angiogenic-osteogenic coupling. *J Mol Med (Berl).* 2009;87(6):583-90. doi: 10.1007/s00109-009-0477-9
- [46] Chan MC, Holt-Martyn JP, Schofield CJ, Ratcliffe PJ. Pharmacological targeting of the HIF hydroxylases—a new field in medicine development. *Mol Aspects Med.* 2016;47:54-75. doi: 10.1016/j.mam.2016.01.001

- [47] Komatsu DE, Warden SJ. The control of fracture healing and its therapeutic targeting: Improving upon nature. *J Cell Biochem.* 2010;109(2):302-11. doi: 10.1002/jcb.22418
- [48] Mac Gabhann F, Qutub AA, Annex BH, Popel AS. Systems biology of pro-angiogenic therapies targeting the VEGF system. *Wiley Interdiscip Rev Syst Biol Med.* 2010;2(6):694-707. doi: 10.1002/wsbm.92
- [49] Lu C, Saleh N, Wang X, Sinha A, Decker S, Kazakia G, et al. The role of oxygen during fracture healing. *Bone.* 2013;52(1):220-9. doi: 10.1016/j.bone.2012.09.037
- [50] Hankenson KD, Zimmerman G, Marcucio R. Biological perspectives of delayed fracture healing. *Injury.* 2014;45(Suppl 2):S8-S15. doi: 10.1016/j.injury.2014.04.003
- [51] D'Angelo G, Duplan E, Boyer N, Vigne P, Frelin C. Hypoxia up-regulates prolyl hydroxylase activity: A feedback mechanism that limits HIF-1 responses during reoxygenation. *J Biol Chem.* 2003;278(40):38183-7. doi: 10.1074/jbc.M302244200
- [52] del Peso L, Castellanos MC, Temes E, Martin-Puig S, Cuevas Y, Olmos G, et al. The von Hippel Lindau/hypoxia-inducible factor (HIF) pathway regulates the transcription of the HIF-proline hydroxylase genes in response to low oxygen. *J Biol Chem.* 2003;278(49):48690-5. doi: 10.1074/jbc.M308862200
- [53] Utting JC, Robins SP, Brandao-Burch A, Orriss IR, Behar J, Arnett TR. Hypoxia inhibits the growth, differentiation and bone-forming capacity of rat osteoblasts. *Exp Cell Res.* 2006;312(10):1693-702. doi: 10.1016/j.yexcr.2006.02.007
- [54] Lechler P, Klein SM, Prantl L, Englert C, Renkawitz T, Grifka J. Hypoxic downregulation of cellular proliferation and loss of phenotype stability in human osteoblasts is mediated by HIF-1alpha. *Clin Hemorheol Microcirc.* 2011;49(1-4):279-86. doi: 10.3233/CH-2011-1478
- [55] Komatsu D, Hadjiargyrou M. Activation of the transcription factor HIF-1 and its target genes, VEGF, HO-1, iNOS, during fracture repair. *Bone.* 2004;34(4):680-8. doi: 10.1016/j.bone.2003.12.024
- [56] Tazzyman S, Murdoch C, Yeomans J, Harrison J, Muthana M. Macrophage-mediated response to hypoxia in disease. *Hypoxia (Auckl).* 2014;2:185-96. doi: 10.2147/HP.S49717
- [57] Segar CE, Ogle ME, Botchwey EA. Regulation of angiogenesis and bone regeneration with natural and synthetic small molecules. *Curr Pharm Des.* 2013;19(19):3403-19. doi: 10.2174/1381612811319190007.
- [58] Hu K, Olsen BR. The roles of vascular endothelial growth factor in bone repair and regeneration. *Bone.* 2016;91:30-8. doi: 10.1016/j.bone.2016.06.013
- [59] Towler DA. The osteogenic-angiogenic interface: Novel insights into the biology of bone formation and fracture repair. *Curr Osteoporos Rep.* 2008;6(2):67. doi: 10.1007/s11914-008-0012-x
- [60] Schipani E, Maes C, Carmeliet G, Semenza GL. Regulation of osteogenesis-angiogenesis coupling by HIFs and VEGF. *J Bone Miner Res.* 2009;24(8):1347-53. doi: 10.1359/jbmr.090602
- [61] Arnett TR. Acidosis, hypoxia and bone. *Arch Biochem Biophys.* 2010;503(1):103-9. doi: 10.1016/j.abb.2010.07.021
- [62] Knowles H, Athanasou N. Canonical and non-canonical pathways of osteoclast formation. *Histol Histopathol.* 2009;24(3):337-46. doi: 10.14670/HH-24.337
- [63] Knowles H. Hypoxic regulation of osteoclast differentiation and bone resorption activity. *Hypoxia.* 2015. doi: 10.2147/hp.S95960
- [64] Wang Y, Wan C, Deng L, Liu X, Cao X, Gilbert SR, et al. The hypoxia-inducible factor alpha pathway couples angiogenesis to osteogenesis during skeletal development. *J Clin Invest.* 2007;117(6):1616-26. doi: 10.1172/JCI1581
- [65] Wan C, Gilbert SR, Wang Y, Cao X, Shen X, Ramaswamy G, et al. Activation of the hypoxia-inducible factor-1alpha pathway accelerates bone regeneration. *Proc Natl Acad Sci U S A.* 2008;105(2):686-91. doi: 10.1073/pnas.0708474105
- [66] Wan C, Shao J, Gilbert SR, Riddle RC, Long F, Johnson RS, et al. Role of HIF-1alpha in skeletal development. *Ann N Y Acad Sci.* 2010;1192:322-6. doi: 10.1111/j.1749-6632.2009.05238.x
- [67] Zou D, Zhang Z, Ye D, Tang A, Deng L, Han W, et al. Repair of critical-sized rat calvarial defects using genetically engineered bone marrow-derived mesenchymal stem cells overexpressing hypoxia-inducible factor-1alpha. *Stem Cells.* 2011;29(9):1380-90. doi: 10.1002/stem.693
- [68] Schofield CJ, Ratcliffe PJ. Oxygen sensing by HIF hydroxylases. *Nat Rev Mol Cell Biol.* 2004;5(5):343-54. doi: 10.1038/nrm1366
- [69] Nagel S, Talbot NP, Mecinović J, Smith TG, Buchan AM, Schofield CJ. Therapeutic manipulation of the HIF hydroxylases. *Antioxid Redox Signal.* 2010;12(4):481-501. doi: 10.1089/ars.2009.2711
- [70] Appelhoff RJ, Tian YM, Raval RR, Turley H, Harris AL, Pugh CW, et al. Differential function of the prolyl hydroxylases PHD1, PHD2, and PHD3 in the regulation of hypoxia-inducible factor. *J Biol Chem.* 2004;279(37):38458-65. doi: 10.1074/jbc.M406026200
- [71] Fraisl P, Aragonés J, Carmeliet P. Inhibition of oxygen sensors as a therapeutic strategy for ischaemic and inflammatory disease. *Nat Rev Drug Discov.* 2009;8(2):139-52. doi: 10.1038/nrd2761

- [72] Shen X, Wan C, Ramaswamy G, Mavalli M, Wang Y, Duvall CL, et al. Prolyl hydroxylase inhibitors increase neoangiogenesis and callus formation following femur fracture in mice. *J Orthop Res.* 2009;27(10):1298-305. doi: 10.1002/jor.20886
- [73] Ding H, Gao YS, Wang Y, Hu C, Sun Y, Zhang C. Dimethylxaloylglycine increases the bone healing capacity of adipose-derived stem cells by promoting osteogenic differentiation and angiogenic potential. *Stem Cells Dev.* 2014;23(9):990-1000. doi: 10.1089/scd.2013.0486
- [74] Wu C, Zhou Y, Chang J, Xiao Y. Delivery of dimethylxallyl glycine in mesoporous bioactive glass scaffolds to improve angiogenesis and osteogenesis of human bone marrow stromal cells. *Acta Biomater.* 2013;9(11):9159-68. doi: 10.1016/j.actbio.2013.06.026
- [75] Donneys A, Farberg AS, Tchanque-Fossuo CN, Deshpande SS, Buchman SR. Deferoxamine enhances the vascular response of bone regeneration in mandibular distraction osteogenesis. *Plast Reconstr Surg.* 2012;129(4):850-6. doi: 10.1097/PRS.0b013e31824422f2
- [76] Drager J, Ramirez-GarciaLuna JL, Kumar A, Gbureck U, Harvey EJ, Barralet JE. Hypoxia Biomimicry to Enhance Monetite Bone Defect Repair. *Tissue Eng Part A.* 2017;23(23-24):1372-81. doi: 10.1089/ten.tea.2016.0526
- [77] Stewart R, Goldstein J, Eberhardt A, Chu T-MG, Gilbert S. Increasing vascularity to improve healing of a segmental defect of the rat femur. *J Orthop Trauma.* 2011;25(8):472. doi: 10.1097/BOT.0b013e31822588d8
- [78] Cho EA, Song HK, Lee SH, Chung BH, Lim HM, Lee MK. Differential *in vitro* and cellular effects of iron chelators for hypoxia inducible factor hydroxylases. *J Cell Biochem.* 2013;114(4):864-73. doi: 10.1002/jcb.24423
- [79] Wu C, Zhou Y, Fan W, Han P, Chang J, Yuen J, et al. Hypoxia-mimicking mesoporous bioactive glass scaffolds with controllable cobalt ion release for bone tissue engineering. *Biomaterials.* 2012;33(7):2076-85. doi: 10.1016/j.biomaterials.2011.11.042
- [80] Huang J, Liu L, Feng M, An S, Zhou M, Li Z, et al. Effect of CoCl₂ on fracture repair in a rat model of bone fracture. *Mol Med Rep.* 2015;12(4):5951-6. doi: 10.3892/mmr.2015.4122
- [81] Nangaku M, Izuhara Y, Takizawa S, Yamashita T, Fujii-Kuriyama Y, Ohneda O, et al. A novel class of prolyl hydroxylase inhibitors induces angiogenesis and exerts organ protection against ischemia. *Arterioscler Thromb Vasc Biol.* 2007;27(12):2548-54. doi: 10.1161/ATVBAHA.107.148551
- [82] Mancini L, Moradi-Bidhendi N, Becherini L, Martineti V, MacIntyre I. The biphasic effects of nitric oxide in primary rat osteoblasts are cGMP dependent. *Biochem Biophys Res Commun.* 2000;274(2):477-81. doi: 10.1006/bbrc.2000.3164
- [83] Wimalawansa SJ. Nitric oxide and bone. *Ann N Y Acad Sci.* 2010;1192:391-403. doi: 10.1111/j.1749-6632.2009.05230.x
- [84] Van't Hof RJ, Ralston SH. Nitric oxide and bone. *Immunology.* 2001;103(3):255-61. doi: 10.1046/j.1365-2567.2001.01261.x
- [85] Diwan AD, Wang MX, Jang D, Zhu W, Murrell GA. Nitric oxide modulates fracture healing. *J Bone Miner Res.* 2000;15(2):342-51. doi: 10.1359/jbmr.2000.15.2.342
- [86] Krausz A, Friedman AJ. Nitric oxide as a surgical adjuvant. *Future science OA.* 2015;1(1). doi: 10.4155/fso.15.56
- [87] Nichols SP, Storm WL, Koh A, Schoenfish MH. Local delivery of nitric oxide: Targeted delivery of therapeutics to bone and connective tissues. *Adv Drug Deliv Rev.* 2012;64(12):1177-88. doi: 10.1016/j.addr.2012.03.002
- [88] Kim-Shapiro DB, Gladwin MT. Nitric oxide pathology and therapeutics in sickle cell disease. *Clin Hemorheol Microcirc.* 2018;68(2-3):223-37. doi: 10.3233/CH-189009
- [89] Rangaswami H, Schwappacher R, Marathe N, Zhuang S, Casteel DE, Haas B, et al. Cyclic GMP and protein kinase G control a Src-containing mechanosome in osteoblasts. *Sci Signal.* 2010;3(153):ra91. doi: 10.1126/scisignal.2001423
- [90] R. Silva B, D. Paula T, Paulo M, M. Bendhack L. Nitric Oxide Signaling and the Cross Talk with Prostanoids Pathways in Vascular System. *Medicinal Chemistry.* 2017;13(4):319-33. doi: 10.2174/1573406412666161228115627
- [91] Spitler R, Schwappacher R, Wu T, Kong X, Yokomori K, Pilz RB, et al. Nitrosyl-cobinamide (NO-Cbi), a new nitric oxide donor, improves wound healing through cGMP/cGMP-dependent protein kinase. *Cell Signal.* 2013;25(12):2374-82. doi: 10.1016/j.cellsig.2013.07.029
- [92] Wang H, Zhang R, Wen S, McCafferty DM, Beck PL, MacNaughton WK. Nitric oxide increases Wnt-induced secreted protein-1 (WISP-1/CCN4) expression and function in colitis. *J Mol Med (Berl).* 2009;87(4):435-45. doi: 10.1007/s00109-009-0445-4
- [93] Teixeira CC, Agoston H, Beier F. Nitric oxide, C-type natriuretic peptide and cGMP as regulators of endochondral ossification. *Dev Biol.* 2008;319(2):171-8. doi: 10.1016/j.ydbio.2008.04.031
- [94] Kim SF, Huri DA, Snyder SH. Inducible nitric oxide synthase binds, S-nitrosylates, and activates cyclooxygenase-2. *Science.* 2005;310(5756):1966-70. doi: 10.1126/science.1119407
- [95] Hao Y-J, Tang Y, Chen F-B, Pei F-X. Different Doses of Nitric Oxide Donor Prevent Osteoporosis in Ovariectomized Rats. *Clin Orthop Rel Res.* 2005;&NA;(435):226-31. doi: 10.1097/01.blo.0000153990.74837.73

- [96] Saura M, Tarin C, Zaragoza C. Recent insights into the implication of nitric oxide in osteoblast differentiation and proliferation during bone development. *ScientificWorldJournal*. 2010;10:624-32. doi: 10.1100/tsw.2010.58
- [97] Rajfer RA, Kilic A, Neviasser AS, Schulte LM, Hlaing SM, Landeros J, et al. Enhancement of fracture healing in the rat, modulated by compounds that stimulate inducible nitric oxide synthase: Acceleration of fracture healing via inducible nitric oxide synthase. *Bone Joint Res*. 2017;6(2):90-7. doi: 10.1302/2046-3758.62.BJR-2016-0164.R2
- [98] Zhu W, Diwan AD, Lin JH, Murrell GA. Nitric oxide synthase isoforms during fracture healing. *J Bone Miner Res*. 2001;16(3):535-40. doi: 10.1359/jbmr.2001.16.3.535
- [99] Zhu W, Murrell GA, Lin J, Gardiner EM, Diwan AD. Localization of nitric oxide synthases during fracture healing. *J Bone Miner Res*. 2002;17(8):1470-7. doi: 10.1359/jbmr.2002.17.8.1470
- [100] Kalyanaraman H, Schall N, Pilz RB. Nitric oxide and cyclic GMP functions in bone. *Nitric Oxide*. 2018. doi: 10.1016/j.niox.2018.03.007
- [101] Bechmann N, Kniess T, Kockerling M, Pigorsch A, Steinbach J, Pietzsch J. Novel (pyrazolyl)benzenesulfonamides with a nitric oxide-releasing moiety as selective cyclooxygenase-2 inhibitors. *Bioorg Med Chem Lett*. 2015;25(16):3295-300. doi: 10.1016/j.bmcl.2015.05.059
- [102] Kalyanaraman H, Ramdani G, Joshua J, Schall N, Boss GR, Cory E, et al. A Novel, Direct NO Donor Regulates Osteoblast and Osteoclast Functions and Increases Bone Mass in Ovariectomized Mice. *J Bone Miner Res*. 2017;32(1):46-59. doi: 10.1002/jbmr.2909
- [103] Miller MR, Megson IL. Recent developments in nitric oxide donor drugs. *Br J Pharmacol*. 2007;151(3):305-21. doi: 10.1038/sj.bjp.0707224
- [104] Vendegh Z, Melly A, Toth B, Wolf K, Farkas T, Kadas I, et al. Calcitonin gene-related peptide, substance P, nitric oxide and epinephrine modulate bone marrow micro circulation of the rabbit tibia and femur. *Clin Hemorheol Microcirc*. 2010;45(1):9-17. doi: 10.3233/CH-2010-1282
- [105] Parker JD. Nitrate tolerance, oxidative stress, and mitochondrial function: Another worrisome chapter on the effects of organic nitrates. *J Clin Invest*. 2004;113(3):352-4. doi: 10.1172/JCI21003
- [106] Rejnmark L, Vestergaard P, Mosekilde L. Decreased fracture risk in users of organic nitrates: A nationwide case-control study. *J Bone Miner Res*. 2006;21(11):1811-7. doi: 10.1359/jbmr.060804
- [107] Wimalawansa SJ. Nitric oxide: Novel therapy for osteoporosis. *Expert Opin Pharmacother*. 2008;9(17):3025-44. doi: 10.1517/14656560802197162
- [108] Frost MC, Reynolds MM, Meyerhoff ME. Polymers incorporating nitric oxide releasing/generating substances for improved biocompatibility of blood-contacting medical devices. *Biomaterials*. 2005;26(14):1685-93. doi: 10.1016/j.biomaterials.2004.06.006
- [109] Amadeu TP, Seabra AB, de Oliveira MG, Monte-Alto-Costa A. Nitric oxide donor improves healing if applied on inflammatory and proliferative phase. *J Surg Res*. 2008;149(1):84-93. doi: 10.1016/j.jss.2007.10.015
- [110] Wimalawansa SJ. Rationale for using nitric oxide donor therapy for prevention of bone loss and treatment of osteoporosis in humans. *Ann N Y Acad Sci*. 2007;1117:283-97. doi: 10.1196/annals.1402.066
- [111] Laube M, Kniess T, Pietzsch J. Development of Antioxidant COX-2 Inhibitors as Radioprotective Agents for Radiation Therapy-A Hypothesis-Driven Review. *Antioxidants (Basel)*. 2016;5(2). doi: 10.3390/antiox5020014
- [112] Chegaev K, Lazzarato L, Tosco P, Cena C, Marini E, Rolando B, et al. NO-donor COX-2 inhibitors. New nitrooxy-substituted 1, 5-diarylimidazoles endowed with COX-2 inhibitory and vasodilator properties. *J Med Chem*. 2007;50(7):1449-57. doi: 10.1021/jm0607247
- [113] Wallace JL, Viappiani S, Bolla M. Cyclooxygenase-inhibiting nitric oxide donors for osteoarthritis. *Trends Pharmacol Sci*. 2009;30(3):112-7. doi: 10.1016/j.tips.2009.01.001
- [114] Velazquez C, Rao PN, McDonald R, Knaus EE. Synthesis and biological evaluation of 3,4-diphenyl-1,2,5-oxadiazole-2-oxides and 3,4-diphenyl-1,2,5-oxadiazoles as potential hybrid COX-2 inhibitor/nitric oxide donor agents. *Bioorg Med Chem*. 2005;13(8):2749-57. doi: 10.1016/j.bmc.2005.02.034
- [115] Hoogstraate J, Andersson LI, Berge O-G, Jonzon B, Öjteg G. COX-inhibiting nitric oxide donors (CINODs)—a new paradigm in the treatment of pain and inflammation. *Inflammopharmacology*. 2003;11(4):423-8. doi: 10.1163/156856003322699591
- [116] Bucă BR, Mititelu-Tartau L, Lupusoru R, Lupusoru CE, Rezus C. New insights into the therapeutic use of nitric oxide-donating non-steroid anti-inflammatory drugs. *MSJ*. 2018;122(2):347-51. issn: 2286-2560.
- [117] Geusens P. Naproxenol, a new cyclooxygenase-inhibiting nitric oxide donor (CINOD). *Expert Opin Biol Ther*. 2009;9(5):649-57. doi: 10.1517/14712590902926071
- [118] Gur S, J Kadowitz P, Can Serefoglu E, JG Hellstrom W. PDE5 inhibitor treatment options for urologic and non-urologic indications: 2012 update. *Curr Pharm Des*. 2012;18(34):5590-606. doi: 10.2174/138161212803307554

- [119] Gong Y, Xu CY, Wang JR, Hu XH, Hong D, Ji X, et al. Inhibition of phosphodiesterase 5 reduces bone mass by suppression of canonical Wnt signaling. *Cell Death Dis.* 2014;5:e1544. doi: 10.1038/cddis.2014.510
- [120] Haider H, Lee YJ, Jiang S, Ahmed RP, Ryon M, Ashraf M. Phosphodiesterase inhibition with tadalafil provides longer and sustained protection of stem cells. *Am J Physiol Heart Circ Physiol.* 2010;299(5):H1395-404. doi: 10.1152/ajpheart.00437.2010
- [121] Togrul G, Arıkan M, Korkusuz P, Hesar RH, Eksioğlu MF. Positive effect of tadalafil, a phosphodiesterase-5 inhibitor, on fracture healing in rat femur. *Eklemler Hastalıkları Cerrahisi.* 2015;26(3):137-44. doi: 10.5606/ehc.2015.29
- [122] Aydın K, Şahin V, Gürsu S, Mercan A, Demir B, Yıldırım T. Effect of pentoxifylline on fracture healing: An experimental study. *Eklemler Hastalıkları Cerrahisi.* 2011;22:160-5.
- [123] Yaman F, Atılğan S, Günes N, Ağacayak S, Günay A, Ucan M, et al. Phosphodiesterase-5 inhibitors may facilitate bone defect recovery. *Eur Rev Med Pharmacol Sci.* 2011;15(11):1301-5.
- [124] Histing T, Marciniak K, Scheuer C, Garcia P, Holstein JH, Klein M, et al. Sildenafil accelerates fracture healing in mice. *J Orthop Res.* 2011;29(6):867-73. doi: 10.1002/jor.21324
- [125] Tokuhara Y, Wakitani S, Imai Y, Nomura C, Hoshino M, Yano K, et al. Local delivery of rolipram, a phosphodiesterase-4-specific inhibitor, augments bone morphogenetic protein-induced bone formation. *J Bone Miner Metab.* 2010;28(1):17-24. doi: 10.1007/s00774-009-0103-5
- [126] Dyke HJ, Montana JG. Update on the therapeutic potential of PDE4 inhibitors. *Expert Opin Investig Drugs.* 2002;11(1):1-13. doi: 10.1517/13543784.11.1.1
- [127] Bäumer W, Hoppmann J, Rundfeldt C, Kietzmann M. Highly selective phosphodiesterase 4 inhibitors for the treatment of allergic skin diseases and psoriasis. *Inflamm Allergy Drug Targets.* 2007;6(1):17-26. doi: 10.2174/187152807780077318
- [128] Kinoshita T, Kobayashi S, Ebara S, Yoshimura Y, Horiuchi H, Tsutsumimoto T, et al. Phosphodiesterase inhibitors, pentoxifylline and rolipram, increase bone mass mainly by promoting bone formation in normal mice. *Bone.* 2000;27(6):811-7. doi: 10.1016/S8756-3282(00)00395-1
- [129] Horiuchi H, Saito N, Kinoshita T, Wakabayashi S, Yotsumoto N, Takaoka K. Effect of phosphodiesterase inhibitor-4, rolipram, on new bone formations by recombinant human bone morphogenetic protein-2. *Bone.* 2002;30(4):589-93. doi: 10.1016/S8756-3282(02)00681-6
- [130] Takami M, Cho ES, Lee SY, Kamijo R, Yim M. Phosphodiesterase inhibitors stimulate osteoclast formation via TRANCE/RANKL expression in osteoblasts: Possible involvement of ERK and p38 MAPK pathways. *FEBS Lett.* 2005;579(3):832-8. doi: 10.1016/j.febslet.2004.12.066
- [131] Morris AJ, Selim S, Salous A, Smyth SS. Blood relatives: Dynamic regulation of bioactive lysophosphatidic acid and sphingosine-1-phosphate metabolism in the circulation. *Trends Cardiovasc Med.* 2009;19(4):135-40. doi: 10.1016/j.tcm.2009.07.005
- [132] Smyth SS, Cheng HY, Miriyala S, Panchatcharam M, Morris AJ. Roles of lysophosphatidic acid in cardiovascular physiology and disease. *Biochim Biophys Acta.* 2008;1781(9):563-70. doi: 10.1016/j.bbali.2008.05.008
- [133] Rosen H, Gonzalez-Cabrera P, Marsolais D, Cahalan S, Don AS, Sanna MG. Modulating tone: The overture of S1P receptor immunotherapeutics. *Immunol Rev.* 2008;223(1):221-35. doi: 10.1111/j.1600-065X.2008.00645.x
- [134] Ryu J, Kim HJ, Chang EJ, Huang H, Banno Y, Kim HH. Sphingosine 1-phosphate as a regulator of osteoclast differentiation and osteoclast-osteoblast coupling. *EMBO J.* 2006;25(24):5840-51. doi: 10.1038/sj.emboj.7601430
- [135] Meshcheryakova A, Mechtcheriakova D, Pietschmann P. Sphingosine 1-phosphate signaling in bone remodeling: Multifaceted roles and therapeutic potential. *Expert Opin Ther Targets.* 2017;21(7):725-37. doi: 10.1080/14728222.2017.1332180
- [136] Aronin CS, L.; Tholpady, S.; Tholpady, A.; Sadik, K.; Macdonald, T.; Peirce, S.; Wamhoff, B.; Lynch, K.; Ogle, R.; Botchwey, E. FTY720 promotes local microvascular network formation and regeneration of cranial bone defects. *Tissue Eng Part A.* 2010;16(Number 6). doi: 10.1089=ten.tea.2009.0539
- [137] Le Stunff H, Milstien S, Spiegel S. Generation and metabolism of bioactive sphingosine-1-phosphate. *J Cell Biochem.* 2004;92(5):882-99. doi: 10.1002/jcb.20097
- [138] Pyne NJ, Pyne S. Sphingosine 1-phosphate and cancer. *Nat Rev Cancer.* 2010;10(7):489-503. doi: 10.1038/nrc2875
- [139] Pitson SM, Xia P, Leclercq TM, Moretti PA, Zebol JR, Lynn HE, et al. Phosphorylation-dependent translocation of sphingosine kinase to the plasma membrane drives its oncogenic signalling. *J Exp Med.* 2005;201(1):49-54. doi: 10.1084/jem.20040559
- [140] Spiegel S, Milstien S. Functions of the multifaceted family of sphingosine kinases and some close relatives. *J Biol Chem.* 2007;282(4):2125-9. doi: 10.1074/jbc.R600028200
- [141] Okada T, Ding G, Sonoda H, Kajimoto T, Haga Y, Khosrowbeygi A, et al. Involvement of N-terminal-extended form of sphingosine kinase 2 in serum-dependent regulation of cell proliferation and apoptosis. *J Biol Chem.* 2005;280(43):36318-25. doi: 10.1074/jbc.M504507200

- [142] Maceyka M, Sankala H, Hait NC, Le Stunff H, Liu H, Toman R, et al. SphK1 and SphK2, sphingosine kinase isoenzymes with opposing functions in sphingolipid metabolism. *J Biol Chem*. 2005;280(44):37118-29. doi: 10.1074/jbc.M502207200
- [143] Mendelson K, Evans T, Hla T. Sphingosine 1-phosphate signalling. *Development*. 2014;141(1):5-9. doi: 10.1242/dev.094805
- [144] Kawahara A, Nishi T, Hisano Y, Fukui H, Yamaguchi A, Mochizuki N. The sphingolipid transporter spns2 functions in migration of zebrafish myocardial precursors. *Science*. 2009;323(5913):524-7. doi: 10.1126/science.1167449
- [145] Takabe K, Spiegel S. Export of sphingosine-1-phosphate and cancer progression. *J Lipid Res*. 2014;55(9):1839-46. doi: 10.1194/jlr.R046656
- [146] Spiegel S, Milstien S. The outs and the ins of sphingosine-1-phosphate in immunity. *Nat Rev Immunol*. 2011;11(6):403-15. doi: 10.1038/nri2974
- [147] Ishii M, Egen JG, Klauschen F, Meier-Schellersheim M, Saeki Y, Vacher J, et al. Sphingosine-1-phosphate mobilizes osteoclast precursors and regulates bone homeostasis. *Nature*. 2009;458(7237):524-8. doi: 10.1038/nature07713
- [148] Doring A, Penel G, Hardouin P. Understanding the local actions of lipids in bone physiology. *Prog Lipid Res*. 2015;59:126-46. doi: 10.1016/j.plipres.2015.06.002
- [149] Roelofsen T, Akkers R, Beumer W, Apotheker M, Steeghs I, Van de Ven J, et al. Sphingosine-1-phosphate acts as a developmental stage specific inhibitor of platelet-derived growth factor-induced chemotaxis of osteoblasts. *J Cell Biochem*. 2008;105(4):1128-38. doi: 10.1002/jcb.21915
- [150] Kihara Y, Maceyka M, Spiegel S, Chun J. Lysophospholipid receptor nomenclature review: IUPHAR Review 8. *Br J Pharmacol*. 2014;171(15):3575-94. doi: 10.1111/bph.12678
- [151] Aronin CEP, Shin SJ, Naden KB, Rios PD, Jr., Sefcik LS, Zawodny SR, et al. The enhancement of bone allograft incorporation by the local delivery of the sphingosine 1-phosphate receptor targeted drug FTY720. *Biomaterials*. 2010;31(25):6417-24. doi: 10.1016/j.biomaterials.2010.04.061
- [152] Spiegel S, Milstien S. Sphingosine-1-phosphate: An enigmatic signalling lipid. *Nat Rev Mol Cell Biol*. 2003;4(5):397-407. doi: 10.1038/nrm1103
- [153] Milstien S, Spiegel S. Targeting sphingosine-1-phosphate: A novel avenue for cancer therapeutics. *Cancer Cell*. 2006;9(3):148-50. doi: 10.1016/j.ccr.2006.02.025
- [154] Hla T. Physiological and pathological actions of sphingosine 1-phosphate. *Semin Cell Dev Biol*. 2004;15(5):513-20. doi: 10.1016/j.semcdb.2004.05.002
- [155] Sato C, Iwasaki T, Kitano S, Tsunemi S, Sano H. Sphingosine 1-phosphate receptor activation enhances BMP-2-induced osteoblast differentiation. *Biochem Biophys Res Commun*. 2012;423(1):200-5. doi: 10.1016/j.bbrc.2012.05.130
- [156] Kono M, Mi Y, Liu Y, Sasaki T, Allende-ML, Wu YP, et al. The sphingosine-1-phosphate receptors S1P1, S1P2, and S1P3 function coordinately during embryonic angiogenesis. *J Biol Chem*. 2004;279(28):29367-73. doi: 10.1074/jbc.M403937200
- [157] Ishii M, Kikuta J. Sphingosine-1-phosphate signaling controlling osteoclasts and bone homeostasis. *Biochim Biophys Acta*. 2013;1831(1):223-7. doi: 10.1016/j.bbali.2012.06.002
- [158] Heilmann A, Schinke T, Bindl R, Wehner T, Rapp A, Haffner-Luntzer M, et al. Systemic treatment with the sphingosine-1-phosphate analog FTY720 does not improve fracture healing in mice. *J Orthop Res*. 2013;31(11):1845-50. doi: 10.1002/jor.22426
- [159] Kong Y, Wang H, Lin T, Wang S. Sphingosine-1-phosphate/S1P receptors signaling modulates cell migration in human bone marrow-derived mesenchymal stem cells. *Mediators Inflamm*. 2014;2014:565369. doi: 10.1155/2014/565369
- [160] Sefcik LS, Aronin CE, Awojoodu AO, Shin SJ, Mac Gabhann F, MacDonald TL, et al. Selective activation of sphingosine 1-phosphate receptors 1 and 3 promotes local microvascular network growth. *Tissue Eng Part A*. 2011;17(5-6):617-29. doi: 10.1089/ten.TEA.2010.0404
- [161] Ogle ME, Sefcik LS, Awojoodu AO, Chiappa NF, Lynch K, Peirce-Cottler S, et al. Engineering *in vivo* gradients of sphingosine-1-phosphate receptor ligands for localized microvascular remodeling and inflammatory cell positioning. *Acta Biomater*. 2014;10(11):4704-14. doi: 10.1016/j.actbio.2014.08.007
- [162] Kono M, Proia RL. Imaging S1P1 activation *in vivo*. *Exp Cell Res*. 2015;333(2):178-82. doi: 10.1016/j.yexcr.2014.11.023
- [163] Wacker BK, Scott EA, Kaneda MM, Alford SK, Elbert DL. Delivery of sphingosine 1-phosphate from poly(ethylene glycol) hydrogels. *Biomacromolecules*. 2006;7(4):1335-43. doi: 10.1021/bm050948r
- [164] Sefcik LS, Petrie Aronin CE, Wieghaus KA, Botchwey EA. Sustained release of sphingosine 1-phosphate for therapeutic arteriogenesis and bone tissue engineering. *Biomaterials*. 2008;29(19):2869-77. doi: 10.1016/j.biomaterials.2008.03.017

- [165] Tengood JE, Kovach KM, Vescovi PE, Russell AJ, Little SR. Sequential delivery of vascular endothelial growth factor and sphingosine 1-phosphate for angiogenesis. *Biomaterials*. 2010;31(30):7805-12. doi: 10.1016/j.biomaterials.2010.07.010
- [166] Sartawi Z, Schipani E, Ryan KB, Waeber C. Sphingosine 1-phosphate (S1P) signalling: Role in bone biology and potential therapeutic target for bone repair. *Pharmacol Res*. 2017;125:232-45. doi: 10.1016/j.phrs.2017.08.013
- [167] Osada M, Yatomi Y, Ohmori T, Ikeda H, Ozaki Y. Enhancement of sphingosine 1-phosphate-induced migration of vascular endothelial cells and smooth muscle cells by an EDG-5 antagonist. *Biochem Biophys Res Commun*. 2002;299(3):483-7. doi: 10.1016/S0006-291X(02)02671-2
- [168] Brinkmann V, Billich A, Baumruker T, Heining P, Schmouder R, Francis G, et al. Fingolimod (FTY720): discovery and development of an oral drug to treat multiple sclerosis. *Nat Rev Drug Discov*. 2010;9(11):883-97. doi: 10.1038/nrd3248
- [169] Paugh SW, Payne SG, Barbour SE, Milstien S, Spiegel S. The immunosuppressant FTY720 is phosphorylated by sphingosine kinase type 2. *FEBS Letters*. 2003;554(1-2):189-93. doi: 10.1016/s0014-5793(03)01168-2
- [170] Mechtcheriakova D, Wluchos A, Sobanov J, Bornancin F, Zlabinger G, Baumruker T, et al. FTY720-phosphate is dephosphorylated by lipid phosphate phosphatase 3. *FEBS Lett*. 2007;581(16):3063-8. doi: 10.1016/j.febslet.2007.05.069
- [171] Bandhuvula P, Tam YY, Oskouian B, Saba JD. The immune modulator FTY720 inhibits sphingosine-1-phosphate lyase activity. *J Biol Chem*. 2005;280(40):33697-700. doi: 10.1074/jbc.C500294200
- [172] Huang C, Das A, Barker D, Tholpady S, Wang T, Cui Q, et al. Local delivery of FTY720 accelerates cranial allograft incorporation and bone formation. *Cell Tissue Res*. 2012;347(3):553-66. doi: 10.1007/s00441-011-1217-3
- [173] Das A, Segar CE, Chu Y, Wang TW, Lin Y, Yang C, et al. Bioactive lipid coating of bone allografts directs engraftment and fate determination of bone marrow-derived cells in rat GFP chimeras. *Biomaterials*. 2015;64:98-107. doi: 10.1016/j.biomaterials.2015.06.019
- [174] Wang T, Krieger J, Huang C, Das A, Francis MP, Ogle R, et al. Enhanced osseous integration of human trabecular allografts following surface modification with bioactive lipids. *Drug Deliv Transl Res*. 2016;6(2):96-104. doi: 10.1007/s13346-015-0244-0
- [175] Das A, Segar CE, Hughley BB, Bowers DT, Botchwey EA. The promotion of mandibular defect healing by the targeting of S1P receptors and the recruitment of alternatively activated macrophages. *Biomaterials*. 2013;34(38):9853-62. doi: 10.1016/j.biomaterials.2013.08.015
- [176] Das A, Tanner S, Barker DA, Green D, Botchwey EA. Delivery of S1P receptor-targeted drugs via biodegradable polymer scaffolds enhances bone regeneration in a critical size cranial defect. *J Biomed Mater Res A*. 2014;102(4):1210-8. doi: 10.1002/jbm.a.34779
- [177] Hughes JE, Srinivasan S, Lynch KR, Proia RL, Ferdek P, Hedrick CC. Sphingosine-1-phosphate induces an anti-inflammatory phenotype in macrophages. *Circ Res*. 2008;102(8):950-8. doi: 10.1161/CIRCRESAHA.107.170779
- [178] Awjoodu AO, Ogle ME, Sefcik LS, Bowers DT, Martin K, Brayman KL, et al. Sphingosine 1-phosphate receptor 3 regulates recruitment of anti-inflammatory monocytes to microvessels during implant arteriogenesis. *Proc Natl Acad Sci U S A*. 2013;110(34):13785-90. doi: 10.1073/pnas.1221309110
- [179] LaMontagne K, Littlewood-Evans A, Schnell C, O'Reilly T, Wyder L, Sanchez T, et al. Antagonism of sphingosine-1-phosphate receptors by FTY720 inhibits angiogenesis and tumor vascularization. *Cancer Res*. 2006;66(1):221-31. doi: 10.1158/0008-5472.CAN-05-2001
- [180] Oo ML, Thangada S, Wu MT, Liu CH, Macdonald TL, Lynch KR, et al. Immunosuppressive and anti-angiogenic sphingosine 1-phosphate receptor-1 agonists induce ubiquitylation and proteasomal degradation of the receptor. *J Biol Chem*. 2007;282(12):9082-9. doi: 10.1074/jbc.M610318200
- [181] Ji F, Mao L, Liu Y, Cao X, Xie Y, Wang S, et al. K6PC-5, a novel sphingosine kinase 1 (SphK1) activator, alleviates dexamethasone-induced damages to osteoblasts through activating SphK1-Akt signaling. *Biochem Biophys Res Commun*. 2015;458(3):568-75. doi: 10.1016/j.bbrc.2015.02.007
- [182] Bigaud M, Guerini D, Billich A, Bassilana F, Brinkmann V. Second generation S1P pathway modulators: Research strategies and clinical developments. *Biochim Biophys Acta*. 2014;1841(5):745-58. doi: 10.1016/j.bbalip.2013.11.001
- [183] Blackburn J, Mansell JP. The emerging role of lysophosphatidic acid (LPA) in skeletal biology. *Bone*. 2012;50(3):756-62. doi: 10.1016/j.bone.2011.12.002
- [184] Sheng X, Yung YC, Chen A, Chun J. Lysophosphatidic acid signalling in development. *Development*. 2015;142(8):1390-5. doi: 10.1242/dev.121723
- [185] Moolenaar WH, van Meeteren LA, Giepmans BN. The ins and outs of lysophosphatidic acid signaling. *Bioessays*. 2004;26(8):870-81. doi: 10.1002/bies.20081
- [186] Mansell JP, Nowghani M, Pabbruwe M, Paterson I, Smith A, Blom A. Lysophosphatidic acid and calcitriol co-operate to promote human osteoblastogenesis: Requirement of albumin-bound LPA. *Prostaglandins*. 2011;95(1-4):45-52. doi: 10.1016/j.prostaglandins.2011.05.003

- [187] Binder BY, Williams PA, Silva EA, Leach JK. Lysophosphatidic Acid and Sphingosine-1-Phosphate: A Concise Review of Biological Function and Applications for Tissue Engineering. *Tissue Eng Part B Rev.* 2015;21(6):531-42. doi: 10.1089/ten.TEB.2015.0107
- [188] Karagiannis SA, Chrisler WB, Bollinger N, Karin NJ. Lysophosphatidic acid-induced ERK activation and chemotaxis in MC3T3-E1 preosteoblasts are independent of EGF receptor transactivation. *J Cell Physiol.* 2009;219(3):716-23. doi: 10.1002/jcp.21720
- [189] Gardell SE, Dubin AE, Chun J. Emerging medicinal roles for lysophospholipid signaling. *Trends Mol Med.* 2006;12(2):65-75. doi: 10.1016/j.molmed.2005.12.001
- [190] Lee H, Goetzl EJ, An S. Lysophosphatidic acid and sphingosine 1-phosphate stimulate endothelial cell wound healing. *Am J Physiol-Cell Physiol.* 2000;278(3):C612-C8. doi: 10.1152/ajpcell.2000.278.3.C612
- [191] Xu KP, Yin J, Yu FS. Lysophosphatidic acid promoting corneal epithelial wound healing by transactivation of epidermal growth factor receptor. *Invest Ophthalmol Vis Sci.* 2007;48(2):636-43. doi: 10.1167/iovs.06-0203
- [192] David M, Machuca-Gayet I, Kikuta J, Ottewill P, Mima F, Leblanc R, et al. Lysophosphatidic acid receptor type 1 (LPA1) plays a functional role in osteoclast differentiation and bone resorption activity. *J Biol Chem.* 2014;289(10):6551-64. doi: 10.1074/jbc.M113.533232
- [193] Liu YB, Kharode Y, Bodine PV, Yaworsky PJ, Robinson JA, Billiard J. LPA induces osteoblast differentiation through interplay of two receptors: LPA1 and LPA4. *J Cell Biochem.* 2010;109(4):794-800. doi: 10.1002/jcb.22471
- [194] Salles JP, Laurencin-Dalicioux S, Conte-Auriol F, Briand-Mésange F, Gennero I. Bone defects in LPA receptor genetically modified mice. *Biochim Biophys Acta.* 2013;1831(1):93-8. doi: 10.1016/j.bbailip.2012.07.018
- [195] Lin CI, Chen CN, Huang MT, Lee SJ, Lin CH, Chang CC, et al. Lysophosphatidic acid upregulates vascular endothelial growth factor-C and tube formation in human endothelial cells through LPA(1/3), COX-2, and NF-kappaB activation- and EGFR transactivation-dependent mechanisms. *Cell Signal.* 2008;20(10):1804-14. doi: 10.1016/j.cellsig.2008.06.008
- [196] Woclawek-Potocka I, Kondraciuk K, Skarzynski DJ. Lysophosphatidic acid stimulates prostaglandin E2 production in cultured stromal endometrial cells through LPA1 receptor. *Exp Biol Med (Maywood).* 2009;234(8):986-93. doi: 10.3181/0901-RM-36
- [197] Tomsig JL, Snyder AH, Berdyshev EV, Skobeleva A, Mataya C, Natarajan V, et al. Lipid phosphate phosphohydrolase type 1 (LPP1) degrades extracellular lysophosphatidic acid *in vivo*. *Biochem J.* 2009;419(3):611-8. doi: 10.1042/BJ20081888
- [198] Sims SM, Panupinthu N, Lapierre DM, Pereverzev A, Dixon SJ. Lysophosphatidic acid: A potential mediator of osteoblast-osteoclast signaling in bone. *Biochim Biophys Acta.* 2013;1831(1):109-16. doi: 10.1016/j.bbailip.2012.08.001
- [199] Lapierre DM, Tanabe N, Pereverzev A, Spencer M, Shugg RP, Dixon SJ, et al. Lysophosphatidic acid signals through multiple receptors in osteoclasts to elevate cytosolic calcium concentration, evoke retraction, and promote cell survival. *J Biol Chem.* 2010;285(33):25792-801. doi: 10.1074/jbc.M110.109322
- [200] David M, Wannecq E, Descotes F, Jansen S, Deux B, Ribeiro J, et al. Cancer cell expression of autotaxin controls bone metastasis formation in mouse through lysophosphatidic acid-dependent activation of osteoclasts. *PLoS One.* 2010;5(3):e9741. doi: 10.1371/journal.pone.0009741
- [201] Chen X, Wang Z, Duan N, Zhu G, Schwarz EM, Xie C. Osteoblast-osteoclast interactions. *Connect Tissue Res.* 2018;59(2):99-107. doi: 10.1080/03008207.2017.1290085
- [202] Karagiannis SA, Karin NJ. Lysophosphatidic acid induces osteocyte dendrite outgrowth. *Biochem Biophys Res Commun.* 2007;357(1):194-9. doi: 10.1016/j.bbrc.2007.03.121
- [203] Salous AK, Panchatcharam M, Sunkara M, Mueller P, Dong A, Wang Y, et al. Mechanism of rapid elimination of lysophosphatidic acid and related lipids from the circulation of mice. *J Lipid Res.* 2013;54(10):2775-84. doi: 10.1194/jlr.M039685
- [204] Yu ZL, Jiao BF, Li ZB. Lysophosphatidic Acid Analogue rather than Lysophosphatidic Acid Promoted the Bone Formation *In Vivo*. *Biomed Res Int.* 2018;2018:7537630. doi: 10.1155/2018/7537630
- [205] Rothe R, Schulze S, Neuber C, Hauser S, Rammelt S, Pietzsch J. Adjuvant drug-assisted bone healing: Part III – Further strategies for local and systemic modulation. *Clin Hemorheol Microcirc.* in press.
- [206] Nakajima C, Haffner P, Goerke SM, Zurhove K, Adelman G, Frotscher M, et al. The lipoprotein receptor LRP1 modulates sphingosine-1-phosphate signaling and is essential for vascular development. *Development.* 2014;141(23):4513-25. doi: 10.1242/dev.109124
- [207] Gennero I, Laurencin-Dalicioux S, Conte-Auriol F, Briand-Mésange F, Laurencin D, Rue J, et al. Absence of the lysophosphatidic acid receptor LPA1 results in abnormal bone development and decreased bone mass. *Bone.* 2011;49(3):395-403. doi: 10.1016/j.bone.2011.04.018

- [208] Ishii M, Kikuta J, Shimazu Y, Meier-Schellersheim M, Germain RN. Chemorepulsion by blood S1P regulates osteoclast precursor mobilization and bone remodeling *in vivo*. *J Exp Med*. 2010;207(13):2793-8. doi: 10.1084/jem.20101474
- [209] Kim YH, Tabata Y. Recruitment of mesenchymal stem cells and macrophages by dual release of stromal cell-derived factor-1 and a macrophage recruitment agent enhances wound closure. *J Biomed Mater Res A*. 2016;104(4):942-56. doi: 10.1002/jbm.a.35635
- [210] Kim Y-H, Furuya H, Tabata Y. Enhancement of bone regeneration by dual release of a macrophage recruitment agent and platelet-rich plasma from gelatin hydrogels. *Biomaterials*. 2014;35(1):214-24. doi: 10.1016/j.biomaterials.2013.09.103
- [211] Selma JM, Das A, Awojoodu AO, Wang T, Kaushik AP, Cui Q, et al. Novel Lipid Signaling Mediators for Mesenchymal Stem Cell Mobilization During Bone Repair. *Cell Mol Bioeng*. 2018;11(4):241-53. doi: 10.1007/s12195-018-0532-0
- [212] Jamal SA, Cummings SR, Hawker GA. Isosorbide mononitrate increases bone formation and decreases bone resorption in postmenopausal women: A randomized trial. *J Bone Miner Res*. 2004;19(9):1512-7. doi: 10.1359/JBMR.040716
- [213] Nabhan AF, Rabie NH. Isosorbide mononitrate versus alendronate for postmenopausal osteoporosis. *Int J Gynaecol Obstet*. 2008;103(3):213-6. doi: 10.1016/j.ijgo.2008.07.011
- [214] Kdolsky RK, Mohr W, Savidis-Dacho H, Beer R, Puig S, Reihnsner R, et al. The influence of oral L-arginine on fracture healing: An animal study. *Wien Klin Wochenschr*. 2005;117(19-20):693-701. doi: 10.1007/s00508-005-0431-y
- [215] Baecker N, Boese A, Schoenau E, Gerzer R, Heer M. L-arginine, the natural precursor of NO, is not effective for preventing bone loss in postmenopausal women. *J Bone Miner Res*. 2005;20(3):471-9. doi: 10.1359/JBMR.041121
- [216] Wimalawansa S. Restoration of ovariectomy-induced osteopenia by nitroglycerin. *Calcif Tissue Int*. 2000;66(1):56-60. doi: 10.1007/s002230050011
- [217] Wimalawansa S, Chapa T, Fang L, Yallampalli C, Simmons D, Wimalawansa S. Frequency-dependent effect of nitric oxide donor nitroglycerin on bone. *J Bone Miner Res*. 2000;15(6):1119-25. doi: 10.1359/jbmr.2000.15.6.1119
- [218] Wimalawansa SJ. Nitroglycerin therapy is as efficacious as standard estrogen replacement therapy (Premarin) in prevention of oophorectomy-induced bone loss: A human pilot clinical study. *J Bone Miner Res*. 2000;15(11):2240-4. doi: 10.1359/jbmr.2000.15.11.2240
- [219] Baldik Y, Talu U, Altinel L, Bilge H, Demiryont M, Aykac-Toker G. Bone healing regulated by nitric oxide: An experimental study in rats. *Clin Orthop Relat Res*. 2002;404:343-52. doi: 10.1097/01.blo.0000022182.66847.8b
- [220] Lin IC, Smartt JM, Jr., Nah HD, Ischiropoulos H, Kirschner RE. Nitric oxide stimulates proliferation and differentiation of fetal calvarial osteoblasts and dural cells. *Plast Reconstr Surg*. 2008;121(5):1554-66; discussion 67-9. doi: 10.1097/PRS.0b013e31816c3bd7
- [221] Aguirre J, Buttery L, O'Shaughnessy M, Afzal F, de Martcorena IF, Hukkanen M, et al. Endothelial nitric oxide synthase gene-deficient mice demonstrate marked retardation in postnatal bone formation, reduced bone volume, and defects in osteoblast maturation and activity. *Am J Pathol*. 2001;158(1):247-57. doi: 10.1016/S0002-9440(10)63963-6.
- [222] Armour KE, Armour KJ, Gallagher ME, Gödecke A, Helfrich MH, Reid DM, et al. Defective bone formation and anabolic response to exogenous estrogen in mice with targeted disruption of endothelial nitric oxide synthase. *Endocrinology*. 2001;142(2):760-6. doi: 10.1210/endo.142.2.7977
- [223] Meesters DM, Neubert S, Wijnands KAP, Heyer FL, Zeiter S, Ito K, et al. Deficiency of inducible and endothelial nitric oxide synthase results in diminished bone formation and delayed union and nonunion development. *Bone*. 2016;83:111-8. doi: 10.1016/j.bone.2015.11.006
- [224] Baldik Y, Diwan AD, Appleyard RC, Fang ZM, Wang Y, Murrell GA. Deletion of iNOS gene impairs mouse fracture healing. *Bone*. 2005;37(1):32-6. doi: 10.1016/j.bone.2004.10.002
- [225] van't Hof RJ, Macphee J, Libouban H, Helfrich MH, Ralston SH. Regulation of bone mass and bone turnover by neuronal nitric oxide synthase. *Endocrinology*. 2004;145(11):5068-74. doi: 10.1210/en.2004-0205
- [226] Huyut Z, Bakan N, Yıldırım S, Alp HH. Effects of the Phosphodiesterase-5 (PDE-5) Inhibitors, Avanafil and Zaprinast, on Bone Remodeling and Oxidative Damage in a Rat Model of Glucocorticoid-Induced Osteoporosis. *Med Sci Monit Basic Res* 2018;24:47-58. doi: 10.12659/msmbr.908504
- [227] Dincel YM, Alagoz E, Arıkan Y, Çağlar AK, Doğru SC, Ortes F, et al. Biomechanical, histological, and radiological effects of different phosphodiesterase inhibitors on femoral fracture healing in rats. *J Orthop Surg (Hong Kong)*. 2018;26(2):2309499018777885. doi: 10.1177/2309499018777885
- [228] Alp HH, Huyut Z, Yildirim S, Basbugan Y, Ediz L, Sekeroglu MR. The effect of PDE5 inhibitors on bone and oxidative damage in ovariectomy-induced osteoporosis. *Exp Biol Med (Maywood)*. 2017;242(10):1051-61. doi: 10.1177/1535370217703352
- [229] Wang L, Jia H, Tower RJ, Levine MA, Qin L. Analysis of Short-term Treatment with the Phosphodiesterase Type 5 Inhibitor Tadalafil on Long Bone Development in Young Rats. *Am J Physiol Endocrinol Metab*. 2018. doi: 10.1152/ajpendo.00130.2018

- [230] Horiuchi H, Saito N, Kinoshita T, Wakabayashi S, Tsutsumimoto T, Takaoka K. Enhancement of bone morphogenetic protein-2-induced new bone formation in mice by the phosphodiesterase inhibitor pentoxifylline. *Bone*. 2001;28(3):290-4. doi: 10.1016/S8756-3282(00)00450-6
- [231] Atalay Y, Gunes N, Guner MD, Akpolat V, Celik MS, Guner R. Pentoxifylline and electromagnetic field improved bone fracture healing in rats. *Drug Des Devel Ther*. 2015;9:5195-201. doi: 10.2147/DDDT.S89669
- [232] Vashghani Farahani MM, Ahadi R, Abdollahifar M, Bayat M. The effects of pentoxifylline administration on fracture healing in a postmenopausal osteoporotic rat model. *Lab Anim Res*. 2017;33(1):15-23. doi: 10.5625/lar.2017.33.1.15

Corrected Proof