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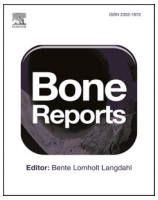

Fracture healing research: Recent insights

Lena Steppe [a], Michael Megafu [b], Miriam E.A. Tschaffon-Müller [a], Anita Ignatius [a], Melanie Haffner-Luntzer [a,*]

[a] Institute of Orthopaedic Research and Biomechanics, University Medical Center Ulm, Germany
[b] A.T. Still University Kirksville College of Osteopathic Medicine, USA

A B S T R A C T

Bone has the rare capability of scarless regeneration that enables the complete restoration of the injured bone area. In recent decades, promising new technologies have emerged from basic, translational and clinical research for fracture treatment; however, 5–10 % of all bone fractures still fail to heal successfully or heal in a delayed manner. Several comorbidities and risk factors have been identified which impair bone healing and might lead to delayed bone union or non-union. Therefore, a considerable amount of research has been conducted to elucidate molecular mechanisms of successful and delayed fracture healing to gain further insights into this complex process. One focus of recent research is to investigate the complex interactions of different cell types and the action of progenitor cells during the healing process. Of particular interest is also the identification of patient-specific comorbidities and how these affect fracture healing. In this review, we discuss the recent knowledge about progenitor cells for long bone repair and the influence of comorbidities such as diabetes, postmenopausal osteoporosis, and chronic stress on the healing process. The topic selection for this review was made based on the presented studies at the 2022 annual meeting of the European Calcified Tissue Society (ECTS) in Helsinki.

1. Introduction

Bone healing is a dynamic multi-step process that requires coordination of many cell types and signaling networks and proceeds through different phases, namely the inflammation, repair and remodeling phase. These phases show distinct cellular and molecular characteristics, but do also partly overlap with each other. Initially in the inflammatory phase, a fracture hematoma is formed which is rich in mediators that recruit immune cells including neutrophils, macrophages and lymphocytes. These cells are responsible for the removal of necrotic tissue and inducing angiogenesis as well as for the recruitment of progenitor cells from the endosteum and periosteum (Kawanami et al., 2009; Matthews et al., 2014; Colnot, 2009; Roberts et al., 2015). Activated upon fracture, progenitor cells like mesenchymal stroma cells (MSCs) and skeletal stem cells (SSCs) are essentially involved in bone regeneration. They have the potential to expand and undergo chondrogenic and osteogenic differentiation to form the fracture callus. During the repair phase of endochondral fracture healing, the fracture gap is first bridged by cartilaginous tissue that is formed by chondrocytes arising from osteochondro-progenitor cells. Chondrocytes become hypertrophic and cartilage is transformed into bone. In this process, cartilage resorption and chondrocyte apoptosis are crucial for allowing ossification by invading osteoprogenitor cells. It is also suggested that some chondrocytes directly transdifferentiate into osteoblasts, thereby promoting bone formation (Bahney et al., 2014). Finally at the remodeling phase, the bony callus is remodeled by osteoclasts to reconstruct the pre-injury geometry of the bone and to replace woven bone by mature lamellar bone. Multiple rounds of remodeling are needed to generate a strong cortical bone at the fracture site which is similar to the original bone structure. However, 5–10 % of fractures still do not heal successfully, leading to delayed bone union or non-union (Bhandari et al., 2003; Zura et al., 2016). Besides clinical fracture management failures, this has been mostly attributed to comorbidities and risk factors, which are well established to negatively influence the healing outcome. Basic and translational research was conducted to uncover more about potential patient-specific risk factors and how these might influence bone regeneration. Several studies also attempted to unravel the complex interactions of different cell types during the healing process and to gain insights into the regenerative potential of different progenitor cell populations. This review focuses on recently gained knowledge about progenitor cells for long bone repair and on the influence of comorbidities such as diabetes, osteoporosis and chronic stress on the healing process. The topic selection for this review was made based on the presentations at the 2022 annual meeting of the European Calcified Tissue Society (ECTS) in Helsinki, Finland.

* Corresponding author at: Helmholtzstraße 14, 89081 Ulm, Germany.
E-mail address: melanie.haffner-luntzer@uni-ulm.de (M. Haffner-Luntzer).

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2. Progenitor cells for long bone repair

During bone regeneration, the presence of active progenitor cells accounts for the regenerative potential of bone because they substantially contribute to all stages of fracture repair (Bianco, 2014; Bianco et al., 2008). Studies provided evidence that the periosteum hosts many of these cells, because its removal was shown to impair bone healing (Utvag et al., 1996; Murao et al., 2013) and that periosteal tissue transplantations were shown to improve bone healing (Chang and Knothe Tate, 2012). The periosteum includes mature Collagen-1 expressing osteoblasts and early progenitor cells expressing Prx1, the latter being shown to contribute to fracture callus development (Utvag et al., 1996; Murao et al., 2013). Therefore, in this chapter, we discuss the nature of adult skeletal stem cells, mainly mesenchymal stroma/stem cells (MSCs) and skeletal stem cells (SSCs) (Table 1) and what is known about the signaling pathways that are involved in regulating the cell fate decision during bone regeneration. Establishing a full understanding of progenitor cells offers great potential for the development of advanced cell-based therapies for promoting fracture repair.

MSCs were first described in the 1990s as adult multipotent progenitor cells because they were shown to differentiate into the adipogenic, osteogenic and chondrogenic lineages (Dominici et al., 2006; Pittenger et al., 1999). It was first assumed that MSCs only resides in the bone marrow. This assumption was later revised, when studies reported MSC isolation from adipose tissue (Zuk et al., 2002), periosteum (De Bari et al., 2006; Miura et al., 1994) or muscle (Lavasani et al., 2013) and that they had the same trilineage differentiation potential as bone marrow-derived MSCs. MSCs are now known to be ubiquitously distributed and are characterized by their differentiation capacity into several lineages. Furthermore, MSCs can be identified by their surface marker expression, for example, CD73, CD90 and CD105. Regarding fracture healing, the recent knowledge points more to a regulatory function by the secretion of growth factors and cytokines, thereby controlling the local inflammatory environment (Xu et al., 2020) and less a direct contribution to bone formation by differentiation into the osteoblast lineage. Additionally, MSCs secrete angiogenic factors to stimulate endothelial cells to generate new blood vessels during bone healing (S. Zhang et al., 2022). MSCs are recruited to the site of injury mainly by chemokines. Chemokines bind to specific receptors on the surface of the stem cells, initiating a signaling cascade that leads to changes in the cytoskeleton and ultimately, cell migration. One chemokine that has been shown to be important in stem cell recruitment during bone fracture healing is stromal cell-derived factor-1 (SDF-1). SDF-1 is expressed by bone marrow stromal cells and is upregulated in response to bone fracture. SDF-1 binds to its receptor CXCR4, which is expressed on the surface of stem cells, leading to their migration towards the site of injury (Kitaori et al., 2009).

Another important progenitor cell type which might display an even greater regenerative potential are SSCs. Because an intact periosteum was shown to be essential for bone regeneration, studies aimed to identify which cells within the periosteal layer contribute to bone repair. In contrast to MSCs, SSCs reside only in the bone marrow, periosteum, endosteum and adjacent muscle (Julien et al., 2021; Abou-Khalil et al., 2015; Park et al., 2012; Bianco and Robey, 2015). They are activated in response to bone fracture, resulting in cell proliferation and increased migration to the fracture site (Chan et al., 2015). SSCs have the potential of self-renewal and display exclusive differentiation capacities towards the skeletal lineages. This has been elucidated by experimental approaches that combined bone grafting and lineage tracing. It was demonstrated that periosteal SSCs have an osteochondral potential, whereas those derived from the endosteum can only give rise to the osteogenic lineage. By contrast, SSCs from skeletal muscle mostly contributed to cartilage formation (Julien et al., 2022). Therefore, SSCs have been considered to be a heterogeneous cell population due to their origin-dependent differentiation capacity. Although MSCs and SSCs derive from a common embryonic mesenchymal lineage (Duchamp de Lageneste et al., 2018), SSCs exhibit an overall greater regenerative potential due to a higher proliferation rate and differentiation capacity (Agata et al., 2007; Ho-Shui-Ling et al., 2018; Lu et al., 2019). Furthermore, they also possess a particular transcriptional signature that differs from MSCs and strongly depends on their tissue source (Debnath et al., 2018).

It has been a major challenge to establish unique surface markers for the identification and tracking of SSC populations during fracture healing. Their high heterogeneity in particular complicates their identification. To date, a precise definition of SSCs remains lacking, but several phenotypic markers were proposed for distinguishing SSC populations from different origins. Nestin (Nes), a marker for periosteum development, was initially utilized to label early progenitor cells, but later studies revealed that Nes+ cells might not differentiate into the osteoblast lineage or that they lack the key aspect of self-renewal *in vivo*, therefore, Nes is most probably not a selective label of SSCs (Tournaire et al., 2020; Worthley et al., 2015; Kurenkova et al., 2020). Nevertheless, Nes-expressing cells were shown to participate in bone regeneration and can give rise to osteogenic cells at the fracture site (Tournaire et al., 2020). Interestingly, Debnath et al. found that the well-established osteoclast marker cathepsin K labels periosteal SSCs involved in intramembranous bone healing, but can also convert to endochondral-competent cells, thereby contributing to endochondral repair (Debnath et al., 2018). Another study identified periostin as an important fracture-induced key regulator that was shown to be upregulated by SSCs (Duchamp de Lageneste et al., 2018). Furthermore, periostin appears to be crucial for the self-renewal capacity of SSCs, because in periostin knockout (KO) mice bone repair and SSC function were impaired. Within the periosteum of long bones, glioma-associated oncogene 1 (Gli1) was reported to be expressed by a subpopulation of SSCs which were shown to proliferate in response to fracture and participate in callus formation (Xia et al., 2020). By lineage tracing approaches, αSMA was shown to reliably label two osteoprogenitor subsets in the periosteum that contribute to osteoblast formation in response to loading (Matthews et al., 2020). A combination of two markers, namely Msx1 and αSMA was described by Ortinau et al. to identify SSCs (Ortinau et al., 2019). They provided strong evidence that when using both markers, periosteal SSCs were precisely labelled, showing self-renewing properties and participation in bone healing. Recently, it was demonstrated that aside from the platelet-derived growth factor receptor β (PDGFR β) (Bohm et al., 2019), PDGFRα is expressed by a periosteal progenitor cell population that profoundly

Table 1
Overview of the key features of mesenchymal stroma cells (MSCs) and skeletal stem cells (SSCs) (Bianco, 2014; Bianco et al., 2008; Utvag et al., 1996; Murao et al., 2013; Ortinau et al., 2019; Bohm et al., 2019).

	MSCs	SSCs
Location	Ubiquitously	Skeleton
Skeletal lineage restricted	No	Yes
Markers	CD73, CD90 and CD105	CD73, CD146, CD164, CD200 PDPN Periosteum: Prx1, CTSK, Gli1, αSMA, Mx1, Grem1, Sox9, Col2, Aggrecan, LepR Endosteum: CTSK, Mx1 Periostin
Regenerative capacity	+	++
Stability	Low	High
Chemokine/receptor interaction during attraction to fracture site	SDF-1/CXCR4	CCL5/CCR5, PDGF/PDGFRs
Contribution to fracture callus	Indirect effects by immunomodulation	Direct effects by contributing to cartilage and bone cells

contributes to cortical bone formation and fracture repair. In addition, Prx1 expression defines a subset of SSCs which is activated upon bone fracture and contributes to callus formation (Esposito et al., 2020). Since several markers for SSCs were reported, it has been shown that they partially overlap, for example, Msx1+ cells also express Prx1 (Ortinau et al., 2019). Future studies are needed to enhance our knowledge about the hierarchy and subpopulations of periosteal stem cells and by which marker combination they can be truly identified.

Regarding the molecular mechanisms involved in SSC migration towards the injury site, it was demonstrated that nearly all SSCs positive for Mx1 and αSMA express the receptor for the chemokine CCL5, CCR5 (Ortinau et al., 2019). Ortinau et al. further revealed that CCL5 induced periosteal SSC migration *in vitro* and *in vivo*. Confirming this, CCL5 deletion or inhibition delayed bone regeneration, whereas local CCL5 treatment improved bone healing (Ortinau et al., 2019). Also, PDGFRβ interaction with PDGF seems to be crucial for stem cell recruitment to the fracture site after bone injury (Bohm et al., 2019).

To promote SSCs differentiation into osteoblasts and chondrocytes at the injury site, bone morphogenic protein 2 (BMP2) was shown to regulate the stepwise downregulation of Prx1 *via* controlling C-X-C motif-ligand-12 (CXCL12) signaling (Esposito et al., 2020). Intriguingly, BMP2 mediated signaling was also demonstrated to be essential for the osteogenic capability of periosteum-derived cells (Bolander et al., 2020). Additionally, in another study, BMP signaling was found to regulate the coordinated activation of periosteal and skeletal muscle SSCs in response to bone fracture (Julien et al., 2022). Further evidence for the importance of BMPs for bone regeneration derived from experiments that used *in vitro* expanded SSCs that were implanted together with a BMP2-delivering hydrogel in a calvarial defect model. In this model, BMP-2 increased the regenerative capacity (Papageorgiou et al., 2019). Activated SSCs were demonstrated to undergo fibrogenesis before differentiating in the chondrogenic direction, while BMP inactivation compromised bone healing and decreased SSC functions upon bone regeneration (Julien et al., 2022). During the early phase of the healing process, Xia et al. further demonstrated the importance of TGF-β/Smad2 signaling in $Gli1^+$ periosteal SSCs (Xia et al., 2020). This was confirmed by inducible deletion of TGF-β expression in $Gli1^+$ SSCs and by the usage of neutralizing antibody against TGF-β1, while both approaches resulted in impaired bone and cartilage formation and compromised SSC proliferation (Xia et al., 2020). In addition, Notch signaling in αSMA^+ SSCs has been reported to accelerate fracture healing, while Notch1 inhibition had the opposite effect, leading to a higher cartilaginous ratio accompanied by a lower bone formation rate (Novak et al., 2020).

Besides biochemical signals, stem cells are also able to react to their biomechanical environment. It has been shown that stresses and strains in the fracture callus (*e.g.* shear stress, compression, micromovements, fluid flow, hydrostatic pressure) shape the response of stem cells to the tissue injury and therefore are critical for tissue differentiation at the fracture site (Zhang et al., 2012; Ghimire et al., 2018; Miramini et al., 2023; Liu et al., 2023; Augat et al., 2003; Augat et al., 2005; Haffner-Luntzer et al., 2016). The mechanical environment and therefore stem cell response is highly dependent on fracture geometry, fracture fixation, movement and bodyweight of the patient and are subjected to change during the healing process. The underlying mechanism of biomechanical influence on fracture healing is described in Pauwel's theory of "causal histogenesis" (Pauwels, 1960). He postulated the profound influence of the mechanical environment on tissue differentiation. In more detail, Claes et al. demonstrated in 1998, that if there are high stresses at the fracture area, mesenchymal cells are likely to form fibrous tissue, whereas osseous tissue is generated under low stress conditions. At intermediate stresses, mesenchymal cells will differentiate into chondrocytes and initiate cartilaginous callus formation, which initially bridges the fracture gap (Claes and Heigele, 1999; Claes et al., 2009; Claes et al., 1998).

Taken together, recent research revealed that SSC activation and recruitment is a crucial step in injury repair that promotes callus formation and regeneration (Perrin and Colnot, 2022). Mechanistically, several molecular pathways and the biomechanical environment have been identified to be involved in MSC and SSC recruitment and/or bone and cartilage formation at the injury site, among them BMP signaling and chemokine pathways (CCL5, CCR5, PDGF) as well as TGF-β/Smad2 and Notch signaling. Further studies are needed to understand the complex interplay of these signaling pathways to develop strategies for progenitor expansion and differentiation to facilitate bone regeneration.

3. The influence of comorbidities on fracture healing

3.1. Diabetes mellitus

Diabetes is a metabolic disorder and a major public health challenge of the 21st century. The global prevalence of diabetes in 2019 was estimated to represent 9.3 % of adults (463 million people) and is expected to increase to 10.2 % (578 million) by 2030 and 10.9 % (700 million) by 2045 (American Diabetes, 2014). Diabetes is a chronic medical condition caused by insufficient insulin secretion and/or insulin resistance in target tissue (Saeedi et al., 2019). It can be differentiated in two forms: type 1 and type 2. Type 1 diabetes mellitus (T1DM) is a consequence of autoimmune destruction of the beta cells of the pancreas, which results in the necessity of providing exogenous insulin to control blood sugars in the body. By contrast, type 2 diabetes mellitus is a consequence of an acquired resistance to the peripheral action of insulin by cells and organs that develops over years. Many research studies have identified numerous complications associated with diabetes, including kidney disease, retinopathy, peripheral neuropathy, coronary heart disease, heart failure, stroke, peripheral vascular disease, cancer, infections, liver disease, cognitive disability, functional disability and affective disorders (Bertoni et al., 2004; Chatterjee et al., 2016; Einarson et al., 2018; Harding et al., 2019; Pearson-Stuttard et al., 2016; Shah et al., 2015; Tolman et al., 2007; Tsilidis et al., 2015). Most notably, recent evidence has demonstrated a significant association between diabetes type 2, reduced bone quality and poor fracture healing (Chen et al., 2022; Tanios et al., 2022; Tomic et al., 2022). Diabetes is a proinflammatory metabolic condition which exacerbates hematoma formation and prolongs callus development, contributing to an overall delay in fracture healing. Callus formation and angiogenic processes are disrupted, causing poor quality of the new bone formed at the fracture site (Mosyagina and Astrakhantsev, 2022). The underlying molecular and cellular mechanisms are poorly understood and are currently a focal point in fracture healing research. Most used research models mimic type 2 diabetes. The metabolic nature of diabetes anticipates a hyperglycemic state which leads to biochemical changes such as oxidative stress, signaling pathway activation or inhibition, inflammatory reactions, adipogenesis/osteogenesis transformation imbalance and microvascular changes in the bone marrow (Dhaliwal et al., 2022; Napoli et al., 2017). The accumulation of senescent cells can also accelerate bone aging and result in impaired bone formation (Khosla et al., 2021). Disturbances in inflammation can also lead to compromised bone healing, specifically, in the interactions among osteoblasts, adipocytes, bone marrow stem cells and the bone marrow environment (Murray and Coleman, 2019). Studies are investigating advanced glycation end products (AGEs) as potential mediators of the pathophysiological mechanisms underlying fracture healing impairment in diabetics (Jiao et al., 2015). Although the mechanisms involving AGEs are not thoroughly understood, the chronic hyperglycemic state of diabetes predisposes a rapid AGEs formation and increases the generation of brittle bone (Samakkarnthai et al., 2020). This allows a downstream effect to increase receptors for advanced glycation end products and increase inflammation and local resorption, creating a porous bone within diabetics. Also stem cells are negative affected by diabetes during fracture healing. Diabetes affects SSC niche, their proliferation and differentiation capacity and their migratory behavior (Fu et al., 2023; Rao et al., 2021). Tevlin et al. investigated the effects of diabetes on the

skeletal stem cell niche during bone healing (Tevlin et al., 2017). They found that high serum concentrations of tumor necrosis factor-α directly repressed the expression of Indian hedgehog (Ihh) in murine SSCs and in their downstream cell offspring. Exogenous Ihh was able to rescue the negative effects of diabetes on SSCs and therefore enhanced fracture healing.

The clinical management of diabetes has focused primarily on controlling blood glucose. The non-pharmacological approach to diabetes essentially constitutes lifestyle interventions such as consistent physical activity and a well-balanced diet, but treatment plan adherence is a major part of ensuring the success of this approach (He et al., 2009; Tan et al., 2019). Pharmacological agents include insulin, metformin, sulfonylureas, thiazolidinediones, dipeptidyl peptidase-4 inhibitors, glucagon-like peptide 1 receptor agonists, and sodium-glucose cotrasnsporter-2 inhibitors (He et al., 2009; Tan et al., 2019). These agents target different aspects of glucose metabolism to exert an overall glucose-lowering effect in diabetic patients. Insulin injection is one of the main treatment regiments for T1DM and replenishes the deficient insulin within the pancreas, regularizing glucose and HbA1c levels. Metformin (from the drug class biguanides) is the most prescribed drug particularly in obese and overweight individuals, which increases insulin sensitivity, boosting glucose uptake *via* GLUT-enhancer factor phosphorylation and hepatic gluconeogenesis suppression (He et al., 2009; Lin et al., 2018). Metformin can also aid in lowering weight, serum triglycerides and serum LDL cholesterol levels. However, Metformin is also prescribed in non-obese patients suffering from monogenetic forms of diabetes. While all of these pharmacological agents are used to control glucose-mediated effects in diabetic patients, their influence on delayed fracture healing in these patients is unclear. The influence of metformin on fracture healing is strongly debated, with studies showing controversial effect (Jeyabalan et al., 2013; La Fontaine et al., 2016; Yang et al., 2019). One recent study investigated the effects of a modified version of metformin on bone healing. It was demonstrated that the drug induced the AMPK signaling cascade and led to pro-osteoblastic effects, suggesting its potential positive effect on fracture healing (Mu et al., 2018). Other research studies have looked at TNF-alpha as a potential target for the treatment of diabetes and its ability to aid in fracture healing processes. Low TNF-alpha doses have been shown to augment fracture healing while TNF-alpha receptor-deficient mice displayed significantly disturbed bone healing (Jiao et al., 2015; Gerstenfeld et al., 2001; Gerstenfeld et al., 2003; E. Zhang et al., 2022). A recent study by Zhang et al. suggested that inhibition of TNF-alpha signaling might be able to counteract the diabetes-induced delay in bone healing, because diabetic mice displayed increased TNF-alpha levels during fracture healing (E. Zhang et al., 2022). Further research has investigated MSCs as possible treatment targets for diabetic fracture healing. A study by Zhang et al. investigated the possible effects of leptin signaling on BMP9-induced osteogenic MSC differentiation and demonstrated that exogenous leptin potentiates BMP9-induced MSC osteogenic differentiation *in vitro* and *in vivo* and inhibits the BMP9-induced adipogenic differentiation (Zhang et al., 2020). Several studies also investigated Trb3 as a potential molecular target to regulate adipo-osteogenic differentiation of MSCs in bone formation and diabetic fracture healing (Avery et al., 2010; Du et al., 2003; Staines et al., 2014). Most notably, Fan et al. investigated the reciprocal role of Trb3 in regulating MSC osteogenic and adipogenic differentiation and observed that local *in vivo* Trb3 administration *via* a polymer scaffold stimulated bone regeneration (Fan et al., 2017; Fan et al., 2021). These studies indicated that MSCs play an important role in regulating tissue differentiation during diabetic fracture healing and might therefore be a possible therapeutic target.

In conclusion, diabetes induces delayed fracture healing. The effect of pharmacological agents to treat diabetes (*e.g.* metformin) on fracture healing is widely unclear. Experimental studies focus on different signaling pathways as targets to accelerate diabetic fracture healing, however, further research is needed to prove the translational relevance of these findings in humans.

3.2. Postmenopausal osteoporosis

Osteoporosis is a metabolic disease leading to loss of bone mass and microstructural deterioration of bone tissue. Especially the decline of estrogen levels after menopause is an important trigger for the loss of bone mass, since the sex hormone estrogen has important functions in bone metabolism. Osteoporotic patients do not only have an increased risk for fractures because of the weak bone quality, also the healing process is often delayed. After an osteoporotic hip fracture, 10–15 % of patients die within the first year and 20–30 % remain permanently invalid (Schurch et al., 1996). Orthopaedic complications after osteoporotic fractures frequently result from the reduced bone quantity and quality, which hinder proper fixation of osteosynthesis devices and decrease the primary stability of fracture fixation. But also the biological processes of regeneration are often disturbed in osteoporotic bone (Nikolaou et al., 2009). Therefore, osteoporotic fractures lead to a high degree of suffering for the patient and a dramatic socio-economic burden for society. New treatment strategies to prevent delayed osteoporotic fracture healing are urgently needed. Regarding this aim, a deeper understanding of the molecular mechanisms underlying disturbed bone regeneration is necessary, which can be gained by experimental studies. A commonly used model for postmenopausal osteoporosis is ovariectomy (OVX) in rodents. OVX induced estrogen depletion in rats and mice leads to a decreased mechanical competence and bone formation in the late fracture callus (Namkung-Matthai et al., 2001; Meyer Jr. et al., 2001; Hao et al., 2007). The number of osteoclasts was significantly increased during callus development (Islam et al., 2005). This might be explained by the general effects of the hormone estrogen on bone by increasing osteoclast and decreasing osteoblast apoptosis (Nakamura et al., 2007; Kousteni et al., 2001; Yang et al., 2013), and by stimulating the recruitment, proliferation and differentiation of skeletal progenitor cells (Cheung et al., 2016; Shang et al., 2014). Estrogen has been shown to enhance the proliferation and differentiation of SSCs (Cheung et al., 2016; Shang et al., 2014), leading to increased bone formation. It does so by binding to estrogen receptors on the surface of the cells, triggering a cascade of molecular events that ultimately promote bone formation. Studies have also demonstrated that estrogen deficiency can lead to a decrease in skeletal stem cell activity and an increase in bone loss. Interestingly, the effects of estrogen on skeletal stem cells appear to be sex-specific, with females experiencing a greater response to estrogen than males. This may partially explain why postmenopausal women/OVX rodents are at a higher risk for delayed fracture union. Besides direct effects of estrogen, it was also demonstrated that aging increases stem cell senescence, leading to an impaired stem cell pool for fracture healing (Goodnough and Goodman, 2022). Analysis of the intermediate phase of endochondral fracture healing in OVX mice further demonstrated a decreased cartilaginous callus area (Beil et al., 2010) and a reduced expression of cartilage markers (Hatano et al., 2004) and angiogenic factors (Li et al., 2015). But also the first phase after fracture, the inflammatory phase, was shown to be disturbed by postmenopausal osteoporosis (Haffner-Luntzer et al., 2017; Fischer et al., 2018). OVX mice displayed significantly more inflammatory cytokines and immune cells like neutrophils and mast cells in the early fracture hematoma. Especially mast cells were shown to play an important role during compromised fracture healing (Ragipoglu et al., 2022a; Ragipoglu et al., 2022b).

One novel therapeutic approach which could target the alterations in the various stages of bone regeneration in osteoporotic patients is vibration treatment. Particularly whole-body vibration is proposed to be a promising non-invasive and non-pharmacological therapy for osteoporosis (Lau et al., 2010). Indeed, low-magnitude, high-frequency vibration (LMHFV) was shown to stimulate osteoanabolic effects on the skeleton, dependent on the vibration settings, in many experimental and clinical studies in both healthy and osteoporotic subjects (Bemben et al.,

2010; von Stengel et al., 2011; Tezval et al., 2011; Rubin et al., 2004). LMHFV was also proposed as a suitable means to improve fracture healing. However, the few existing experimental studies reported contradictory results. Analysing these studies, LMHFV appears to act beneficially on fracture healing in estrogen-deficient rodents (Stuermer et al., 2010; Chung et al., 2014; Wei et al., 2016; Shi et al., 2010), whereas no or negative effects were observed in estrogen-competent animals (Stuermer et al., 2010; Chung et al., 2014; Wehrle et al., 2015; Wehrle et al., 2014). Recent studies have revealed that LMHFV treatment ameliorated the altered inflammatory response after fracture in OVX rodents (Cui et al., 2022) and boosted bone formation after fracture in estrogen-deficient mice, the latter being mediated by estrogen receptor alpha signaling specifically on osteoblasts (Steppe et al., 2021). LMHFV was also shown to increase MSC recruitment in osteoporotic fracture healing through the SDF-1/CXCR4 pathway (Wei et al., 2016). However, because LMHFV has also shown negative effects of fracture healing in estrogen-competent animals, caution is needed to determine the specific patient population who could benefit from this type of treatment.

In conclusion, postmenopausal osteoporosis leads to various alterations in all of the different phases of fracture healing. Estrogen-deficient rodents display an imbalanced inflammation after fracture as well as a prolonged healing time. One novel therapeutic approach to counteract these negative effects of estrogen-deficiency on bone regeneration is the application of whole-body vibration. Because this method for external biomechanical stimulation is already applied in osteoporotic patients without fracture, it holds promise to be a clinically relevant treatment approach. Indeed, there is one clinical trial registered on clinicaltrails.gov which aims to investigate the effects of vibration on fracture healing in humans, however no data regarding that has been published yet.

3.3. Chronic stress

Chronic stress is an increasing problem in our modern society (Jackson, 2014). Psychological stress is defined as negative emotional experience which is accompanied by physiological, biochemical and behavioral changes (Baum, 1990). Stress causes activation of different pathways, including the hypothalamus-pituitary-adrenal (HPA) axis and catecholaminergic systems (the sympatho-adrenomedullary pathway and the sympatho-neural system), following in a cascade of events which leads to a complex stress response (Kvetnansky et al., 2009; Mariotti, 2015). While short-term physiological adaptations due to stress hormones are of advantage for acute stress situations (Dhabhar, 2009), long-term chronic stress has adverse effects on our health. It increases the risk for cardiovascular diseases, such as hypertension, myocardial ischemia and myocardial infarction (Dimsdale, 2008; Gullette et al., 1997; Spruill, 2010). Furthermore, it can increase the risk for infections due to the immunosuppressive function of stress hormones (Dhabhar, 2009). By contrast, it also increases the risk for inflammatory diseases including asthma, allergies, autoimmune diseases and inflammatory bowel disease (Dhabhar, 2009; Langgartner et al., 2015; Liu et al., 2017). Furthermore, stress increased the risk for mental diseases like depression or posttraumatic stress disorders (PTSD). Additionally, chronic stress may have adverse effects on bone, because people who experienced repeated mental traumatization during childhood are from shorter stature (Batty et al., 2009) and because depression and PTSD have been associated with osteoporosis and increased fracture risk (Calarge et al., 2014; Gebara et al., 2014; Glaesmer et al., 2011; Zong et al., 2016). Moreover, there is some clinical evidence for delayed fracture healing in PTSD patients (Thayer et al., 2015). Preclinical studies demonstrated in a mouse model of chronic psychosocial stress (chronic subordinate colony housing) disturbed endochondral ossification during long bone growth (Foertsch et al., 2017) (Table 2). Furthermore, stressed mice showed disturbed fracture healing (Haffner-Luntzer et al., 2019) (Table 2). Stressed mice displayed a significantly reduced flexural rigidity of the fractured femur as well as a decreased bone volume-to tissue volume ratio and tissue mineral density in the fracture callus. Persisting cartilage in the fracture callus and decreased Runx2 expression in the cartilage-to-bone transition zone of the fracture callus indicate a disturbed endochondral ossification also during fracture healing. Furthermore, stressed mice displayed a dysregulated immune response after fracture. Increased tyrosine hydroxylase (TH) expression was observed in the early fracture callus of stressed mice, indicating that an increased local catecholamine production contributes to the stress effects on fracture healing because TH is the rate-limiting enzyme in catecholamine synthesis. Blockade of the beta-adrenoreceptor (AR) signaling by injecting the unspecific beta-blocker propranolol was able to inhibit the negative effects of stress on fracture healing. This indicates an important role of catecholamine signaling on bone fracture healing, particularly under stress conditions. Indeed, the expression of several ARs has been shown on osteoblasts, osteoclasts and chondrocytes (Foertsch et al., 2017; Aitken et al., 2009; Huang et al., 2009; Lorenz et al., 2016; Opolka et al., 2012; Takarada et al., 2009). Among β-ARs, the β_2-AR is the predominantly expressed subtype in bone cells (Aitken et al., 2009; Elefteriou et al., 2014; Hajifathali et al., 2014). In murine chondrocytes, only the β_2-AR is expressed among all β-AR subtypes (Lai and Mitchell, 2008). The effects of catecholamine-mediated signaling on bone cells seem to be highly dependent on catecholamine concentration and the involved receptor subtype. Although epinephrine (EPI), norepinephrine (NE) and the β-AR agonist isoproterenol have been shown to increase proliferation of the osteoblast cell line MC3T3-E1 and EPI increases ALP activity in these cells *in vitro* (Suzuki et al., 1998), genetic inactivation of the β-ARs *in vivo*, particularly of the β_2-AR, increased bone mass in mice and β-blocker application appeared to increase the bone mineral density and reduce the fracture risk in osteoporotic patients (Elefteriou et al., 2014). This indicates a negative effect of β_2-AR-signaling on bone tissue. NE also alters chondrogenic differentiation of human MSCs and chondrogenic progenitor cells *via* β-AR signaling (Jenei-Lanzl et al., 2014) and the differentiation of murine chondrocytes has been shown to be negatively affected by β_2-AR signaling (Lai and Mitchell, 2008; Mitchell et al., 2011). This explains the positive effects of AR-blockers on fracture healing which have been shown in several experimental studies (Knapstein et al., 2022). Interestingly, pharmacological blockade of NE reuptake improved late stage fracture healing, but led to reduced bone mass in the intact skeleton (Donat et al., 2022). Regarding the effects of stress on bone, Tschaffon et al. recently reported that catecholamines produced locally by myeloid cells are responsible for the delayed fracture healing in stressed mice (Tschaffon et al., 2022).

Another way how chronic stress might interfere with bone is *via* disturbances of the gut microbiome and interactions with the immune system. Early life stress (ELS) is known to have long term and sex-dependent impact on the gut microbiota and the immune system in

Table 2
Effects of chronic stress on bone and fracture healing (Foertsch et al., 2017; Haffner-Luntzer et al., 2019).

	Effects of stress on bone	Effects of stress on fracture healing
Endochondral ossification	Disturbed during long bone growth	Disturbed during callus formation
Osteoblast numbers	No effect	Minor effects
Osteoclast numbers	No effect	Minor effects
Runx2 expression in hypertrophic chondrocytes	Reduced	Reduced
Inflammation	Systemic low-grade chronic inflammation	Reduced expression of inflammatory cytokines in the hematoma, increased presence of myeloid cells
Catecholamine signaling	Increased in the growth plate	Increased in the fracture hematoma

rodents (Langgartner et al., 2020; Donoso et al., 2020; O'Mahony et al., 2009; Rincel et al., 2019; Rincel and Darnaudéry, 2020). For instance, while ELS in male mice has been shown to affect the abundance of taxa belonging to Lachnospiraceae and Porphyromonadaceae families or other unclassified Firmicutes, but also Bacteroides, Lactobacillus and Alloprevotella genera, ELS effects in females were mainly restricted to Lactobacillus and Mucispirillum genera. This might also explain while females are in general more susceptible for hyperinflammation after ELS. Support for a critical role of the microbiota in ELS-induced immune and bone effects comes from own earlier studies showing that psychosocial stress during adulthood affects the composition of the gut microbiome and that rectal infusions of control feces into psychosocially stressed mice ameliorated the stress-induced systemic immune activation and changes in bone metabolism (Reber et al., 2016; Reber et al., 2008; Langgartner et al., 2018).

In summary, chronic stress is a global health problem with increasing prevalence. Experimental studies demonstrated the negative effects of chronic stress on bone healing and showed that catecholamine signaling might be involved. These data offer translational potential to treat stressed fracture patients to ensure uneventful healing.

4. Conclusion

Fractures are a common occurrence in both humans and animals. While most fractures heal without complications, a significant number of patients experience delayed or non-unions, which can result in long-term disability and a decreased quality of life. Therefore, understanding the process of fracture healing and its disturbances is essential in

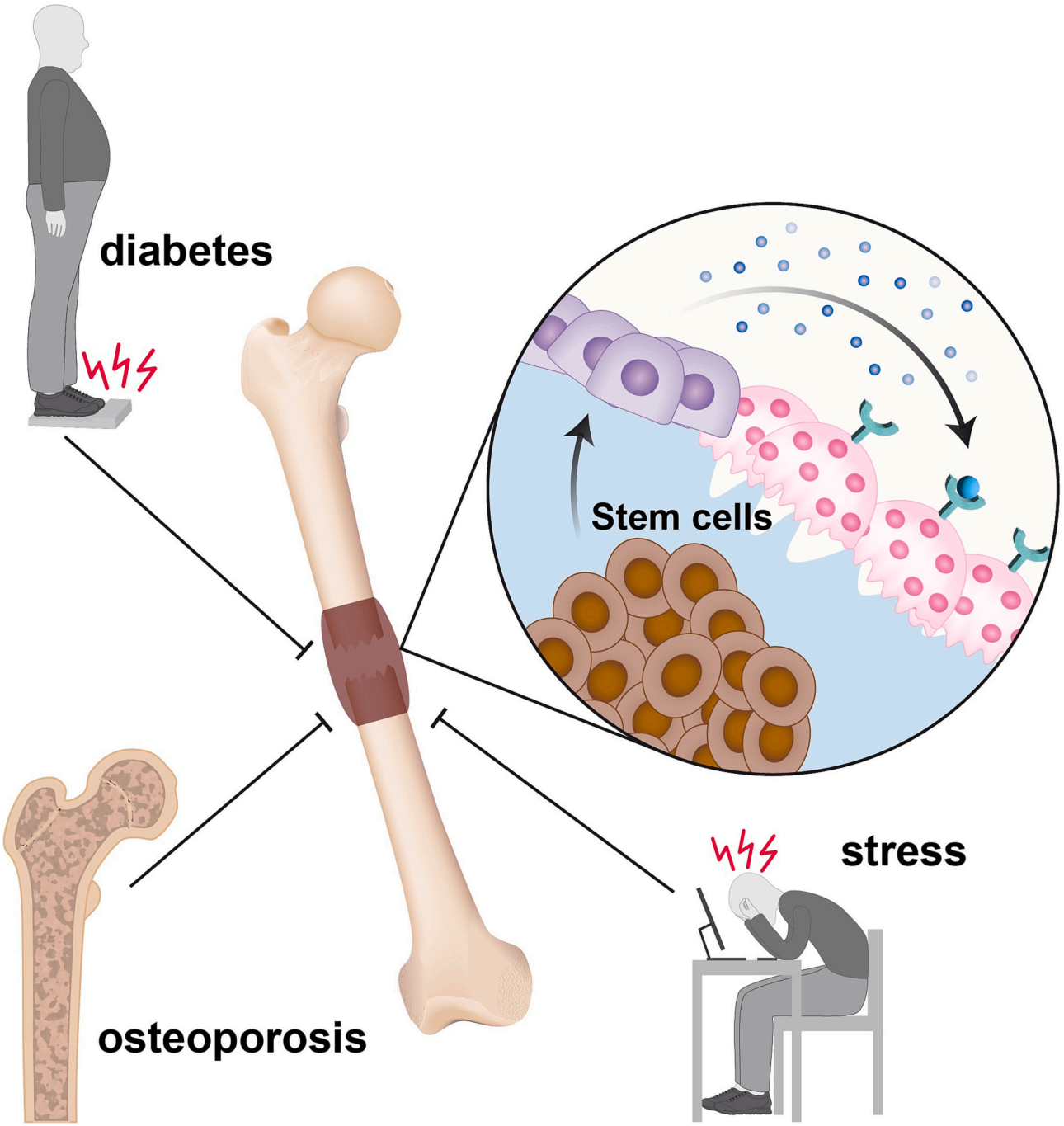

Fig. 1. Disturbance factors for fracture healing summarized in this review.

developing effective treatments for these injuries. Basic/translational research, which involves studies conducted in animals or cell cultures, is a critical step in investigating the fracture healing process. Recent studies in fracture healing research have gained insights into how different progenitor cells contribute to bone regeneration and have generated hypotheses and new research ideas on how to utilize these cells to benefit the patient. Furthermore, studies have shown the significant influence of comorbidities like diabetes, osteoporosis and chronic stress on the bone healing process and the involved molecular mechanisms (Fig. 1). However, to transfer these findings into clinical practice, more research is needed to specify patient cohorts for different treatments and to investigate safety and efficacy of novel therapeutic interventions.

CRediT authorship contribution statement

Lena Steppe: Investigation, Methodology, Writing – original draft, Writing – review & editing. **Michael Megafu:** Investigation, Writing – original draft, Writing – review & editing. **Miriam E.A. Tschaffon-Müller:** Investigation, Writing – original draft, Writing – review & editing. **Anita Ignatius:** Supervision, Writing – review & editing. **Melanie Haffner-Luntzer:** Conceptualization, Funding acquisition, Investigation, Methodology, Supervision, Visualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

We do not have a conflict of interest regarding that manuscript. The work was done in the framework of projects funded from the German Research Foundation (HA 8470/1-1 and 251293561 SFB1149).

Data availability

No data was used for the research described in the article.

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