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What do we know about bone morphogenetic proteins and osteochondroprogenitors in inflammatory conditions?

Nina Lukač[1,2], Vedran Katavić[1,2], Sanja Novak[3], Alan Šućur[1,4], Maša Filipović[1,4], Ivo Kalajzić[3], Danka Grčević[1,4], Nataša Kovačić[1,2]

[1]Laboratory for molecular immunology, University of Zagreb School of Medicine, Zagreb, Croatia

[2]Department of Anatomy, University of Zagreb School of Medicine, Zagreb, Croatia

[3]Department of Reconstructive Sciences, University of Connecticut Health Center, Farmington, Connecticut, USA

[4]Department of Physiology and Immunology, University of Zagreb School of Medicine, Zagreb, Croatia

Corresponding author: Prof. Nataša Kovačić, address: Department of Anatomy, University of Zagreb Medical School, Šalata 11, 10000 Zagreb, Croatia, telephone: +385 1 4566 846, e-mail: natasa.kovacic@mef.hr

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Abstract

Osteochondroprogenitors are crucial for embryonic bone development and postnatal processes such as bone repair in response to fracture injury, and their dysfunction may contribute to insufficient repair of structural damage in inflammatory arthritides. In the fracture healing, the early inflammatory phase is crucial for normal callus development and new bone formation. This process involves a complex interplay of many molecules and cell types, responsible for recruitment, expansion and differentiation of osteochondroprogenitor populations. In inflammatory arthritides, inflammation induces bone resorption and causes insufficient bone formation, which leads to local and systemic bone loss. While bone loss is a predominant feature in rheumatoid arthritis, inflammation also induces pathologic bone formation at enthesial sites in seronegative spondyloarthropaties. Bone morphogenetic proteins (BMP) are involved in cell proliferation, differentiation and apoptosis, and have fundamental roles in maintenance of postnatal bone homeostasis. They are crucial regulators of the osteochondroprogenitor pool and drive their proliferation, differentiation and lifespan during bone regeneration. In this review, we summarize the effects of inflammation on osteochondroprogenitor populations during fracture repair and in inflammatory arthritides, with special focus on inflammation-mediated modulation of BMP signaling. We also present data in which we describe a population of murine synovial osteochondroprogenitor cells, which are reduced in arthritis, and characterize their expression of genes involved in regulation of bone homeostasis, emphasizing the up-regulation of BMP pathways in early progenitor subset. Based on the presented data, it may be concluded that during an inflammatory response, innate immune cells induce osteochondroprogenitors by providing signals for their recruitment, by producing BMPs and other osteogenic factors for paracrine effects, and by secreting inflammatory cytokines that may positively regulate osteogenic pathways. On the other hand, inflammatory cells may secrete cytokines that interfere with osteogenic pathways, proapoptotic factors that reduce the pool of osteochondroprogenitor cells, as well as BMP and Wnt antagonists. The net effect is strongly context-dependent and influenced by the local milieu of cells, cytokines, and growth factors. Further elucidation of the interplay between inflammatory signals and BMP-mediated bone formation may provide valuable tools for therapeutic targeting.

Key words

osteochondroprogenitors, bone morphogenic proteins, inflammation, inflammatory arthritis, fracture

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1. Introduction

Development of the skeletal system, bone growth, remodeling and regeneration after injury all depend on a pool of bone/cartilage progenitor cells and mechanisms regulating their differentiation. The bone morphogenetic protein (BMP) pathway is crucial for postnatal bone homeostasis and regeneration. Although the role of BMPs is well described in the regulation of osteochondroprogenitor proliferation, differentiation and apoptosis, data on specific changes under inflammatory conditions are still scarce. The inflammatory process has, paradoxically, a dual effect on bone tissue, with anabolic action described during fracture healing and enthesial ossifications, as well as catabolic and anti-anabolic action in rheumatoid arthritis (RA) and other immune-mediated osteoresorptive diseases. Understanding the interplay between the inflammatory response and BMP pathway modulation in osteochondroprogenitor cells promises insight into the pathogenesis of skeletal disorders such as non-unions, different forms of arthritis and osteoporosis, and aids to the development of new approaches for their treatment.

1.1. Definition of bone and cartilage progenitor populations

The concept of a postnatal multipotent self-renewing bone marrow stromal progenitor cell, able to differentiate into adipocytes, chondroblasts and osteoblasts emerged in 1988 (1) and the cell was termed mesenchymal stem cell shortly after its discovery (2). In 2006, the International Society for Cellular Therapy (ISCT) proposed a change in terminology into mesenchymal stromal cell (MSC), and defined minimal criteria for their definition based on their plastic-adherence in standard culture conditions, expression of surface markers, and their capability of trilineage differentiation *in vitro* (3). Since the establishment of these criteria, an overwhelming amount of new research has been published, characterizing the MSCs isolated from different tissues by various surface markers, urging scientists to call for additional, more stringent criteria (4). While cells from multiple anatomical locations that fit ISCT criteria are capable of *in vitro* trilineage differentiation after appropriate growth factor supplementation, not all of them inherently express osteo- or chondrogenic genes and pose this potency *in vivo* (5, 6). This prompted a further change in terminology from MSCs, now reserved for cells of ubiquitous tissue sources complying with ISCT criteria, to skeletal stem cells (SSCs), cells capable of self-renewal, present within the skeleton, with the ability to differentiate into osteoblasts, chondroblasts, bone marrow stroma, and adipocytes (5, 7). Recently, only chondrogenic and osteogenic differentiation have been proposed as sufficient

(8). The usage of transgenic strains for *in vivo* cell fate mapping is responsible for the extensive characterization of skeletal progenitor populations in mice. SSCs have been identified in bone marrow, periosteum, growth plates, metaphyses and in cranial sutures (9). Murine mesenchymal progenitor populations express various combinations of surface markers. Morikawa et al. characterized SSCs in perivascular regions in bone marrow as $CD45^-TER119^-Sca\text{-}1^+CD140a^+$ cells (10). Ambrosi et al. showed that $CD45^-CD31^-Sca\text{-}1^+CD24^+$ cells from the same compartment are able to differentiate into more committed adipogenic and osteochondrogenic progenitors ($CD45^-CD31^-Sca\text{-}1^-CD140a^+$) (11). Recent work by Chan et al. identified murine SSCs (mSSCs), contained within the $CD45^-Ter119^-Tie2^-CD51^+Thy^-6C3^-CD105^-CD200^+$ subset of whole neonatal bone samples (12). The authors proposed a hierarchical model of differentiation into bone, cartilage and bone marrow stromal cells, where further stages of more committed progenitors are defined based on the presence or absence of CD90, 6C3, CD105 and CD200 surface markers (12, 13) (Fig 1).

At the gene expression level, bone marrow-residing SSCs were highlighted by the expression of *Nes* (neuroectodermal stem cell marker, nestin) (14), *Acta2* (alpha smooth muscle actin, αSma) (15), *Mx1* (myxoma resistance 1) (16), *Hox11* (homeobox gene 11) (17) and *Prrx1* (paired related homeobox 1) (18). The majority of αSMA-labelled cells (15) exhibit the surface markers SCA-1, CD51 and CD90, $Mx1^+$ cells abundantly possess CD140a, CD105, CD29, CD44 and CD133 (16), $Hox11^+$ cells bear CD140a and CD51, whereas $Prrx1^+$ cells express *Pdgfra*, *Grem1* (gremlin 1) and *Nes* (18). Simultaneous expression of *Mx1* and *Acta2* selectively labels a subset of periosteal SSC, most of which are $CD105^+CD140a^+$, and express *LepR* and *Grem1* (19). Substantial subset of growth plate SSCs are $PTHrP^+$ (20). In adult tissues, *LepR* (leptin receptor) expression was linked to a postnatal progenitor subset residing in the bone marrow and on endosteal surfaces (21-23).

Human SSCs (hSSC) have been characterized by multi-color panels, not overlapping with murine markers (Fig 1). Sacchetti et al. described a population capable of self-renewal, osteogenic differentiation and hematopoietic stem cell (HSC) niche support as $CD146^+$ perivascular cells residing in bone marrow (24). Tormin et al. described perivascular $CD146^+CD271^+$ progenitors and a distinct $CD146^{-/low}CD271^+$ subset located in endosteal regions (25). Li et al. identified a $CD140a^{-/low}CD271^+$ population residing in the perisinusoidal space in bone marrow, which highly expresses CD90, CD105, CD140b and STRO-1 (26). Although they proposed CD140a as a marker of exclusion, a $CD140a^+CD51^+$ perivascular population was described as a pool of multipotent progenitors with trilineage

potential by Pinho et al. (27). Chan et al. identified SSCs residing at different locations in the human fetal and adult skeleton, especially enriched in the growth plate. Similar to the murine model, they suggested a hierarchy of human skeletal progenitors, where hSSCs are $PDPN^{+}CD73^{+}CD164^{+}CD146^{-}$, capable of osteochondrogenic differentiation, but like its murine counterpart lack adipogenic potential (28). The multipotent hSSCs are $CD146^{-}$, but osteo/chondro-directed progenitors gain CD146 expression.

While there is a great overlap of currently described populations, some progenitor subsets, especially those at distinct skeletal locations, also differ by their surface marker or gene expression, the ability to support the HSC niche, their osteochondroadipogenic or osteochondrogenic potency, and the ability to form bone through endochondral or intramembranous ossification, suggesting there is more than one type of SSC.

1.2. Signaling pathways driving differentiation of bone and cartilage progenitors

Proliferation, differentiation, and apoptosis of both chondrocytes and osteoblasts is regulated by many genes, signaling pathways, as well as local and systemic factors. The major pathways/molecules governing the fate of these cells are typically recognized as the Indian hedgehog (Ihh), homologous wingless (wg)/integrated-1 (Wnt), BMPs, parathyroid hormone-related protein (PTHrP), fibroblast-growth factors (FGFs), and Notch.

Ihh is a protein from the evolutionary highly conserved Hedgehog family (Indian, Desert and Sonic Hedgehog), produced by pre-hypertrophic and early hypertrophic chondrocytes. It binds and thus, inactivates its cell-surface receptor Patched-1 (PTC-1), leading to the activation (or rather, un-inhibition) of Smoothened (SMO, a seven-transmembrane protein) which then transmits a signal to the cytoplasmic transcription factors Gli to regulate chondrocyte proliferation and maturation during endochondral ossification (29).

The Wnts are a large highly conserved family (of 19 members in both human and murine genomes) (30). There are three pathways that are typically recognized for Wnt signaling (31): 1) the canonical Wnt/β-catenin pathway (dominant in bone biology); 2) the non-canonical Wnt-planar cell polarity pathway (Wnt/PCP); and 3) the Wnt-calcium pathway ($Wnt/Ca2^{+}$). Various agonistic (e.g. Norrin, RSPO) and antagonistic ligands (e.g. dickkopf1 – DKK1, sclerostin, and Wise), as well as molecules that act as Wnt sequesters (secreted frizzled-related proteins, SFRPs and Wnt inhibitory factor 1, WIF1) influence both the canonical and non-canonical signaling pathways.

BMPs, another highly conserved group of proteins, have fundamental roles in embryonic development, postnatal bone homeostasis and fracture healing. BMPs bind as dimers to type I and type II serine-threonine kinase receptors. These tetrameric (oligomeric) receptor complexes can transduce signals to the canonical, Smad-dependent, signaling pathway and the non-canonical, Smad-independent pathway (p38-MAPK) (32). Of the 14 BMPs, only BMP-2, -4, -5, -6, -7, and -9 have a high osteogenic activity (33, 34). BMPs that exert osteogenic signals bind to three type I receptors (BMPR1A/ALK3, BMPR1B/ALK6, and type I activin receptor – AcvR1/ALK2). BMP-3 opposes osteogenic activities of other BMPs by transducing type IIB activin receptor (AcvRIIB)-Smad2/3 signaling (35). The canonical (Smad-dependent) signaling involves phosphorylation of R-Smads (Smad 1, 5 or 8) to create complexes with their partner Smad 4 and then they co-translocate to the nucleus to recruit RUNX2, which regulates gene expression of, e.g. Osterix (OSX) (36). Smads 6 and 7 (I-Smad) negatively regulate the canonical pathway by preventing R-Smad phosphorylation. The non-canonical (Smad-independent) signaling involves MKK-p38 or MKK-ERK1/2 signaling cascades (32). There are several subfamilies of BMP antagonists (DAN family; Twisted gastrulation; Chordin and Noggin), which directly associate with BMPs to block their receptor binding (37).

PTHrP, secreted by proliferating chondrocytes (38), acts in bone by binding to the PTH receptor 1 (PTHR1) which also functions as a PTH receptor. PTHR1 is expressed at high levels in pre-hypertrophic and early hypertrophic chondrocytes (39), as well as in most tissues that express PTHrP (40). There, it interferes with locally produced Ihh keeping the chondrocytes of the growth plate in the proliferative pool (41, 42).

FGFs are a large family of evolutionary highly conserved proteins (43) that regulate both the endochondral as well as the intramembranous bone formation, acting through four receptors (FGFRs) (44). Their actions involve regulation of proliferation, migration, differentiation, as well as angiogenesis. FGF signaling starts with a dimerization (including two heparin sulfate chains) of two FGFs that bind to two FGFRs (45) leading to activation of the RAS/MAP kinase pathway that regulates cell proliferation and differentiation. In addition, FGFs utilize the PI3 kinase/AKT pathway (regulating cell survival) and the PLCγ pathway (regulating cell morphology and migration) (46-48).

The Notch signaling pathway regulates bone development and regeneration (49-52). It is activated by binding of Notch ligands (Jagged 1 and 2, and Delta-like ligands 1, 3, and 4) to Notch receptors (1-4) on the neighboring cells. Ligand binding triggers a conformational change of the receptor, which leads to proteolytic cleavage of the Notch intracellular domain

(NICD) by the γ-secretase complex (53). Cleaved NICD enters the nucleus where it binds to the recombination signal-binding protein for the Ig-jκ region (RBPjκ). NICD in complex with RBPjκ removes co-repressors, binds mastermind-like (Maml), and activates transcription of downstream target genes including the *Hes* and *Hey* family of transcriptional repressors (53). In addition to the described RBPjκ-dependant canonical pathway, Notch also signals through a RBPjκ-independent non-canonical pathway, which involves interactions with IKKα/NF-κB and Wnt/β-catenin pathways (54). Notch actions in bone affect both osteoblasts and osteoclasts, and are highly context-dependent. Activation of Notch signaling in immature osteoblast lineage cells inhibits cell differentiation and causes cancellous bone osteopenia due to impaired bone formation (55). However, stimulation of Notch pathway is osteocytes leads to secretion of osteoprotegerin and thus, inhibits bone resorption (56).

In addition to major signaling pathways, several transcription factors are indispensable for bone homeostasis. SOX9 (SRY-Box Transcription Factor 9) is a chondrocyte lineage-specific transcription factor indispensable for early chondrocyte development (57), regulated by FGFs (58). It is expressed until the pre-hypertrophic stage of chondrocyte maturation and blocks osteoblastic differentiation of growth plate chondrocytes (59). It is regulated by several osteogenic pathways (BMPs, PTH/PTHrP, insulin-like growth factor (IGF), and Notch) and interacts with a multitude of transcription factors and regulatory proteins, influencing many osteoblast-specific genes, such as bone sialoprotein (BSP) and osteocalcin (OC). OSX is a downstream target of RUNX2 needed for early osteoblast differentiation (60), but also at later stages of the osteoblast lineage (61). Besides RUNX2, OSX may be regulated by other transcription factors, e.g. p53 (suppression of osteoblast differentiation) and NFATC1 (stimulation of osteoblast differentiation). Ihh pathways may also be involved in regulating RUNX2 and OSX transcription and action. Furthermore, all of the described pathways – Ihh, FGF, Wnt, PTH/PTHrP, Notch, and BMP are involved in active cross-talks between skeletal cells – chondrocytes, osteoblasts, osteocytes and osteoclasts (31).

2. Effects of inflammation on osteochondroprogenitor populations during fracture repair

Fracture healing is a sequential process that requires a complex interaction between different cell lineages, including immune cells, resident tissue cells, and osteochondroprogenitor cells. Early after fracture, a hematoma forms and inflammatory cells infiltrate the fracture site, producing cytokines/chemokines and modulating the microenvironment to promote healing.

MSCs residing within the periosteal layer rapidly expand after the fracture, and differentiate to chondrocytes and osteoblasts to form a fracture callus (62). As healing progresses, the soft callus mineralizes and remodels until the original bone structure is restored (63).

During a stabilized bone fracture without injury to the periosteum, bones heal by intramembranous ossification. Instability and subsequent movements of the fragments at the fracture site induce the activation of periosteal progenitors and formation of a fibrocartilaginous callus, which is similar to endochondral ossification. The position of osteochondroprogenitors alters their contribution to the fracture callus. In stabilized fractures, periosteal progenitors are the greatest contributors to the outer callus, while bone marrow-derived progenitors mostly contribute to callus formation at the proximal fracture area, as well as on the endosteal surface and in the bone marrow. In open fractures, muscle-derived progenitors (muscle satellite cells) also contribute to the regeneration process (64). Further characterization of these progenitor populations with regard to their regulation of the BMP pathways is required for better understanding their role in the fracture healing sequence.

2.1. Osteochondroprogenitor populations contributing to bone repair

The key population responsible for fracture healing is a subset of non-hematopoietic mesenchymal progenitors with tri-lineage potential (65). These cells reside in the perivascular niche within the bone marrow (66, 67) and periosteum (62). Surface-marker profiling identified an injury-induced $CD45^{-}Ter119^{-}Tie2^{-}CD51^{+}CD90^{-}6C3^{-}CD105^{-}$ population, capable of self-renewal as well as *in vitro* and *in vivo* bone/cartilage/stromal differentiation (68, 69). Compared to its uninjured counterpart, this callus-expanded population specifically expresses the integrin subunit α6 (CD49f) and exhibits enhanced bone formation, reduced apoptotic activity and increased Ihh signaling. In a tibial-fracture mouse model, substantial proportion of non-hematopoietic callus progenitors expressed CD51 and CD140b (70).

In recent years, a variety of markers have been proposed to identify MSCs in their native environment, utilizing visual transgenes and lineage tracing models, including *Prrx1* (71-73), *Acta2/αSMA* (15, 50, 74, 75), *Gli1* (76, 77), *Grem1* (78), *Osx*/*Sp7* (79, 80), and *Cxcl12* (79). Contributions of Nes^{+} (81), $Sox9^{+}$ (82), $Col2.3^{+}$ (73), and transcortical progenitor channel cells (83) to fracture regeneration were also suggested. Cells expressing the mesenchymal marker *Prrx1* have been detected in the periosteum, perichondrium and, to a lesser extent, endosteum and bone marrow (72). Sorted $Prxx1^{+}$ cells differentiate into chondrocytes and

osteoblasts, and their differentiation, induced by BMP-2, is superior to the *Prrx1*$^{-}$ subset (72). Wang et al. used the fracture model in *Prrx1*CreERT2-GFP;Rosa-tdTomato mice to confirm numerous GFP^{+}tdTomato^{+} cells (indicating active *Prrx1* expression) and GFP^{-}tdTomato^{+} cells (indicating cell maturation) in the periosteum, cartilaginous and hard callus, contributing to fracture healing (73). Our group developed αSMACreERT2/Ai9-tdTomato mice to identify a rare population of perivascular αSMA-labeled cells in the periosteum and bone marrow, which expresses mesenchymal markers (Sca-1, CD140a, CD140b and LepR) and rapidly expands early after fracture (day 2), in parallel to periosteum thickening (15, 74). Six days after the fracture, αSMACre-labeled cells extensively contributed to the callus tissue (distal and proximal to the fracture site), and expressed chondrogenic (*Col2a1*, *Sox9*, *Aggrecan*) and osteogenic (*Bsp*, *Osx*) markers (50). Recently, *Gli1* was suggested as a postnatal osteochondroprogenitor marker (77), with tri-lineage potential *in vitro* (76). Lineage-traced *Gli1*$^{+}$ cells from *Gli1*CreERT2/Ai9-tdTomato mice, expressing mesenchymal markers CD44, CD106, CD146, CD140a, CD140b, and αSMA, were present in the articular cartilage, metaphysis, and periosteum but not in bone marrow or endosteum (77). Upon fracture, around half of them gained the expression of Aggrecan and OC, and significantly contributed to the fracture callus. Cells expressing the BMP antagonist *Grem1* are considered osteochondroreticular (OCR) progenitors. Lineage-traced *Grem1*$^{+}$ cells from *Grem1*CreERT;R26-LSL-tdTomato mice were detected adjacent to the growth plate, trabecular bone, and bone marrow, and possessed self-renewing and tri-lineage potential (78). They contributed to callus formation, with around 28% of them becoming osteoblasts (Col2.3GFP^{+}) and 14% chondrocytes (Sox9^{+}) by day 7 after fracture. *Grem1*$^{+}$ OCR cells are distinct from *Nes*$^{+}$ cells, but some of them co-express αSMA. In comparison to *Grem1*$^{-}$ cells, sorted *Grem1*$^{+}$ cells overexpressed *BMP-2/5/6*, and BMP receptor *Acvr1*. Lineage traced *Osx*-expressing cells exhibited *LepR*, CD140a and CD140b, with a substantial fibroblast colony-forming activity and tri-lineage potential. In a semi-stabilized tibial fracture model, *Osx*$^{+}$ cells were present within the cartilage tissue and around the pin insertion, whereas in a model of cortical bone regeneration, *Osx*$^{+}$ osteocytes were detected in the healing bone 8 weeks after injury (79). CXCL12 (stromal cell-derived factor 1, SDF-1) is a chemokine expressed by a subset of quiescent osteoprogenitors (79). Using *Cxcl12*CreER/Ai9-tdTomato mice, Matsushita et al. observed a subset of CXCL12^{+} resting cells within the diaphyseal marrow space, which are readily induced by different injury stimuli (drill hole and bone

marrow ablation) to replenish the chondrocyte, osteoblast, and osteocyte pool within the injured site.

Normal BMP expression is important for fracture healing. Global or conditional inactivation of BMPs presents a range of phenotypes, from embryonically lethal, through phenotypes characterized by various skeletal defects, to non-detectable phenotypes. Conditional deletion of *Bmp-7* in Prrx1-expressing mesenchymal lineage cells did not exert noticeable effects on the healing process (84), which was characterized by unaltered periosteal activation, bridging of callus by day 10, and callus mineralization by day 20, comparable to the control littermates. Conditional inactivation of *Bmp-2* in the limb mesenchyme (using *Prrx1*CreBMP-2 mice) results in spontaneous fractures with inability to heal (85). Inactivation of *Bmp-2* in chondrocytes (using *Col2Cre*) increases the proportion of cartilage within the callus, but it's inefficient mineralization eventually results in weaker bones (86). In contrast, inactivation of *Bmp-2* in osteoblasts (using *Col1Cre* mice), does not affect the fracture healing, suggesting that *Bmp-2* expression in osteoblasts is not crucial for callus formation. Global *Gdf5* and *Bmp-14* (87) knockout mice have similar fracture phenotypes, characterized by decreased chondrogenesis, delayed fracture healing, and more prominent callus infiltration with inflammatory cells.

2.2 Effects of fracture-associated inflammation on BMP signaling

BMPs are involved in cell proliferation, differentiation, and apoptosis (88), so their effects on osteochondroprogenitor cells during regeneration are crucial. The expression of BMP-2, BMP-4, Noggin, and Chordin is evident in healing tissues, confirming the central role of BMP signaling in progenitor recruitment and endochondral ossification during bone repair. Therefore, BMPs have therapeutic potential to enhance the process of fracture healing (89). Although treatments with BMP-2 and -7 have been FDA-approved, treatments with BMPs are accompanied with undesirable side effects due to the supraphysiological dosages used. Understanding the interactions between different signaling pathways during fracture healing is important to optimize the treatment dosage of BMPs (90).

Fracture injury affects the bone as well as the periosteum layer, blood vessels, and the surrounding soft tissue. The inflammatory phase is initiated when disruption of the vasculature leads to a hematoma formation around the fracture site, which becomes infiltrated by inflammatory cells (91). Within 24 hours, neutrophils predominate within the fracture hematoma, providing chemotactic signals for subsequent macrophage homing. In the next

several days, the fracture hematoma becomes intrinsically osteogenic and angiogenic, due to recruitment of MSCs and accumulation of osteogenic/angiogenic factors. Finally, T lymphocytes are recruited into the fracture hematoma and subsequent granulation tissue. Highly regulated inflammatory responses, as well as the release of cytokines and growth factors, are crucial for fracture healing. Infiltrating macrophages and neutrophils secrete cytokines (IL-1, IL-6, TNF-α), chemokines (CCL2, CCL4, CCL5, CXCL12), and several growth factors (BMP-2, BMP-7, TGF-β, PDGF etc.). In the absence of inflammatory cytokines, such as IL-6 and TNF-α (using IL-6 or TNF-α knockout mice), fracture healing is delayed (92, 93). Administration of an anti-IL-6 antibody in the early phase after fracture reduces systemic inflammation, recruitment of immune cells, and bone regeneration, thus impairing fracture healing (94). It was further observed that classic signaling (membrane IL-6R/membrane gp130) and not trans-signaling (soluble IL-6R/membrane gp130) is essential for bone repair. Mice that lack the proinflammatory molecule PTX3 showed a delayed sequence of callus mineralization (70). NOD/scid-IL2Rγ_c^{null} mice with impaired innate and adaptive immune responses have an increased cartilaginous area suggesting defective endochondral ossification (95). Nevertheless, RAG1$^{-/-}$ mice deficient for T and B lymphocytes showed improved callus mineralization (96). Although this study implies a negative effect of the adaptive immune system on fracture healing, it seems that activated T lymphocytes support osteogenic differentiation (97). Despite the importance of initial acute inflammatory response for fracture healing, prolonged and uncontrolled inflammation leads to poor fracture regeneration, mainly due to overproduction of inflammatory and apoptotic factors as well as tissue destruction and insufficient vascularization (98, 99).

The inflammatory phase of fracture healing is necessary to recruit mesenchymal progenitors by chemotactic signals from the fracture site as well as to activate osteoprogenitor subsets within the periosteal layer (Fig1). Both major subtypes of macrophages – inflammatory M1 and alternative M2 are able to produce BMP-2 (100, 101). In addition, cytokines (TNF-α, IFN-γ, IL-17, TGF-β) produced by T lymphocytes enhance BMP-2 production in MSCs (97). BMPs released by immune and osteoprogenitor cells induce osteogenic differentiation in an autocrine and paracrine manner (91, 102). In the mouse fracture model, BMPs are expressed during the initial phase of callus formation and increase up to 3 weeks post fracture (103). The highest expression of BMP-2 and GDF8 is observed right after injury, during the inflammatory phase, in parallel to the high concentration of proinflammatory cytokines. GDF5 and TGF-β are expressed during chondrocyte expansion and maturation, which is

followed by increased expression of BMP-3a, -4, -7, and -8 along the reparatory phase of fracture healing (104). Secreted BMPs contribute to bone regeneration by promoting angiogenesis (105), progenitor cell recruitment (106) and osteoblast differentiation (98). In human callus tissue, active osteoblasts abundantly express BMP-7 and BMP-3 as well as BMPR-IA, -IB, and -II. Expression of phosphorylated R-Smads confirmed active BMP signal in both osteoblast and cartilage callus cells (107). Noggin is released by immune cells (macrophages and neutrophils) and, along with other BMP antagonists in the callus (Gremlin, Chordin, Smad-6/7, and BAMBI), may contribute to impaired fracture repair (108, 109). PDGF signaling is active in periosteal and callus cells and has the ability to modulate the BMP-2 response during periosteal cell differentiation (110). BMP-induced VEGF release by osteoblasts stimulates vascularization and further delivery of progenitor cells (91). Clearly, complex signaling cascades of many cytokines and growth factors within the callus tissue determine the net shape of the healing process, whose precise interactive network is yet to be fully determined.

3. Effects of inflammation on osteochondroprogenitor populations in arthritis

The inflammatory milieu is often associated with alterations of skeletal remodeling, affecting both osteoclasts and osteoblasts. Among the most common diseases accompanied by inflammation-induced bone disorders are inflammatory rheumatic diseases. They are generally divided into: 1) a seropositive RA, characterized by production of autoantibodies such as rheumatoid factor and/or anti-citrullinated protein antibodies; and 2) seronegative spondyloarthritis (SpA), including ankylosing spondylitis (AS), psoriatic arthritis (PsA), reactive arthritis, arthritis associated with inflammatory bowel disease, and juvenile SpA. In RA, bone damage typically occurs at joint margins, where the inflamed synovium produces focal erosions of cortical bone. Progression of bone erosions causes a loss of the subchondral bone and contributes to destruction of the articular cartilage. Finally, patients exhibit systemic bone loss in the form of osteopenia or osteoporosis involving the axial and appendicular skeleton remote from the synovial inflammation. Patients with SpA may develop erosions of articular bone as well as erosions of sacroiliac joints. In addition, inflammation affects the spine at enthesial sites in the form of concurrent bone erosion and induced bone formation. Disease progression may eventually lead to bony fusion (ankylosis) of sacroiliac joints and syndesmophyte formation between vertebral bodies (111-118). In this

review, we specifically discuss the effects of rheumatic diseases on osteoblast lineage cells, while the osteoclastogenic effects are extensively reviewed elsewhere (111, 112, 119, 120).

3.1. Osteochondroprogenitor populations within the synovial compartment

Progenitor populations from periarticular tissues such as synovia, cartilage or subchondral bone have been suggested to participate in bone regeneration during arthritis. However, apart from murine and human bone-residing progenitors described before, most of the cells from other locations have not been stringently assessed for their self-renewing capacity and *in vivo* trilineage potential (8, 121). De Bari et al. first isolated potential progenitors from the human synovial membrane termed "SM-derived MSCs" and confirmed their ability to proliferate and maintain multilineage potential *in vitro* (122). These cells were also identified in the synovial fluid from arthritic patients (123). According to Sakaguchi et al., synovial MSCs had superior chondrogenic and osteogenic potential in comparison to other sources (124). Futami et al. isolated and characterized a similar population from the mouse infrapatellar fat pad synovia (125). These cells express several markers defined by ISCT criteria (122, 124, 125). Developmentally, synovial cells originate from the embryonic mesenchymal joint interzone (JI) cells, which contribute to joint structural elements including the articular cartilage, synovial lining and intra-articular ligaments (126). Roelofs et al. have recently shown that *Gdf5*-expressing JI cells persist in the adult murine synovium, subchondral bone, bone marrow and articular cartilage, and proliferate upon cartilage injury (127). These cells have the ability to form synovial lining cells, chondrocytes and adipocytes, but were not osteogenic, supporting the existence of a distinct osteochondroprogenitor subset within the synovial tissue. However, the developmental origin of such synovia-derived osteochondroprogenitors with *in vitro* multilineage potential has not been precisely determined. The relationship of described MSC populations to fibroblast-like synoviocytes (FLSs), which rapidly proliferate in RA, is also not clear, as their phenotypes largely overlap (128). Similarly as in the synovia, progenitor cells have been reported in normal and osteoarthritic (OA) human articular cartilage (129), but their progenitor properties have not been completely proven.

During joint inflammation, we and other groups have observed increased cellularity and proliferation of stromal cells derived from affected joints, with the ability to differentiate towards osteogenic or chondrogenic lineages (130, 131). This population is a heterogeneous mixture and includes highly proliferative hypertrophic FLSs, requiring further

characterization by surface marker profiling (CD29, CD140b, Sca-1, CD44, CD90.1, CD105, CD51, and CD200). We observed that cells released from collagenase-treated synovial joints showed apparent heterogeneity in the expression of mesenchymal markers amongst control and arthritic mice, reflecting their distinct differentiation and activation status (131). Using a modified panel proposed by Chan et al. (12), we identified a small subset of $CD51^{+}CD200^{+}$ cells amongst non-hematopoietic cells within the synovial compartment and postulated that this population might share progenitor properties of mSSC. This subset was decreased in antigen-induced arthritis (AIA), reflecting an impaired osteogenic and chondrogenic regeneration in arthritis (131). At the single cell level, Chan et al. determined the stage-specific expression of BMP, Wnt, and TGF-β pathways, revealing their differential responses to growth and differentiation stimuli. Co-expression of BMP-2 and its receptor (BMPR1a) in 28% of mSSCs pointed to their autocrine or paracrine signaling in sustaining the progenitor pool or inducing its expansion (12). Committed mSSC progeny expressed antagonists of the BMP2 pathway, such as Gremlin 2 and Noggin, suggesting their ability to control the expansion of mSSCs via a negative feedback loop (12).

We aimed to further characterize the $CD51^{+}CD200^{+}$ population, reduced in arthritis, by next-generation sequencing of the transcriptome of the $CD45^{-}CD31^{-}TER119^{-}CD51^{+}CD200^{+}CD105^{-}$ cell subset from AIA and non-immunized (NI) mice. In addition, we sequenced $CD45^{-}CD31^{-}TER119^{-}CD51^{+}CD200^{-}CD105^{+}$ cells from AIA mice to determine whether they were an expanded committed progeny of $CD51^{+}CD200^{+}$ cells or represented a population of $CD105^{+}$ FLS enriched in arthritis. The comparison of gene expression profiles of $CD200^{+}CD105^{-}$ cells between AIA and NI mice revealed no significant differences, pointing to their preserved functional features in arthritis. However, in AIA, the $CD200^{+}CD105^{-}$ population differed significantly from the $CD200^{-}CD105^{+}$ population, by overexpression of BMP, Wnt, and TGF-β pathways (Table S1). In particular, $CD200^{+}CD105^{-}$ cells exhibit a significantly enhanced expression of *BMP-2*, *-4* and *-6*, *Gdf5,* as well as receptors *BMPR1*, *BMPRII,* and *AcvR2b*. Several components of Wnt and TGF-β pathways were also overexpressed in this population, as well as *Osx* and *Runx2* (Table S1). In addition to *Grem1*, ascribed to SSCs in bone marrow (18), periosteum (19), and long bone metaphyses (78), $CD51^{+}CD200^{+}$ cells also showed an enriched expression of *LepR*, another marker for SSC identification (21), whereas expression of *Nes* was unchanged (Table S1). Zhou et al. also showed that SSCs do not overlap for *LepR* and *Nes* expression. Our results, therefore, suggest that the arthritis-reduced $CD200^{+}CD105^{+}$ population has osteochondroprogenitor properties, whereas the $CD200^{-}CD105^{+}$ population might represent proliferating FLSs. The

transcriptome analysis also revealed a higher expression of Fas in $CD200^{+}CD105^{-}$ cells compared to $CD200^{-}CD105^{+}$ cells, indicating that osteochondroprogenitors are effectively removed by apoptosis under inflammatory conditions, while hypertrophic FLS survive and proliferate, inducing damage to surrounding tissue. Interestingly, we previously observed a preservation of the $CD51^{+}CD200^{+}$ population in AIA mice deficient for a functional Fas receptor, which were protected from arthritis-induced subchondral bone loss (131).

3.2. Effects of joint inflammation on BMP signaling

The inflammatory environment within the synovial compartment, accompanied by hypoxia and reduced pH, is associated with insufficient osteoblast function, often attributed to direct effects of proinflammatory cytokines, such as TNF-α, IL-1 and IL-6, which inhibit their differentiation and maturation (132-135) (Fig1). Clinical studies applying TNF-α or IL-6 receptor blockers documented the persistence of bone erosions despite reduced inflammatory activity in treated patients (136-138). Histological sections of arthritic samples, either from humans or from murine models, have shown the presence of osteoblast lineage cells close to the eroded bone once inflammation resolves (111, 114, 139). However, their regenerative capacity to repair bone erosions is often defective (113, 140). Cartilage's capacity to heal is intrinsically limited (141), and imposes a therapeutic challenge not only in classic inflammatory arthritis but also in other pathological conditions involving cartilage damage. Many studies have demonstrated a close relationship between the synovial inflammation and pathways regulating bone formation, including BMP and Wnt. Increased production of Wnt antagonists such as DKK-1 and SFRP1/2 by inflamed synovial tissue in arthritis impairs osteoblast differentiation and bone formation (139, 142, 143). In contrast to RA, the synovial fluid in SpA contains increased levels of the inflammatory cytokine IL-32g, which enhances osteoblast differentiation via DKK-1 suppression (144). Indeed, lower levels of DKK-1 are found in AS and PsA, promoting abnormal enthesial bone formation (142, 145, 146).

Impairment in BMP signaling has been described at multiple levels in both experimental arthritis and arthritic patients (147-155), but with contrasting results. By analyzing the global gene expression profile in the tarsal joints harvested from mice with collagen-induced arthritis (CIA), Denninger et al. reported complex time-dependent changes in BMP and Wnt pathways, with an early stimulation of genes associated with bone induction (149). Daans et al. also reported increasing activation of the BMP pathway during CIA progression (148), detected immunohistochemically as phosphorylated Smad1/5. The expression pattern

translated from the synovial lining in the early phase, towards deeper layers of invading pannus in the destructive phase. Mild inflammation was associated with appearance of BMP-7 in the synovial lining and superficial articular chondrocytes, whereas arthritis progression was marked by expression in the subintimal layer (148). Furthermore, arthritis induced opposing changes in the expression of *Gdf5*, with an early decrease in the arthritic synovium and a later increase in the articular cartilage (148). Since arthritis destroys the cortical bone barrier and exposes BM to synovial tissue, resulting changes in the bone marrow compartment affect cortical bone remodeling. Gortz et al. found an increased osteoblast number at the endosteal surface, close to cortical lesions, in the human TNF transgenic (hTNFtg) mice. Indeed, accumulated B lymphocytes expressed BMP-6 and -7, contributing to bone induction (150). Amongst negative regulators of the BMP pathway, upregulation of BMP-3 was detected in osteoblasts, in the vicinity of bone erosions of developed AIA, indicating that maturing osteoblasts may suppress differentiation of newly committed progenitors (153). Maeda et al. determined the synovial expression of miRNAs at erosion sites using the K/BxN serum transfer model (152). They identified several downregulated miRNAs targeting inhibitors of Wnt and BMP pathways, including DKK3, GSK3b, sFRP-1/2, Smad7, and Tob1/2, suggesting that decreased miRNAs in the inflamed synovium may contribute to induction of BMP and Wnt antagonists, and limit bone formation at erosion sites.

Alterations in BMP expression have been reported in different tissues from arthritic patients. Our group observed that peripheral blood cells had a reduced expression of *BMP-4*, *BMP-6*, and *RUNX2* in patients with RA. A negative correlation with disease activity was found for *BMP-4* in RA, while a positive correlation was found for *BMP-4* in PsA (151). Within the affected joints, BMP-4 and -5 were reduced in the rheumatoid synovium, and, similar to experimental studies, localized in deeper synovial layers in contrast to their superficial expression in normal synovial lining (147). However, Verschueren et al. reported increased activation of BMP signaling, detected by phosphorylated Smad1/5, in αSMA^+ perivascular cells, $CD90^+$ synovial fibroblasts, and $CD68^+$ synovial macrophages of RA synovium (154).

Proinflammatory cytokines produced by the inflamed synovium and subchondral bone marrow contribute to the modulation of BMP signals. The addition of TNF-α and IL-1β arrested osteoblast differentiation and maturation *in vitro* (132, 156-165). Yamazaki et al. used the MC3T3-E1 cell line to show that the NF-κB subunit p65 is able to associate with the Smad1/4 complex and suggested that TNF-α-induced NF-κB-activation inhibits BMP

signaling by interfering with the DNA binding of Smads (165). However, Sullivan et al. could not prove the involvement of NF-κB in TNF-α- and IL-1β-mediated suppression of bone formation from primary bone marrow MSCs (164). Huang et al. suggested that TNF-α/IL-1β and BMP-2 have opposing roles that converge on Runx2 to regulate osteoblastic differentiation through p38 and ERK1/2 signaling (160). Similarly, IL-6 trans-signaling inhibited differentiation of MC3T3-E1 cells and primary murine calvarial osteoblasts through MEK/Erk and PI3K/Akt2 pathways. An anti-osteoblastogenic effect was detected as reduced alkaline phosphatase (ALP) activity and mineralization as well as down-regulation of osteoblast-specific genes (*Runx2*, *Osx* and *Bglap*) (135). TNF-α stimulates expression of Smurf1 (Smad ubiquitin regulatory factor 1) in the C2C12 myoblast cell line and primary cultured mouse calvarial cells, which can bind to BMP type I receptors and Smad1/5 complex via I-Smads to induce their ubiquitination and degradation (133). Therefore, *in vivo* blocking of TNF-α may be a promising approach for compromised bone healing (166, 167).

However, several studies indicated that cytokines released at localized sites of inflammation, including TNF-α, TGF-β, IFN-γ, and IL-17, may be the driving force for differentiation of mesenchymal progenitors into the osteoblast phenotype (97). Osta et al. investigated the effects of TNF-α and IL-17 on the osteogenic differentiation of isolated human bone marrow-derived mesenchymal cells (168). They revealed that TNF-α and IL-17 increased ALP activity in differentiating osteoblasts, but only TNF-α stimulated BMP-2 expression at early time-points (6 and 12 h). In osteogenic cultures of adipose-derived mesenchymal cells from RA and OA patients, TNF-α potentiated calcium deposition, up-regulated *RUNX2* and *BMP-2*, but down-regulated *COL1a1* and *OPN* expression (169). In cultures of the MC3T3-E1 murine cell line and primary murine calvarial osteoblasts, IL-6 stimulated osteoblast differentiation through the interplay between STAT3 and the Smad/BMP pathway (135). Similar effects were observed in the human osteoblast MG-63 cell line, where IL-6 trans-signaling caused a marked elevation of ALP activity and a decrease in cell proliferation through the JAK/STAT pathway (170). Although it seems that inflammation may have a positive effect on the BMP pathway, the rate of bone formation in RA is insufficient to counteract the greatly induced rate of bone resorption. However, in AS, where chronic inflammation is accompanied with pathologic bone formation, TNF-α induced BMP-2 production in human chondrogenic cells (171, 172). Elevated IL-17 was associated with an increased BMP-2 expression and heterotopic cartilage/bone formation in hyperplastic entheseal tissues of mouse AS (173). Murine and human spinal ligament cells undergo

osteogenic differentiation under BMP-2 treatment (174-176). Therefore, it seems that the control of inflammation may reduce BMP pathway activation and abnormal osteogenic differentiation within entheses.

During inflammation, BMPs do not participate only in altered homeostasis of the skeletal cells, but affect other cells, such as FLSs and macrophages (177). Lories et al. reported increased BMP-2 and -6 expression in different cell types of RA hyperplastic synovia including $CD68^+$ macrophages and $CD90^+$ FLS (178). TNF-α and IL-1β upregulated both BMPs in FLSs, which, in turn, had opposite autocrine effects, with BMP-2 acting as a proapototic and BMP-6 as a protective factor (178). Varas et al. confirmed the expression of BMP ligands (BMP-2, BMP-6, and BMP-7), receptors (mainly BMPRIA, ACTRIA and BMPRII), and signal transducers (Smad1/5 and co-Smad4) in FLSs (179). These cells also expressed BMP antagonists, which were highly upregulated after activation with TNF-α and IL-17, suggesting that inhibition of the autocrine BMP pathway exacerbates the FLS pro-inflammatory phenotype in RA. Wei et al. showed that BMP-2 down-regulated the expression of inflammatory (M1) mediators, including IL-1β, IL-6 and iNOS, and induced the expression of reparative (M2) factors in macrophages, indicating a positive immunoregulatory role of BMP-2 (180). Thus, it may be possible to utilize BMP-2 immunomodulatory properties to manipulate the osteoimmune environment for favorable bone regeneration.

4. Conclusions

The role of inflammation is strongly context dependent, regarding local microenvironment, cell types and mechanical forces, resulting in opposite net effects on bone tissue in different diseases. In fracture healing, the inflammatory response induced immediately after injury is crucial for recruitment of osteochondroprogenitor cells and creation of a microenvironment that favors their expansion and differentiation into mature chondrocytes or osteoblasts. At the site of injury, inflammation induces BMP signaling, and BMPs originate from various cell types, ranging from osteochondroprogenitors themselves to inflammatory cells. BMP signaling is beneficial in proper regeneration after fracture injury and has even been proposed in therapeutical contexts. In inflammatory arthritides, inflammation promotes bone loss in the joints, in periarticular areas, and systemically in RA, but drives bone formation at enthesial and periosteal sites in diseases such as AS. Although most studies focus on the destructive component of rheumatic diseases through the induction of osteoclasts, it has become

increasingly evident that osteoblasts are also targeted by inflammation. We show that murine synovial $CD51^{+}CD200^{+}CD105^{-}$ cells most likely represent osteochondroprogenitors, which are decreased in number in murine arthritis, suggesting that the progenitor pool is impaired. Wnt and BMP signaling pathways have emerged as critical in the regulation of osteoblast function and the net outcome for bone in rheumatic diseases, and these pathways have been implicated in both impaired repair of articular erosions in RA, and in the pathological bone formation in axial SpA. Proinflammatory molecules in the inflamed joint are known to modulate BMP signaling, and altered BMP signaling is not only crucial for osteoblasts and their progenitors, but also affects other cells, such as the FLS and macrophages. Although it seems that inflammation may have a net stimulatory effect on BMP signaling pathway, the rate of bone formation in arthritis is insufficient to counteract the greatly induced rate of bone resorption. Therefore, more specific approaches to assess activation of the BMP pathway in regard to the skeletal system at their various developmental stages might be useful in addressing the question of impaired bone and cartilage regeneration during inflammatory arthritides.

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Figure legends

Figure 1. Murine/human osteochondroprogenitor phenotype and major mechanisms of the inflammation-induced BMP pathway modulation. Murine mesenchymal osteochondoprogenitor populations express various combinations of surface markers such as stem cell antigen 1 (Sca1), endoglin (CD105), differentiation antigen 1 (BP1, 6C3), membrane glycoprotein Thy-1 (CD90), membrane glycoprotein OX-2 (CD200), vascular cell adhesion protein 1 (VCAM-1, CD106), platelet-derived growth factor receptor alpha (PDGFRα, CD140a), integrin alpha-V (CD51, vitronectin receptor), and membrane glycoprotein MUC18 (MCAM, CD146). Human mesenchymal progenitors have been identified by a number of surface markers including transferrin receptor (CD73), CD105, podoplanin (PDPN), CD90, CD51, CD146, endolyn (CD164), low-affinity nerve growth factor receptor (CD271), CD140a, PDGFRβ (CD140b), and stromal cell surface marker-1 (STRO-1). In addition, a variety of progenitor markers have been applied in murine lineage tracing models, including *Acta2*, *Hox11*, *Mx1*, *Nes*, *Gli1*, and *Prrx1*. Osteogenic pathways in progenitor cells are influenced by a variety of modulators produced by surrounding cells, which determine the net effect on their proliferation, differentiation, and lifespan. Under inflammatory conditions, the balance between anabolic factors, cytokines and osteogenic pathway agonists/antagonists may be affected in opposite ways – either enhancing bone formation or suppressing bone repair. The final outcome mostly relies on the fine interplay between major intracellular signaling pathways. Under the anabolic arm, bone morphogenetic proteins (BMPs) act in a paracrine and autocrine manner and together with growth factors (PDGF, VEGF), inflammatory cytokines (IL-6, IL-17, TNF-α), and Wnt-agonists induce transcription of RUNX2 and OSX, further production of BMPs, osteoblast differentiation, alkaline phosphatase activity and matrix mineralization, at the same time suppressing progenitor proliferation and apoptosis (left). Catabolic pathways include production of BMP- and Wnt-antagonists, apoptotic factors (CD95L), and an uncontrolled release of inflammatory cytokines (TNF-α, IL-1β, IL-6) that suppress differentiation, transcription of RUNX2 and OSX, inhibit collagen synthesis and alkaline phosphatase activity, often leading to cell apoptosis or deregulated proliferation (right). For clarity, only the most commonly described pathways that are influenced by inflammatory signals are schematically presented. More data are required to further confirm their *in vivo* importance and possible use as therapeutic targets.

Table S1. **Differences in gene expression of members of BMP family, Wnt and TGF-β pathway and other selected genes between $CD51^+CD200^-CD105^+$ and $CD51^+CD200^-CD105^+$ populations in murine antigen-induced arthritis (AIA).** Arthritis was induced in 12 week old female C57BL6 mice by intra-articular injection of methylated bovine serum albumin (mBSA) in mice previously immunized with mBSA in complete Freund's adjuvant. On day 10 of arthritis, cells were released by collagenase digestion, and 200-500 live $CD45^-CD31^-TER119^-CD51^+CD200^-CD105^+$ and $CD45^-CD31^-TER119^-CD51^+CD200^+CD105^-$ cells were sorted by FACS Aria IIu, pre-amplified and converted to cDNA by Smartseq v4 Ultra® Low Input RNA Kit for Sequencing (TakaRa). Libraries were prepared using Nextera XT DNA Library Preparation Kit (Illumina). Total of five (n=5) libraries for each population separated from individual mouse, with satisfactory concentration and quality were sequenced, with 50 million 2x75bp reads using NextSeq 500 (Illumina) and High Output Kit v2.5 (150 Cycles) (Illumina). Read quality was assessed by FASTQC, reads were trimmed with cutadapt (1), and sequences aligned by HISAT2 (2). Transcripts assembly and quantification was performed by Stringtie (3) and count matrices were normalized with trimmed mean of M values normalization (TMM) in *egdeR* (4). *limma voom* was used to assess the differential gene expression (5). Genes with absolute $\log_2$ of fold change (FC) higher than 1.5 and adjusted p value (BH correction) lower than 0.05 were considered significantly changed. Log_2FC, adjusted p value and average expression $\log_2$ counts per million mapped reads (CPM) of members of BMP family, Wnt and TGF-β pathway and changes in expression of other selected genes encoding for CD200, CD105, CD90, 6C3, Osterix, Runx2, RANKL, Nestin and Fas are shown in the table. All animal protocols were approved by the Ethics Committee of the University of Zagreb, School of Medicine (380-59-10106-15-168/235) and the National ethics committee (EP 07-2/2015), and conducted in accordance with accepted standards of ethical care and use of laboratory animals.

Members of the BMP family					
Gene symbol	**Gene description**	**$\log_2$FC**	**adjusted p value**	**$\log_2$CPM in $CD51^+CD200^+CD105^-$**	**$\log_2$CPM in $CD51^+CD200^-CD105^+$**
Bmp2	bone morphogenetic protein 2	**8.27**	**1.92×10^{-5}**	**4.69**	**-3.52**
Bmp3	bone morphogenetic protein 3	**8.25**	**0.00056**	**4.20**	**-4.30**
Bmp4	bone morphogenetic protein 4	**9.59**	**1.87×10^{-6}**	**7.06**	**-2.96**
Bmp6	bone morphogenetic protein 6	**8.84**	**3.52×10^{-6}**	**5.81**	**-3.12**
Gdf3	growth differentiation factor 3	**-9.37**	**1.72×10^{-7}**	**-4.06**	**5.45**

Gdf6	growth differentiation factor 6	**6.76**	**0.003886**	**2.31**	**-4.30**
Acvr2b	activin receptor IIB	**5.52**	**0.028873**	**2.57**	**-2.93**
Bmpr1a	bone morphogenetic protein receptor, type 1A	**4.72**	**0.006831**	**6.44**	**1.77**
Bmpr2	bone morphogenetic protein receptor, type II	**3.55**	**0.015029**	**6.82**	**3.17**
Smad1	SMAD family member 1	**4.59**	**0.008171**	**5.59**	**1.09**
Smad3	SMAD family member 3	**4.31**	**0.037767**	**4.57**	**0.72**
Grem1	gremlin 1, DAN family BMP antagonist	**6.53**	**0.007645**	**2.98**	**-3.22**
Members of the WNT pathway					
Fzd4	frizzled class receptor 4	**4.69**	**0.00563**	**5.64**	**0.70**
Fzd7	frizzled class receptor 7	**5.43**	**0.018986**	**2.66**	**-2.50**
Fzd8	frizzled class receptor 8	**5.79**	**0.020296**	**2.08**	**-3.45**
Wnt4	wingless-type MMTV integration site family, member 4	**5.75**	**0.024414**	**2.82**	**-2.77**
Members of the TGF-β pathway					
Tgfb2	transforming growth factor, beta 2	**7.10**	**0.000358**	**6.19**	**-0.71**
Tgfbr1	transforming growth factor, beta receptor I	**2.73**	**0.024076**	**6.37**	**3.79**
Tgfbr3	transforming growth factor, beta receptor III	**4.47**	**0.000777**	**7.98**	**3.37**
Other					
CD200	CD200 antigen	**11.29**	**2.66×10^{-6}**	**8.74**	**-2.72**
Eng	CD105, endoglin	**-3.55**	**0.103711**	**-1.38**	**1.88**
Thy1 (CD90.1)	thymus cell antigen 1, theta	**-1.03**	**0.753003**	**0.17**	**1.08**
Enpep (6C3)	glutamyl aminopeptidase	**6.10**	**0.022652**	**2.67**	**-3.10**
Sp7 (Osterix)	Sp7 transcription factor 7	**7.41**	**0.001808**	**3.59**	**-3.49**
Runx2	runt related transcription factor 2	**7.68**	**0.005623**	**2.66**	**-4.30**
Tnfsf11 (RANKL)	tumor necrosis factor (ligand) superfamily, member 11 (RANKL)	**6.03**	**0.024547**	**1.51**	**-4.30**
Nes	Nestin	**1.49**	**0.636425**	**1.18**	**-0.27**
Fas	Fas, CD95	**6.41**	**0.002375**	**4.93**	**-1.68**

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Figure(s)

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