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

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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 ($NO^{\bullet}$) donors, including bi-functional (hybrid) compounds like $NO^{\bullet}$-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	proteinkinase B
Asn	asparagine
ATP/GTP	adenosine/guanosine triphosphate
BMP	bone morphogenetic protein

[1]SR and JP share senior authorship.

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cAMP/cGMP	cyclic adenosine/guanosine monophosphate
CINOD	COX-inhibiting nitric oxide donator
COX	cyclooxygenase
CREB	cAMP response element-binding protein
DFO	deferoxamine
DMOG	dimethyloxaloylglycine
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-3ß	glycogen synthase kinase-3ß
HIF	hypoxia-inducible factor
HRE	hypoxia response element
IκB	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
NFκB	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 κB 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 angiogenesis[1]

Besides inflammation [1], angiogenesis is the second important process during bone repair and it is necessary to initiate osteogenesis by supply of oxygen (O_2), 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 PGE_2 (prostaglandin E_2) 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-1β 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-1α is present in almost all tissues, whereas HIF-2α is predominantly expressed by endothelial cells during embryogenesis. Moreover, both HIFs are key players in adaption of tumor cells to hypoxia. Comparatively, function of HIF-3α subunit is currently poorly understood, but there are some indications that transcription of this subunit is intensified upon hypoxia. However, HIF-3α 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-1α are hydroxylated by prolyl hydroxylases further recruiting E3 ubiquitin ligase-Von Hippel-Lindau (VHL) complex. Afterwards, HIF-1α 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-1α subunit is stabilized by several kinases and thereby accumulates in cytosol due to prevented hydroxylation and ubiquitin (Ub)-mediated proteasomal degradation. Further, HIF-1α translocates into the nucleus. After heterodimerization with HIF-1β 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 chronical 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-1α 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-1α 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 κB 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-1α in osteoblasts utilizing a murine model [64–66]. Loss of HIF-1α 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-1α 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-1α or stable constitutive HIF-1α 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-1α. They demonstrated that resultant HIF-1α 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-2α as target substrate, whereas PHD2 seems to be predominant in HIF-1α 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 und 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

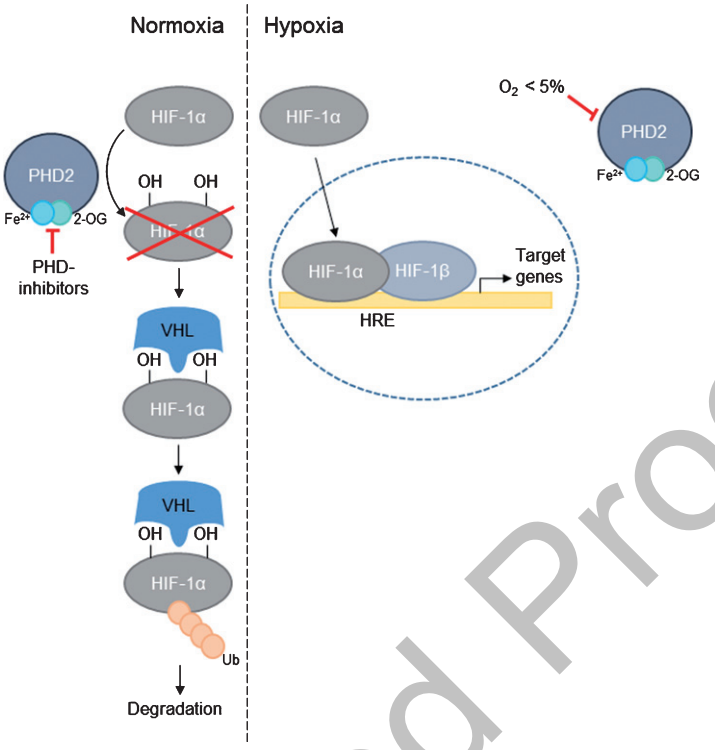

Fig. 1. HIF-1α signaling pathway depends on oxygen availability. Under normoxia, HIF-1α gets hydroxylated by PHD enzymes which PHD-inhibitors can prevent. Further ligase and ubiquitin-mediated (Ub) degradation takes place. On the contrary, during hypoxia PHD enzymes are blocked and HIF-1α is able to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

dimethyloxaloylglycine (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 (Co^{2+}), copper (Cu^{2+}), zinc (Zn^{2+}) and manganese (Mn^{2+}) 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-1α 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-1α 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-1α 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-1α and, consequently, promoted downstream transcription of HIF-1α 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-1α 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 ß-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 μM	–	↑	Karagiosis *et al.*, 2007 [202]
LPA_1 receptor	$LPA_1^{-/-}$ mice	–	–	↓	David *et al.*, 2014 [192]
	$LPA_1^{-/-}$ mice	–	–	↓	Gennero *et al.*, 2011 [207]
LPA_4 receptor	$LPA_4^{-/-}$ mice	–	–	↑	Liu *et al.*, 2010 [193]
S1P and S1P analogs					
	human umbilical vein endothelial cells	5 nmol	PEG hydrogel	↑	Wacker *et al.*, 2006 [163]
S1P	mice	1:400 S1P:PLGA	PLGA-coated implant	↑	Sefcik *et al.*, 2011 [160]
	mice	1800 μM	subcutaneous injected matrigel	↑	Tengood *et al.*, 2010 [165]
	rat	1:400 S1P:PLGA	PLGA scaffold	↑	Petrie Aronin *et al.*, 2010 [136]
	rat	1 mg/ml	PLGA 3D scaffold	↑	Sefcik *et al.*, 2008 [164]
	mice	6 mg/kg	subcutaneous injection	→	Heilmann *et al.*, 2013 [158]
	mice	3 mg/kg/day	intraperitoneal injection	↑	Ishii *et al.*, 2009 [147]
FTY720 (fingolimod)	mice	1:200 FTY720:PLGA	PLGA-coated implant	↑	Sefcik *et al.*, 2011 [160]
	mice	1–10 nM	local injected matrigel	↑	Wang *et al.*, 2016 [174]
	rat	1:200 FTY720:polymer	PCL/PLGA nanofiber implant	↑	Das *et al.*, 2013 [175]
	rat	1:200 FTY720:PLGA	PLGA-coated allograft	↑	Das *et al.*, 2014 [176]/2015 [173]
	rat	1:40 or 1:200 FTY720:PLGA	PLGA-coated allograft	↑	Huang *et al.*, 2012 [172]
FTY720 (fingolimod)	rat	1:200 FTY720:PLGA	PLGA scaffold	↑	Petrie Aronin *et al.*, 2010 [136, 151]
	rat	1.5 mg/0.6 ml	coated allograft	↑	Wang *et al.*, 2016 [174]
JTE-013 (pyrazolopyridine derivative)	mice	3 mg/kg	intraperitoneal injection	↑	Ishii *et al.*, 2010 [208]
SEW2871 (oxadiazole derivative)	mice	0.4–1.6 mg SEW2871 micelles	SDF-1 incorporated gelatin hydrogel	↑	Kim *et al.*, 2016 [209]
	rat	7.5–15 μg SEW2871 micelles	PRP incorporated gelatin hydrogel	↑	Kim *et al.*, 2014 [210]
VPC01091	mice	1:200 VPC01091:PLGA	PLGA-coated implant	↑	Sefcik *et al.*, 2011 [160]
(octylphenyl-substituted	rat	1:200 VPC01091:PLGA	PLGA scaffold	↑	Petrie Aronin *et al.*, 2010 [136]
cyclophenyl derivative)	rat	1–5 mg/kg	intraperitoneal injection	↑	Selma *et al.*, 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 *et al.*, 2000 [85]
isosorbide mononitrate	human	5–20 mg/day	oral	↑	Jamal *et al.*, 2004 [212]
	human	20 mg/day	oral	↑	Nabhan *et al.*, 2008 [213]
L-arginine	guinea pig	100 mg/kg	oral	↑	Kdolsky *et al.*, 2005 [214]
L-arginine	human	18 g L-arginine hydrochloride (14.8 g free L-arginine)	oral	→	Baecker *et al.*, 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 *et al.*, 2005 [95]
	rat	0.2 mg 2% nitroglycerin	dermal (ointment)	↑	Wimalawansa *et al.*, 2000 [216]
	rat	0.2 mg 2% nitroglycerin once, twice or three times a day	dermal (ointment)	↑ (once daily) ↓ (higher frequency)	Wimalawansa *et al.*, 2000 [217]
	human	15 mg/day	percutaneous (ointment)	↑	Wimalawansa *et al.*, 2000 [218]
nitrosobovine serum albumin	rat	0.3 mmol/l	local	↑	Baldik *et al.*, 2002 [219]
nitrosyl-cobinamide	mice	10 mg/kg/day	intraperitoneal injection	↑	Kalyanaraman *et al.*, 2017 [102]
NOC-18/DETA-NONOate (slower release/mimicking eNOS)	calvarial osteoblasts	1–10 μM	–	↑	Lin *et al.*, 2008 [220]
	primary rat osteoblast-enriched Cultures	10–100 μM	–	↑	Mancini *et al.*, 2000 [82]
S-nitrosoglutathione	rat	100 μmol/l in hydrogel 8 nmol/application	local hydrogel	↑	Amadeu *et al.*, 2008 [109]
sodium nitroprusside (rapid release/mimicking iNOS)	primary rat osteoblast-enriched cultures	10–100 μM	–	↓	Mancini *et al.*, 2000 [82]
NOS and NOS inhibitors					
eNOS	eNOS$^{-/-}$ mice	–	–	↓	Aguirre *et al.*, 2001 [221]
	eNOS$^{-/-}$ mice	–	–	↓	Armour *et al.*, 2001 [222]
	eNOS$^{-/-}$ mice	–	–	↓	Meesters *et al.*, 2016 [223]

(*Continued next page*)

Table 1
(*Continued*)

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

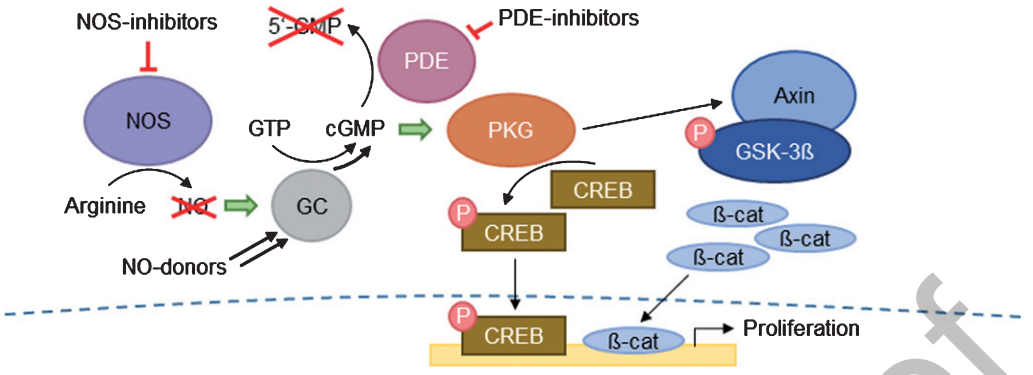

Fig. 2. Intracellular NO• pathway. NO• production by NOS enzymes is based on L-Arginine. NO•, in line with the effect of NO-donors, activates further downstream signaling pathways. Activation of GC, PKG, and the main part of Wnt signaling cascade stimulates proliferation-enhanced transcription via CREB and ß-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 (Fe^{2+}) 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 $NO^{\bullet}$ 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) – ß-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 $NO^{\bullet}$-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 donators (CINODs) display anti-inflammatory and protective effects regarding gastrointestinal tract due to released $NO^{\bullet}$ 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 ($NO^{\bullet}$-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-live of about 17.5 h) provide $NO^{\bullet}$-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 3β (glycogen synthase kinase-3ß) results in phosphorylated cytosolic ß-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 Aydín 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 $NO^{\bullet}$-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 polyethylenglycol (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 $S1P_4$ and $S1P_5$ are expressed in immune and neuronal cells [156]. Osteoblasts express the receptor subtypes $S1P_{1–3,}$ while expression of $S1P_{1\text{-}2}$ is seen in osteoclasts [134, 157, 158]. $S1P_1$ 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, $S1P_2$ inhibits Rac and its mediated migratory effects. Instead, $S1P_2$ 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 $S1P_2$, $S1P_3$ 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 $S1P_3$-mediated signals is comparable to those of $S1P_1$ [136, 143, 159]. Since both $S1P_1$ and $S1P_3$ 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; $S1P_1$ agonist), VPC01091 (octylphenyl-substituted cyclophenyl derivative; $S1P_1$ agonist and $S1P_3$ antagonist), and JTE-013 (pyrazolopyridine derivative [167]; $S1P_2$ 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

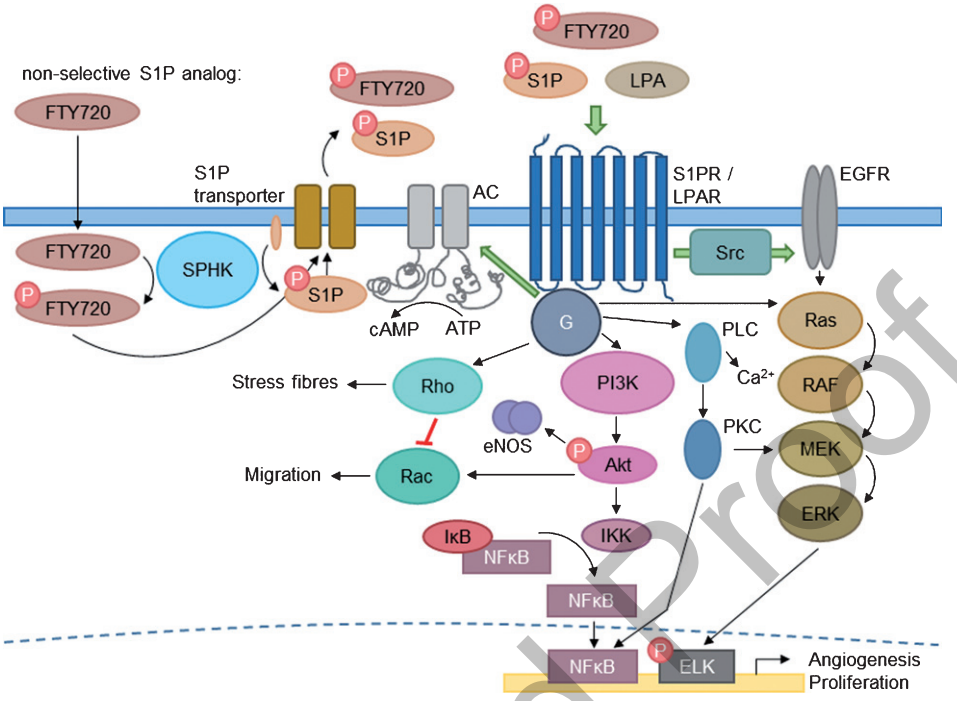

Fig. 3. Lipid-mediated signaling. Characteristic for G-protein coupled lipid receptor (S1PR or LPAR)-mediated downstream signaling pathways, initiated via S1P, its analog FTY720 or LPA, is the activation of certain cytosolic mediators like AC, PI3K – Akt – eNOS, IKK or Rac, Rho, PLC – PKC. Likewise, 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]).

phosphor-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 postoperatively 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 immunemodulatory 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 Awojoodu 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 NFκB (nuclear factor 'kappa-light-chain-enhancer' of activated B-cells), which leads to a reduction in the iNOS expression and $NO^{\bullet}$ 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 $S1P_1$ receptor based on polyubiquitinylation. Downregulation of $S1P_1$ 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 (proteinkinase 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 $S1P_1$ 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. LPA_1 and LPA_2 can also control cell migration through regulation of Rho and ROCK via the coupled $G_{12/13}$ protein. LPA_1 and LPA_3 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. LPA_4 is also able to activate G_s, in addition to the previously mentioned G protein subtypes, in turn stimulating AC (adenylate cyclase). Presumably, LPA_1 and LPA_4 mainly determine bone metabolism, perhaps in opposite ways, as LPA_1 promotes osteoblast differentiation, whereas LPA_4 inhibits this [57, 150, 183, 184, 192–195]. LPA_5 transduces pleiotropic effects of the LPA ligand through $G_{12/13}$ or G_q whereas LPA_6 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 PGE_2 production as a result of LPA administration and LPA_1-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 LPA_1 activity leads to detrimental effects regarding bone metabolism because of reduced osteogenesis. On the contrary, LPA_4 knockout results in an opposite effect, more precisely in increased bone mass due to osteoblastic differentiation [184, 187]. LPA_1 is not only involved in osteoblastogenesis, but also in osteoclastogenesis, suggesting a balancing role of LPA_1-mediated signals in bone remodeling [57, 192]. LPA is believed to stimulate survival of osteoclasts directly via LPA_1-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, LPA_1 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, LPA_3 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 LPA_1 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 metabolization. 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, $NO^{\bullet}$ 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 $NO^{\bullet}$ by small molecule bi-functional (hybrid) drugs like $NO^{\bullet}$-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.

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