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Bone Physiology as Inspiration for Tissue Regenerative Therapies

Diana Lopes[#1], **Cláudia Martins-Cruz**[#1], **Mariana B. Oliveira**[1,*], and **João F. Mano**[1,*]

[1]Department of Chemistry, CICECO – Aveiro Institute of Materials, University of Aveiro, 3810 193 Aveiro, Portugal

[#] These authors contributed equally to this work.

Abstract

The development, maintenance of healthy bone and regeneration of injured tissue in the human body comprise a set of intricate and finely coordinated processes. However, an analysis of current bone regeneration strategies shows that only a small fraction of well-reported bone biology aspects has been used as inspiration and transposed into the development of therapeutic products. Specific topics that include inter-scale bone structural organization, developmental aspects of bone morphogenesis, bone repair mechanisms, role of specific cells and heterotypic cell contact in the bone niche (including vascularization networks and immune system cells), cell-cell direct and soluble-mediated contact, extracellular matrix composition (with particular focus on the non-soluble fraction of proteins), as well as mechanical aspects of native bone will be the main reviewed topics. In this Review we suggest a systematic parallelization of (i) fundamental well-established biology of bone, (ii) updated and recent advances on the understanding of biological phenomena occurring in native and injured tissue, and (iii) critical discussion of how those individual aspects have been translated into tissue regeneration strategies using biomaterials and other tissue engineering approaches. We aim at presenting a perspective on unexplored aspects of bone physiology and how they could be translated into innovative regeneration-driven concepts.

Keywords

bone physiology; bone microenvironment; biomaterials; biomimetics

1 Introduction

Bone physiology involves the coordinated regulation of a myriad of biological processes that lead tissue development, homeostasis and repair upon trauma [1]. Bone regenerative processes can thus be highly complicated to emulate, partially due to the numerous and multifactorial cues contributing for the regulation of its niche. Those include the tissue's complex composition and intricate associated pathways involving aspects like bone's soluble microenvironment, extracellular matrix (ECM) insoluble proteins and glycoprotein composition and renewal, cell-cell and cell-ECM interactions mechanical stimulation, or the

[*]Corresponding author. M.B. Oliveira: mboliveira@ua.pt ; J.F. Mano: jmano@ua.pt.

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role of microRNAs. The understanding of the individual and coordinated role of each intervenient on bone tissue physiology, as well as their interconnected actions, has inspired the design of a plethora of biomimetic and/or bioinspired bone regeneration approaches. In fact, there is an urgent need for the development of new, effective and compatible treatments for bone-related injuries. Age-related fractures are expected to increase in the United States from 2.1 million in 2005 to over 3 million fractures in 2025, solely considering the elderly/aging population at risk [2]. In Europe, the annual number of fractures is estimated to rise 28% from 2010 to 2025, with an absolute increase from 3.5 million to 4.5 million injuries, respectively [3]. The application of concepts learnt from nature for the emulation of the structure and physiology of healthy bone, however, requires insightful and careful approaches. Although mimicking bone's structure and function based on its physiology is an attractive idea, the implementation of effective reproducible therapies is most often dependent on achieving a balance between sufficient complexity to warrant function, along with ease/speed of manufacturing and regulatory compliance.

The study of individual factors in biologically non-representative environments in most available literature concerning bone regeneration may be hindering the disclosure of unprecedented valuable results. The effects of (bio)chemical (e.g. presence of soluble cytokines, as bone morphogenic proteins – BMPs), structural and chemical properties (e.g. different biomaterials chemistries and architectures), or externally applied mechanical stimuli (e.g. compressive stress or flow perfusion) on bone regeneration strategies have been explored in a unifactorial fashion, focusing on single stimuli for the design of biomaterials and other regenerative approaches. Interestingly, though, a growing body of knowledge comprising both fundamental and applied studies focusing on bone's response to engineered cues have undoubtedly proven that bone is a complex and dynamic system, in which different biological processes and structural characteristics play complementary roles towards the successful regeneration and maintenance of bone's healthy behavior [1]. The advent of stem cells biology, as well as the progressive know-how on the structural, biophysical and biochemical role of the ECM components and the scrutiny of immune cells crosstalk supported recent advances in the design of biomaterials targeting bone regeneration [4]. The knowledge of such complexity may be an effective manner to pave the way for the design of multifactorial strategies targeting bone regeneration and disease treatment [5]. Some high-throughput studies have approached multifactorial aspects of bone regeneration as crosstalk between different cell types (including mineral-forming cells, reabsorption cells, immune cells and vascular cells) [6, 7], the combinatorial role of ECM proteins [8, 9], the effect of physical factors as biomaterials bioactivity, stiffness and viscoelasticity [10, 11], as well as the role of extrinsic mechanical forces actuating in the native bone (e.g., compression and shear stress) [12]. Although some key aspects of bone physiology are well studied and have led the development of therapies, several aspects still remain poorly understood and explored as potential added-value assets to enhance regeneration. Some examples include the crosstalk of bone resident cells with immune cells, the role of hematopoietic stem cells (HSC) on regenerative phenomena, or the tight control of time- and space-coordinated biochemical and biophysical signaling [13].

This Review sets the objective of establishing an unprecedented correlation between (i) well-known and recently reported bone physiology phenomena and (ii) the use of this know-how

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for the development of well-established or proof-of-concept bone-healing therapeutic approaches. A thorough analysis is performed to identify aspects of bone biology lacking exploitation for regenerative therapies, which may represent a source of innovative ideas for novel and impactful future developments on the field.

1.1 The need for regenerative medicine therapies – analysis of critical aspects to achieve bone regeneration

While bone tissue trauma normally heals by itself, the so-called “critical” defects - with average diameters of 2 cm or higher in humans - do not show this spontaneous ability [14]. Such defects with poor healing ability often derive from tumor ablation, serious injury and orthopedic diseases [15]. Specifically considering physically-caused injuries, including a range of fractures from standard injury to open tibial defects, bone healing repair failure percentage can go from 10 to 50% [16]. Failure on bone healing will ultimately culminate in the suppression of blood supply to the tissue, which will result in the non-union of the bone due to ischemia, osteonecrosis and bone loss [17].

Efforts to repair bone defects excluding the ones that specifically target bone regeneration can be divided in two main segments: (i) implantation of bone grafts (of autologous or allogenic origin) or (ii) development of synthetic permanent bone substitution grafts [18]. Limitations have been reported for both therapeutic approaches. Although commonly applied in clinics and known to foster bone repair, autologous bone grafts, inflict morbidity of the donor’s extraction site [18, 19]. Allogeneic bone grafts may potentially be rejected due to host-to-graft immune response. Moreover, the implantation of allografts requires a complex implantation technique that involves the achievement of constant vascular supply to the site, as well as a maintenance of an adequate mechanical environment to promote vessels formation [20]. Permanent substitution grafts, manufactured from non-degradable materials, have been associated with unwanted side effects including bone resorption, poor osseointegration and triggering of adverse (e.g. allergenic) reactions on the host [18]. Current strategies based on synthetic grafts for bone healing are out of the scope of this Review. Thorough reviews of this important topic related to bone repair therapies can be found in References [21, 22].

Well-reported problems associated with the current bone repair approaches show that there is a dire need for the development and marketplace implementation of new and more efficient strategies. In opposition to previously mentioned techniques focused on tissue repair, tissue engineering seeks the complete regeneration of damaged tissues through the *in vitro* and/or *in vivo*-synthesis of novel biological matrices with equivalent properties to those of the original healthy tissue. Four main pillars may be used separately or in combined design strategies to promote bone regeneration: (i) biomaterials, (ii) biomolecules, (iii) cells and (iv) externally-applied stimuli. The following sections of this Review will exploit bioinspired rationales behind the use of different tissue engineering players through a parallelization strategy with native bone’s physiological phenomena.

2 An Inter-Scale View of Bone Structure

Bone is the anatomic structure responsible for the movement, protection, maintenance of mineral homeostasis and structural support of the human body. A fully-grown adult's skeleton is composed of 206 individual bones. Human bones are divided in five major categories, which include long (aimed at supporting body weight; e.g. clavicles, raddi, metacarpals, tibiae, phalanges, femurs, humeri, metatarsals, fibulae and ulnae), short (providing movement and stability; e.g. tarsal and carpal), flat (targeted at internal organs protection; e.g. skull, sternum, mandible, ribs and scapulae), irregular (e.g. vertebrae, coccyx, sacrum and hyoid) and sesamoid bones (embedded at tendons; e.g. patella) [23].

During tissue formation, two bone types can be identified: (i) the woven/primary bone, which appears during the embryonic development and fracture repair, and consists of osteoid (unmineralized ECM) with collagen fibers that show little or no determined orientation in the three-dimensional (3D) space, along with a random distribution of cells [24, 25]. This is a transient structure, which is later replaced by mature lamellar bone; and (ii) the lamellar/secondary bone, which constitutes the adult skeleton and consists of highly organized sheets of mineralized osteon [26]. This structure is stronger and more rigid, and less elastic than the woven bone [24].

Lamellar bone is constituted by both cortical/compact and trabecular/cancellous bone. The first one is dense, solid and located in the most outer part of the tissue; on its turn, cancellous bone contains a sponge-like structure with interconnecting cavities and is located at the internal section of bone. Both cortical and cancellous bones are composed of osteons. The ratio of both bone types varies according to the anatomical site (e.g. femoral head with a 50:50 cortical to trabecular ratio; vertebra with 25:75; radial diaphysis with 95:5; and overall, the human skeleton with a 80:20 ratio) [1]. On a structural perspective, in cortical bone the lamellae with about 3 μm thickness, is organized into concentric circles, surrounding a vertical Haversian canal containing blood vessels and nerves. This entire structure is designated the osteon or Haversian system, and is the functional unit of bone. The system is formed when osteoclasts remodel existing bone, leaving cylindrical cavities that are subsequently filled with osteoblasts that produce lamellae around the Haversian canal [24, 27]. Osteocytes are located between lamellae, within small cavities known as lacuna, interconnected by a series of tunnels called canaliculi [1, 24]. The trabecular bone is composed of large spaces, with a honeycomb-like network of trabecular plates. The matrix consists of a 3D network of fine columns that crosslink to form the trabeculae [1, 24]. This results in a light and porous bone that is strong against multidirectional forces, and crucial to enable body movement. The spaces between trabeculae are filled with bone marrow. A fibrous connective tissue layer, called periosteum, surrounds the external surface of cortical bone, while in the inner section, a membranous structure - the endosteum - covers the surface of cortical and trabecular bone. The latter is also in contact with the bone marrow space, blood vessels canals and is composed by blood vessels, osteoblasts and osteoclasts [1]. Both periosteum and endosteum have inspired the development of two-dimensional (2D) membranes to mimic these anatomical structures and assist tissue regeneration processes [28].

On a nanoscale perspective, bone is constituted by a plethora of structural proteins and polysaccharides, which main composition is collagen fibrils with diameters between 35 and 60 nm, and up to 1 μm length, organized with a 67 nm periodicity and 40 nm gaps [29–31]. These fibrils are mineralized by the anisotropic and extremely stiff inorganic component - hydroxyapatite crystals [32] - that lay in the collagen gaps [31]. Interestingly, despite the variations on bone structural shapes depending on bone types and throughout different species, the mineralized collagen fibrils observed in humans are highly conserved across species and bone types [33]. Bone organic and inorganic phases are thought to have an interplay that allows load transfer. Gupta *et al.* [34] showed that both components deform elastically during initial loading, although to different degrees. This specific deformation pattern is hypothesized to culminate in fibril-matrix decoupling targeting the protection of the brittle mineral phase, improving the effective redistribution of strain energy in the tissue. Nanostructured scaffolds based on nanomaterials capable of closely resembling the native ECM structure have been designed. According with some authors, such constructs demonstrate a beneficial effect concerning the formation of functional tissue due to an improvement of the interactions at the cellular and protein level [29, 30]. Although the macro- and microstructure of bone closely replicated using porous scaffolds, the emulation of the osteon organization is dependent on a nanometric control of materials distribution. In fact, a precise combination of micro- and nanometric aspects of the bone structure is fundamental for the development of truly mimetic structured biomaterials. Nonetheless, the effective need for highly organized osteon/adult bone interscale mimetic biomaterials is not unanimous. Effective biomaterials capable of triggering osteogenic differentiation and bone repair have been often based on biophysical and biochemical cues inspired on bone developmental processes, instead of adult bone structural features. These strategies are composed of much simpler units than the adult completely formed bone itself. The role of nanostructured biomaterials as cue providers targeting bone regeneration must not be overlooked. Platforms exhibiting, for example, topographic, pro-differentiation and pro-mineralization cues through techniques as nanopatterning, electrospinning, and development of nanocomposites [30] has led to some of the most interesting outputs in the field. Complete reviews correlating (i) nanomaterials and their similarity with the native bone niche and (ii) their bone regeneration outputs can be found in References [30, 35–38].

3 Bone Development Mechanisms

During mammals' fetal development and natural bone repair upon injury, bone formation is achieved through two processes: intramembranous and endochondral ossification [1]. The primary structure for these two mechanisms is the woven (or immature hollow) bone, that is readily replaced by the lamellar/secondary bone (parallel fibrils deposited in opposite directions) [25]. The formation of lamellar bone occurs at a much slower rate than that of woven bone [39]. This structure does not appear only in the fetal life, but every time a bone suffers a non-critical injury [25].

3.1 Intramembranous bone formation

Intramembranous ossification is the most primitive form of ossification, with the first proof of its existence dating to 500 million years BP, in opposition to endochondral ossification,

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with first reported case with 100 million years BP [40]. In intramembranous ossification, mesenchymal stem cells (MSCs) present in mesenchyme or in the medullary cavity - caused by a bone injury - differentiate into osteoblasts. In fetal development, this process is mainly responsible for the formation of the flat skull bones and some parts of the clavicles [25, 41]. Unlike in the endochondral ossification process, in intramembranous ossification the bone is formed without a cartilaginous intermediate. The formation of a nidus – a cluster of undifferentiated MSCs – is the starting point for the intramembranous ossification process. Cells in these clusters stop their proliferation and develop into the osteoprogenitor phenotype, and then eventually differentiate into osteoblasts, through an intermediate pre-osteoblast lineage [41, 42]. *Runx2* is one of the most important early transcription factors responsible for osteoblastic differentiation [43, 44]. The expression of *Runx2* is dependent on the Wnt signaling, which leads to high levels of β-catenin in MSCs. In turn, *Runx2* induces the later expression of the transcription factor gene *Osterix*, also involved in the differentiation of MSCs to osteoblasts [45]. After full differentiation, osteoblasts produce a non-mineralized type I collagen-rich fibrillar ECM: the osteoid. While entrapped in this matrix, osteoblasts differentiate into mature osteocytes, and the matrix is further mineralized. The described mechanism is also considered by most authors as the backbone for the formation of subperiosteal bone and, thus, the process behind the woven and lamellar bone type formation in this region [46]. However, this concept has been challenged, and the mechanism behind periosteum surface ossification was suggested as developmentally distinct [45]. A schematic representation of the intramembranous ossification process can be found in Figure 1a.

3.2 Endochondral ossification

During endochondral ossification chondrocytes from surrounding cartilage tissues initially form a matrix template, the growth plate, and then differentiate into bone structures [43]. This ossification process drives the embryonic formation of long bones. When chondrocytes' morphology is round, these cells synthesize type II collagen [47] and subsequently form a columnar layer, becoming pre-hypertrophic. They eventually differentiate into post-mitotic hypertrophic cells, which release type X collagen, mineralizing the surrounding matrix, leading the formation of the bone structure [47]. During bone formation various cycles of death of hypertrophic chondrocytes occur, which is accompanied by the invasion of blood vessels, leading to the replacement of the initially collagenous matrix by trabecular bone, also known as primary spongiosa [48]. As the process continues, trabecular bone is resorbed, and its center is split into different plates. While chondrocytes are present in the plates the previous process continues [48]. The adequate differentiation of chondrocytes into the hypertrophic phenotype is of extreme importance for the genesis and proliferation of bone tissue [48]. A schematic representation of the endochondral ossification process can be found in Figure 1b.

For endochondral ossification-driven bone formation, some biochemical factors must be present in specific moments of chondrocyte-to-osteoblast differentiation. Those include:

- The Sox Trio: Sox9/5/6. These molecules are responsible for the differentiation of MSCs into the chondrogenic phenotype, as well as for the regulation of the expression of critical genes for the formation of cartilaginous matrix [48–50].

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- Expression of the fibroblast growth factor (FGF) receptor 3 and a membrane-spanning tyrosine kinase receptor by chondrocytes. These cells contain a domain that binds to extracellular ligands, including FGFs, initiating the receptor's autophosphorylation, as well as the stimulation of the tyrosine kinase activity, leading to the inhibition of proliferation and growth of chondrocytes [51, 52];
- Presence of BMPs, which are responsible for the formation of mesenchymal condensations and of joints in initial stages of endochondral ossification. After condensation, when long bones are already formed, BMP-2, -3, -4, -5 and -7 are released in the perichondrium. BMP-2 and -6 are produced by hypertrophic chondrocytes, and BMP-7 by proliferative chondrocytes. BMPs positively regulate chondrocyte proliferation and negatively modulate chondrocyte terminal differentiation [51, 52];
- High level expression of parathyroid hormone-related peptide (PTHrP) by hypertrophic chondrocytes. This peptide binds and activates the receptor parathyroid hormone (PTH)/PTHrP, also activated by PTH (main regulator of calcium/phosphate metabolism and remodulation of the bone). In fact, the PTH/PTHrP complex is the main regulator of bone development and mineral ion homeostasis. The PTH peptide acts by maturing the immature chondrocytes to a hypertrophic phenotype. When the chondrocytes express PTHrP or an activated form of the receptor, a decrease on the cartilage maturation and increase in bone formation is observed [53, 54]. For a successful bone formation, hypertrophic chondrocytes must express high levels of alkaline phosphatase (ALP), osteonectin, osteopontin, bone sialoprotein and osteocalcin [55–60].
- Indian hedgehog homolog (Ihh). This protein, present in the embryogenic patterning, controls the endochondral bone formation by inhibiting the differentiation of hypertrophic chondrocytes, therefore delaying the mineralization of the matrix. The control of growth plate elongation is not a chondrocyte property, but a property of the growth plate module arising from the interaction with chondrocytes involved in the negative feedback loop of Ihh/PTHrP. Ihh also acts as a chondrocyte proliferation stimulator, through a PTHrP-independent pathway [52, 61].
- Runx2, Runx3 and core-binding factor beta subunit (CBFβ). These three transcription factors have been described in the literature as promoters of chondrocytic hypertrophy, complementing each other in the process [62, 63];
- The proteins hypoxia-inducible factor-1 (HIF-1α) and vascular endothelial growth factor (VEGF). These two factors are essential for bone vascularization, in which HIF-1α acts by mediating hypoxic responses, allowing the survival of chondrocytes, and targets VEGF. VEGF is responsible for the stimulation of angiogenesis and vasculogenesis, as well as restoration of the oxygen supply in hypoxic conditions. It is hypothesized that these two proteins act together in a pathway that regulates chondrocytes survival [64, 65].

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3.3 Development-mimetic strategies to engineer bone tissue

A close look at the embryonic development pathways of bone has served as inspiration for the design of finely tuned regenerative approaches, in strategies addressed as “developmental engineering” [66]. Although endochondral ossification is the pathway that gives rise to most of human bones [24], approaches to differentiate stem cells into functional bone cells (namely, osteoblasts) are mostly based on external stimuli provided to undifferentiated cells that include mineralized/mineralizable platforms, which resembles the intramembranous ossification process [67]. This is a much simpler process when compared to endochondral ossification, thus easier to be carried out *in vitro* and easier to trace overtime; however, it often results in poor vascularization and limited-area bone regeneration. Consequently, endochondral ossification has been hypothesized as advantageous over intramembranous process for tissue engineering due to its inherent ability to form vascularized bone due to the release of VEGF and MMPs by hypertrophic chondrocytes, which allow overcoming associated hypoxia in the tissue [68]. Despite the successful generation of bone tissue reported for endochondral ossification-mimetic strategies, the implantation of tailored mineralized biomaterial matrices has also enabled high quality bone regeneration, in which the final tissue recapitulates key characteristics of the native precursor, including vascular networks. Examples of tissue engineering strategies focused on both intramembranous and endochondral developmental pathways will be reviewed in the following Sections 3.3.1 and 3.3.2.

3.3.1 Regenerative strategies based on intramembranous ossification: the role of mineralized biomaterial matrices—Mineralized biomaterials have been reported as effective promoters of intramembranous ossification-analogous pathways [69–71]. Although in initial approaches their utility was mostly reported exclusively for the treatment of small scale injuries due to their inability to autonomously induce MSCs differentiation, seminal work by Yuan *et al.*,in 2010, introduced physicochemical and structural tailoring of calcium phosphate-based ceramics as a way to achieve osteoinductive biomaterials capable of promoting the full regeneration of large-scale defects in sheep and dog ectopic and orthotopic models [72]. Calcium phosphates with different chemical compositions – hydroxyapatite, tricalcium phosphate (TCP), and mixtures of both (biphasic calcium phosphates - BCP) were exposed to different post-synthesis sintering temperatures, so different microstructural features could be obtained (smaller grains for 1150°C, and larger ones for 1300°C). Those materials were tested for their ability to induce *in vitro* MSCs osteogenic differentiation, as well as *in vivo* bone formation. TCP showed the highest osteoinductive effect on *in vitro* cultured MSCs and the strongest ability to induce bone formation, with outputs similar to the implantation of autografts or treatment with recombinant human BMP-2. Moreover, the implantation of TCP avoided the formation of fibrous tissue when compared to the autograft strategy, and promoted a more defect-localized bone formation when compared to BMP-2 administration. An overall analysis of the study by Yuan *et al.* suggested that elevated specific area achieved through a reduction on grain size accompanied by resorbability features may be the key to process efficient bioceramics targeting bone regeneration [72].

Although bioceramics were proven to promote the regeneration of mineralized tissue on bone defects, the analysis of the *de novo* formed tissue is often restricted to bone-specific genes and proteins. However, the formation of a vascular network in bone is of utmost importance to achieve highly functional regenerated tissues. Recently, Díaz *et al.* (2018) evaluated a series of mineralized biomaterials and their ability to induce bone healing in a major cranial defect in the complete absence of growth factors and endogenous cells [73]. Moreover, the invasion of the implanted biomaterials by endothelial cells and the formation of blood vessels was also assessed. Macroporous poly(ethylene glycol)-diacrylate-co-*N*-acryloyl 6-aminocaproic acid (PEGDA-co-A6ACA), poly(ethylene glycol)-diacrylate (PEGDA) and acryloyl 6 aminocaproic acid (A6ACA) hydrogels were mineralized *in vitro* through immersion in a Ca^{2+}/PO_4^{3-} solution and in simulated body fluid (m-SBF). The *in vivo* performance of the hydrogels was tested before and after the mineralization step. Although endogenous cell proliferation and infiltration and blood vessels formation could be observed in both mineralized and non-mineralized porous biomaterials, the presence of bone forming cells, osteoclast precursors and hard tissue formation was only observed in mineralized biomaterials, suggesting the indispensable role of mineral environments for the promotion of osteogenic differentiation using cell-free and growth factor-free biomaterials [73].

Despite the significant advances concerning the application of calcium phosphates as osteoinducers, their interaction with stem cells and the bone defect moiety is still not completely unravelled [69]. The hypothesis that microarchitectural features act as key drivers for osteogenesis led by calcium phosphates gained momentum during the last decade [74, 75]. Moreover, free ions – specifically calcium - possibly released from these materials to the surrounding environment also showed the ability to induce osteogenesis on MSCs through the stimulation of BMP-2 expression [76]. The full elucidation of the pathways driving bone cells invasion of synthetic mineralized biomaterials, mechanisms leading MSCs osteogenic differentiation and the stimulation of neoangiogenesis in bone defects treated with these materials is in great need to promote the design of rationally tailored mineralized/mineralizable bone regenerative matrices.

3.3.2 Regenerative strategies based on endochondral ossification—In 1998, Bianco *et al.* [77] discussed bone formation through the endochondral ossification pathway, by modulating the terminal differentiation of what the authors called the “borderline chondrocyte”. The authors asked whether there was a future for hypertrophic chondrocytes as primary modules for bone regeneration, as these cells induce the differentiation of neighbor MSCs *in vivo* [77]. It has been later hypothesized that the regeneration of bones natively formed by endochondral ossification would benefit from undergoing the same pathway for their regeneration. With the rise of stem cells as important players on regenerative medicine strategies, the discussion about the selection of the most beneficial way to differentiate cells into functional osteoblasts, and even to fully functional tissues, has gained momentum. Ten years after Bianco and co-workers inquired about the pertinence of using hypertrophic prone-to-mineralization chondrocytes as precursors for bone formation, Jukes *et al.* [78] introduced the endochondral ossification into the stem cells world for bone tissue engineering by inducing the formation of ectopic bone on animal models using murine

embryonic stem cells (ESCs) previously stimulated through the endochondral pathway *in vitro*. Despite this breakthrough, the validation of the endochondral route as an effective way to promote bone formation was still restricted to ESCs, which are prone to ethical concerns and considered of poor clinical relevance for that reason [78–80]. Only in 2010, Scotti *et al.* [81] reported the use of clinically relevant and widely available bone marrow-derived MSCs (BMMSCs) to undergo osteogenic differentiation through the remodeling of MSC-originated hypertrophic cartilage templates and generate ectopic bone tissue when implanted in nude mice (Figure 1c). Hypertrophic cartilage in more advanced maturation stages accelerated bone formation, although the implantation of precursor hypertrophic tissues in all stages of maturation rendered bone formation *in vivo*. Interestingly, gene, protein and structural analysis of the developed tissues showed that morphogenesis occurred with a high level of parallelism with the well-known developmental processes observed for endochondral bone formation in embryos, which included the early activation of Ihh signaling and the *in vivo* subsequent development of a bony collar, its vascularization and osteoclastic remodeling of cartilaginous precursors.

The main advantage associated with the recapitulation of endochondral ossification is the possibility of engineering a fully functional bone organ, containing a mature vascularized mineralized matrix, as well as a hematopoietic bone marrow component. Indeed, the implantation of different progenitor and stem cell types has led the *in vivo* recreation of functional hematopoietic niches. Ectopically implanted CD146+ human skeletal progenitor cells were able to induce the formation of a hematopoietic compartment in mice [75], and the formation of a mature HSC niche after embryonic MSCs implantation was reported to be dependent on the endochondral ossification process [83]. The suppression of directly involved factors on the endochondral ossification process, including VEGF and Osterix, inhibited the generation of such hematopoietic niche [83]. The application of tissue engineered constructs as templates for endochondral ossification capable of promoting not only mineralized tissue formation, but also the development of bone-like ossicles featuring vascularization and functional HSCs niche compartments was hypothesized by Scotti *et al.* [84] in 2013. Human BMMSCs seeded onto type I collagen porous scaffolds, cultured *in vitro* for 3 weeks in serum-free chondrogenic medium, and for additional 2 weeks in pro-hypertrophic medium, which contained IL-β1 aimed at the acceleration of cartilage mass remodeling [85]. Indeed, pre-treatment with this cytokine results in higher accumulation of matrix metalloproteinase 13 (MMP-13) and DIPEN (an aggrecan epitope exposed after its degradation) after 5 weeks of implantation. After pre-conditioning, the constructs were implanted into nude mice and, extensive remodeling was indeed observed after 12 weeks. At that stage, the formed tissues were similar to native bone's structure, with an outer layer resembling cortical bone and inner parts with cancellous bone features. Regions identified as hypertrophic cartilage in the first weeks of culture developed into bone marrow and densely mineralized bone tissue. Impressively, mouse sequential bleeding after 1, 2 and 3.5 months after transplantation confirmed the functionality of the ossicle-derived HSCs, capable of multilineage reconstitution.

The achievement of ossicle structures using other sources of adult stem cells, including human adipose-derived stem cells (ASCs), in endochondral ossification-mimetic strategies has been challenging. However, the application of stem cells from abundant and easily

retrievable sources is potentially highly valuable. This hypothesis is reinforced by the failure of primary chondrocyte lineages - including fully mature nasal chondrocytes induced *in vitro* for an hyperthrophic phenotype – on being capable of leading *in vivo* endochondral ossification; in opposition, implanted tissues prepared from hyperthrophic nasal chondrocytes reverted their phenotype into a hyaline status [86]. In 2016, ASCs assembled as 3D cellular micrometric pellets or adhered onto collagen scaffolds were cultured in chondrogenic cell culture media supplemented with early and, optionally, with late hyperthropic supplements administered on later times of *in vitro* culture [87]. Those constructs were implanted in female nude mice, and both early and late endochondral ossification templates underwent cartilaginous remodeling and developed functional bone marrow-specific features incorporated in the newly formed ossicles. Reprogrammed cells may also represent a breakthrough in the future obtaining of scalable cell sources capable of undergoing endochondral ossification. Specifically, dermal fibroblasts directly reprogrammed through into the chondrogenic lineage doxycycline-inducible human Sox9 were capable of promoting endochondral ossification *in vivo* [88, 89].

Despite the clear promise represented by stem or precursor cells as *in vitro*-modulated templates for *in vivo* bone formation mostly in immunologically suppressed animal models, the achievement of effective protocols to directly drive endochondral differentiation in immunocompetent models, avoiding the existing *in vitro* long-term pre-conditioning protocols, would benefit the translational steps needed for the implementation of these techniques into widely available regenerative therapies. Co-culture strategies targeting the understanding of different cell types on hypertrophic cartilage formation and endochondral ossification processes have been a recent matter of interest. Todorov et al. [90] addressed the effect of the presence of monocytes committed to osteoclastogenesis as possible enhancers of tissues' remodeling through chemotaxis of skeletal and vascular cells. However, the presence of such monocytes did not lead to any improvement of cellular chemotaxis *in vivo*. Future studies may elucidate the role of different cells types on the successful induction of endochondral ossification as a bone regeneration targeting system.

4 Adult bone physiology

4.1 General aspects

Bone undergoes longitudinal and radial growth, modeling and remodeling during the whole life of adult (i.e. non-embryonic) individuals [1]. Longitudinal and radial growth occur during childhood and adolescence period. On its turn, bone modeling, an anabolic process involving new bone deposition in response to physiological or mechanical factors is less frequent in adults than remodeling. Contrarily to bone remodeling, where osteoclasts and osteoblasts work sequentially in the same bone remodeling unit, in bone modeling bones are shaped or reshaped by the independent action of osteoblasts and osteoclasts, i. e. the activity of both cells may not be coupled anatomically or temporally [91]. This is achieved by the action of bone osteoprogenitor-derived cells: osteoblasts and osteoclasts [92]. Bone morphogenesis regulated by exposure to mechanical challenges is reviewed in more detail in Section 6. Also, the role of different bone resident or migrating cell types, as well as their

crosstalk during bone healthy state maintenance and during injury/repair processes are reviewed in Section 5 and further ahead on this section.

Remodeling occurs continuously in human adults to form and maintain the complex and functional skeleton structure [1]. This process helps counter the effects of increasing bone fragility throughout life, allowing for the maintenance of the structural stability of the human body [23, 93]. Bone remodeling is increased during adults' middle age and happens in four stages: activation (activation and recruitment of osteoprogenitor cells), resorption (resorption of the osteoprogenitor cells by osteoclasts), reversal (transitional phase from bone resorption to bone formation) and formation (matrix synthesis by osteoblasts) [1, 94]. A detailed explanation of the previously mentioned phases is provided by Clarke [1]. In brief, during bone resorption osteoclasts act by removing the "old bone" packets; afterwards, new synthesized matrix is created, along with mineralized tissue. Bone formation and degradation are tightly kept in equilibrium throughout humans' life by the bone homeostasis and remodeling [1]. Osteoclasts (the promotors of bone resorption) and osteoblasts (involved in bone formation) are the main mediators of this process [1]. The bone unit responsible to maintain this equilibrium is the basic multicellular unit (BMU), composed by osteoclasts, osteoblasts, connective tissue, nerves and blood vessels [6].

4.2 Bone Healing: tissue response upon injury

The maintenance of a fully functional bone system is indispensable for body structural maintenance and organ protection. For that, it is important to have a system that guaranties its integrity [23] through the activation of mechanisms of healing upon fracture avoiding the formation of scar tissues [93]. Fractures are the most common large-organ traumatic injuries in humans. As discussed previously, the repair of bone fractures is a postnatal regenerative process that recapitulates many of the ontological events of embryonic skeletal development [95]. Although fracture repair processes usually restore the damaged skeletal organ to its pre-injury cellular composition, structure and biomechanical function, critical fractures will not heal normally [95]. A complete schematic representation of the process occurring after trauma – bone healing – can be found in Reference [95]. This phenomenon endows several stadia that occur in a sequential manner: (i) inflammation, (ii) soft and hard callus formation and (iii) bone remodeling, which are reviewed in the following topics:

- The (i) **inflammatory phase**, which is characterized by the proliferation and migration of mesenchymal progenitor and blood cells to the healing fracture site [96]. Blood cells that reside in the defect area form a hematoma [97]. Several pro-inflammatory cytokines and growth factors (including tumor necrosis factor-alpha (TNF-α), interleukin-1 (IL-1), IL-6, IL-11 and IL-18) are targeted to the defect site in a temporally and spatially controlled manner [95, 98]. These signals recruit inflammatory cells and promote angiogenesis. At this stage, platelets are recruited and activated in the defect site and produce transforming growth factor-β1 (TGF-β1) and platelet derived growth factor (PDGF) [98]. Simultaneously, recruited osteoprogenitor cells produce BMPs which, in coordination with other factors, promote the local recruitment and osteogenic differentiation of MSCs [96, 98].

- The (ii) **soft callus formation**. After the formation of the blood hematoma, blood cells, fibroblasts and immune cells are recruited to the injury site, forming the granulation tissue [99]. Bone is formed in the peripheric regions of the fractures sites via intramembranous ossification after 7 to 10 days after injury, generating the periosteum [96, 99] The inner parts of the fracture (mechanically less stable [100] and contain the granulation tissue) are subsequently replaced by fibrous tissue (mainly composed of fibroblast cells), fibrocartilage and later cartilage of the soft callus. This structure provides a cartilaginous scaffold within the bone fracture site, that acts as both a fixation and stabilization structure and a template for subsequent mineralization. Within soft callus construct, the differentiation of mesenchymal cells into chondrocytes takes place [99]. These cells then proliferate until complete differentiation into a mature hypertrophic phenotype [99]. At this stage, TGF-β2 and -β3, as well as BMPs, mediate cell differentiation and proliferation at the injury site [99–101]. By the process of endochondral ossification, the soft callus is transformed in the **hard callus**, with a mineralized matrix produced by osteoblasts. At this stage, the formation of the woven bone is initiated [99, 100].
- The formed primary bone is gradually replaced by secondary (lamellar) bone, in the (iii) **bone remodeling process** and osteocytes undergo apoptosis in a reestablishment of the normal bone physiology [99, 100], reaching a physiological status indistinguishable from the pre-fractured condition.

Understanding the fundamental components that make up the ECM and cell components of native bone tissue is vital for the creation of engineered regenerative strategies able to recapitulate the stages of intramembranous and endochondral ossification. Also, unraveling regulatory factors that drive soft callus formation, a key intermediate stage in endochondral ossification, is important when considering strategies to mimic its ECM or in priming its progenitor cells.

A plethora of immune system cells take part of the bone healing process, and their role has been thoroughly reviewed [102, 103]. Macrophages are highly influential of this process, and several studies point to their presence in the healing cascade [98, 99, 104–106]. Their absence in the healing place have been associated to a complete depletion on the regeneration injured tissues [107, 108]. In bone healing, an optimal balance between macrophages with proinflammatory phenotypes - usually addressed as M1 - and pro-healing phenotypes - usually addressed as M2 – is required for an adequate regeneration process. M1 macrophages initiate the inflammatory response [108] and secrete pro-inflammatory cytokines [105], and M2 macrophages are responsible for tissue remodeling, with a phenotype induced by IL-4 and -13, and secreting IL-10 [109]. These two macrophage types work together to start and finish the immune response in an interlocked chain of events. M1 macrophages, not only initiate the inflammatory response but also secrete factors that stimulate the beginning of the angiogenic process [109, 110]. These are gradually substituted by pro-healing M2 macrophages, which promote ECM synthesis, cell proliferation and vessel maturation on the healing site [109, 110]. An unbalance corresponding to a long M1 macrophages permanence at the defect site may lead to an

excessive inflammation, that may compromise fracture healing [101]. Spiller and coworkers developed a bone regeneration system based on the controlled release of interferon-gamma (IFN-γ) and IL-4 through a decellularized bone scaffold to reproduce the *in vivo* transition of M1 to M2 macrophages that ultimately could improve the vascularization of the construct [112]. The rapid release of IFN-γ caused early M1 polarization of macrophages, while the sustained release of IL-4 caused M2 polarization, *in vitro* [112]. This temporal modulation of macrophage phenotype could be advantageous to improve the vascularization of the scaffolds *in vivo*. In fact, 3D printed silicate-β-tricalcium phosphate scaffolds loaded with IFN-γ were able to drive the sequential activation of M1 and M2 macrophage polarization states in a temporally-controlled manner [113]. Through the combined action of released silicate and IFN-γ, timely induction of M1 phenotypes in early time points (one day after implantation) and pro-healing polarization (seven days after implantation) triggered enhanced vascularization of the implanted scaffolds [113].

Other cell types are also present in the fracture location, which include monocytes, neutrophils and natural killer (NK) cells [114]. These cells produce cytokines that are responsible for the recruitment and activation of other cells with differentiation and proliferation potential to regenerate the tissue (e.g. osteoprogenitor MSCs) [114]. When osteoprogenitor cells are recruited to the fracture place, their osteogenic differentiation is partially induced by immune cells present at the injury site [111]. T-lymphocytes are also part of the regenerative process: they act by inhibiting the healing process through the action of cytokines IFN-γ and TNF-α. [115–118]. Conversely, MSCs have been reported to affect the immune response in a plethora of ways, through suppression or inhibition mechanisms. This response is coordinated by the cellular microenvironment and the MSCs-to-T-lymphocytes ratio, with a high ratio inhibiting the immune response, and a low ratio inducing it [114, 119, 120]. The full elucidation of MSCs/T-cells crosstalk is still dependent on further studies. Interactions between immune cells and bone cells are reviewed in Section 5.3.5, and their applications in biomaterial-based tissue regeneration strategies is summarized in Section 5.4.1 and in Table 1.

5 The adult bone niche

5.1 Bone primary stem cell niche

The bone tissue comprises two primary niches: the osteoblastic and the vascular niche [13]. Two stem cells types - HSCs and MSCs – reside in the bone cavity, which is filled with bone marrow and blood vessels [13]. HSCs, which are surrounded by stromal cells in the bone marrow, are responsible for the formation of the immune and blood system, as well as osteoclasts [13, 121]. MSCs also reside in the bone marrow and intervene in the formation of the mesenchymal lineage cells, which include osteoblasts, adipocytes, chondrocytes, fibroblast and other stromal cells. Together, both stem cell types maintain the normal bone homeostasis and cellular generation [13]. Unlike what was thought until recent years, HSCs are not located on the inner surface of the bone. Instead, HSCs were recently described to be on the perivascular niche where they are regulated by growth factors, chemokines and cytokines (e.g.: stem cell factor, chemokine stromal cell-derived factor 1 (CXCL-12) and

angiopoientin-I), secreted through CXCL-12-abundant reticular cells, endothelial cells and MSCs [122].

MSCs, characterized by the expression of PDGFRα, CD51, nestin, CD139, interferon-induced GTP-binding protein MX1 (Mx1), leptin receptor (Lepr) and periaxin (Prx), give rise to osteoprogenitor cells that form the osteoblastic niche. Thereafter, the factors referred previously are released to promote a correct HSC maintenance (reviewed by Yin and Li [13]). The maintenance of a functional microenvironment in the bone niche is dependent on the precise level of the hierarchical lineages of the HSCs and MSCs, so osteoblastogenesis and hematopoiesis can maintain a correct balance of osteoblast and osteoclast production. Importantly, N-cadherin positive osteoblasts interact with HSCs and help the anchoring of these cells to the osteoblastic niche [123].

Cell signaling inside the bone niche, for instance between osteoblasts and B lymphocyte precursors, is well known to determine features of the immune system [114]. Immunogenesis will not be discussed in this Review, and bone niche interactions will be described in the scope of the correct function of bone tissue itself. Nonetheless, the importance of a proper function of bone niches for successful bone development must be emphasized due to its crucial contribution for the continuous exportation of immune cells and tissue progenitor cells to the peripheral immune system, thus sustaining tissue repair and regeneration (Figure 2) [124].

5.2 Bone resident cells

5.2.1 Osteoblasts—The main function of osteoblasts is to synthetize new bone matrix [1]. Different sub-populations of osteoblasts have shown to respond differently to several signals (mechanical, hormonal and from cytokines) [1]. As discussed on Section 3.1, under physiological conditions, MSCs undergo the Wnt/β-catenin pathway to differentiate into the osteoblastic phenotype. Osteoblasts have a cuboidal morphology while proliferating on the bone matrix surfaces, unlike their precursors cells (preosteoblasts), which show a spindle shape [1]. Mature osteoblasts secrete bone ECM proteins, such as type I collagen. Typical gene indicators of osteoblast differentiation are *Runx2*, distal-less homeobox 5 (*Dlx5*), *Osterix*, alias core-binding factor alpha1 (*Cbfa1*), osteoblast specific factor 2 (*Osf2*) and *ColIA1* [125, 126].

Osteoblasts residing in bone tissue can be divided in two types: mesenchymal (MOBL) or surface osteoblasts (SOBL) [25]. In the bone matrix, the undifferentiated MSCs start to differentiate into MOBL, which secrete collagen throughout the matrix, forming a woven structure. After the creation of sufficient woven bone to form a platform-like structure, SOBL secrete collagen fibrils in a parallel way onto the previously made bone structure, creating the highly oriented lamellar bone. Once this process finishes, osteoblasts are matured into osteocytes surrounded by collagen matrix [25] (Figure 2a).

5.2.1.1 Stem cells differentiation into osteoblasts: exploiting physiological sources: Stem cells osteogenic differentiation, which is commonly targeted in tissue engineering strategies, is divided in three main stages: (i) peak in cell number; (ii) cellular differentiation, initiated with the expression and transcription of ALP; (iii) and a terminal

step: production of osteocalcin and osteopontin [92]. In the human body, BMMSCs reside in a specific niche composed of a large variety of support cells that include hematopoietic progenitors; osteoclasts, immune cells and blood cells. The osteogenic differentiation of MSCs is known to be influenced by factors secreted by osteoblasts and osteocytes [6]. This phenomenon occurs through a communication network amongst osteoblasts and osteocytes that enhance a response in the MSCs, when these two bone cell types are in contact [6]. *In vitro*, the co-culture of MSCs with osteocytes showed greater osteogenic differentiation than the ones with osteoblasts, indicating that osteocytes induce MSCs' osteogenesis more effectively than osteoblasts. On the other hand, osteoblasts helped the proliferation of the MSCs [6]. The differentiation of MSCs recruited to injured sites is not solely driven by contact with residing cells. Aspects of the fracture environment known to regulate MSCs fate include the control over the mineralized environment, respective release of ionic cues and the fine temporal variation of mechanical properties of the generated ECM during regeneration. Examples of tissue engineered approaches based on the use of *in vivo*-like mineralized synthetic biomaterials are described in Section 3.3.1. Other aspects such as control over biophysical cues generated throughout the bone repair process, also mimicked through the application of biomaterials with adequate and dynamic mechanical properties, are reviewed in Section 6.2.

Induced osteogenesis of MSCs may provide an important tool for the development of tissue engineering strategies focusing the treatment of large bone defects, which is currently challenging. Stem cells can derive from adult or embryonic sources, or from reprogramed adult cells (human induced pluripotent stem cells; hiPSCs) [127, 128]. Adult human sources of MSCs proven to enable osteogenic differentiation include bone marrow and adipose tissue [129, 130]. Stem cells from postnatal sources can be obtained from placenta, umbilical cord, cord blood and amnion [127, 128]. BMMSCs have been the most commonly used cell type for bone engineering. However, due to the complex and invasive isolation process, the limited cell number and the reduction of differentiation potential with donors age, researchers have been trying to surpass these disadvantages by using other cell sources [131]. Nonetheless, efforts to promote their efficient expansion using non-conventional 3D microcarriers under bioreactor configurations must be addressed. The *in vitro* expansion of MSCs and their use in combined strategies using microcarriers as cell growth supports and implantable scaffolding materials was recently reviewed in detail by Bunpetch *et al*. [132]. Human umbilical cord and adipose tissue are routinely discarded as clinical waste and, in the case of adipose tissue, may be used as noncontroversial MSCs sources [133].

hiPSCs represent a promising tool for bone regeneration [134]. These pluripotent cells closely resemble human embryonic stem cells; however, they are obtained through the reprograming of human somatic cells [135]. Using hiPSCs instead of stem cells derived from other sources, can be advantageous since they can be obtained directly from the patient (patient-specific) and overcome any ethical and immunological issues [134]. Their ability to differentiate into the three germ layers enable them to be reprogrammed into different bone cells, namely osteoblasts and osteoclasts, which highlights their potential to be used in cell-based therapies of bone defects and injuries [136, 137]. Methods commonly employed to differentiate ESCs into the osteogenic lineage have been adapted for iPSCs differentiation [138]. Wu *et al*. recently provided an updated review on the methods used for iPSCs

differentiation targeting bone repair [139], which are classified according to (i) their dependence on the production of intermediate embryonic body structures, (ii) the direct generation of iPSCs-derived mesenchymal precursors, and (iii) the direct differentiation of iPSCs into osteoblasts without intermediate steps. The classical protocol for the osteogenic differentiation of iPSCs, based on a protocol established for embryonic stem cells [140], involves the initial formation of embryonic bodies followed by the harvest of iPSC-derived MSCs present in those structures and, finally, their differentiation into osteoblasts using osteogenic media. This protocol, however, was proven as low-yield, and approaches based on the direct seeding of dissociated embryonic bodies onto osteoinductive biomaterials have risen as effective ways of achieving regenerative systems for bone defects using iPSCs [136, 137, 141–143]. Alternatively to the formation of embryonic bodies, the adaptation of a protocol for embryonic cells differentiation [144] rendered iPSCs differentiation into MSC-like cells (often named iPSC-MSCs), which may be potentially differentiated into any mesenchymal lineage – including the osteogenic one. iPSC-MSCs were proven as valuable for the tissue regeneration field [145–147]. It is known that iPSC-MSCs are less responsive to the traditional chemically induced osteogenic differentiation protocol applied to MSCs (comprising ascorbic acid, β-glycerophosphate and dexamethasone), which has led researchers to find alternative differentiation methods [148]. In 2013, de Peppo *et al.* targeted the use of iPSC-MSCs as components of a tissue engineered system comprising decellularized bone as 3D scaffolds and a perfusion bioreactor. Perfusion conditions led to increasing expression of osteogenic markers, which were kept stable after the subcutaneous implantation of the iPSC in nude mice for 12 weeks [136]. Most of the studies reporting biomaterial-driven osteogenic differentiation of iPSC-MSCs rely on the use of mineralized matrices, either using decellularized bone or synthetic calcium phosphates. In 2018, a strategy to promote localized iPSC-MSCs *in situ* differentiation through localized BMP-2 recruiting was suggested by the co-injection of cells and a BMP-2 antibody in alginate beads [149]. This strategy avoided the formation of ectopic bone, commonly reported for BMP-2 releasing systems, and has the potential to surpass drawbacks associated to growth factors that include short half-life

The use of iPSCs has not been restricted to their differentiation into osteoblasts. A recent study using iPSC-MSCs also brought to light the potential of iPSCs to be differentiated into cell types other than osteoblasts with extreme relevance for the formation of functional bone, such as osteoclasts. Jeon *et al.* [7] used iPSC-MSCs differentiated into osteoblasts and osteoclasts differentiated from iPSCs-derived macrophages. iPSC-MSCs and iPSCs-derived macrophages were co-cultured and differentiated in PLGA/PLLA/hydroxyapatite porous scaffolds, rendering *de novo*-formed bone tissue upon implantation in nude mice.

Although iPSCs osteogenic differentiation relying on mesenchymal-like precursors has received a great deal of attention, the direct differentiation of iPSCs into osteoblasts has also been reported. In a precursor study, Levi *et al.* used biomaterials with the ability to concomitantly release osteogenic cytokines – BMP-2 - and provide biomineralization cues as boosting agents for *in vivo* bone formation through iPSCs differentiation [150]. Subsequent studied used methods based on the use of small molecules [151,152], osteogenic scaffolds [153–154], and gene modification to promote the fast, safe and high-yield differentiation of iPSCs and their application in bone regeneration strategies.

In general, protocols targeting the direct differentiation of iPSCs into osteoblasts depend on the use of cytokines, multi-step approaches using different supplements overtime, or *in situ* cell differentiation seeded onto specific biomaterials. In 2016, Kang *et al.* reported a breakthrough one-step protocol based on the use of adenosine, a natural occurring nucleoside, as a cell culture medium supplement to directly converse hiPSCs into functional osteoblasts [152]. Adenosine-treated cells seeded onto 3D microporous matrices rendered the successful repair of a critical bone defect, which included the formation of vascularized neobone capable of undergoing resorption.

Despite the clear promise represented by iPSCs as easily obtained cells for tissue regeneration, the field is still not free of challenges that include the delayed or low osteogenic differentiation of some cells that are exposed to differentiation protocols developed so far [148, 155], which may culminate in the long-term formation of dangerous teratoma tissues. Comparison between differentiation protocols, namely a robust parallelization between MSC precursor-based methods and direct differentiation protocols, are still in need.

5.2.2 Osteocytes—Osteocytes are fully matured and differentiated osteoblasts which descend from the mesenchymal lineage (Figure 2a). They comprise 90% to 95% of the whole bone cells in adult bone and may live up to decades in their mineralized environment exhibiting a dendritic configuration. Their function is to support the skeleton and bone metabolism. As osteoblasts differentiate into osteocytes, ALP production decreases, while osteocalcin raises [1] (Figure 2a). Other expressed markers, including phosphate-regulating protein with homologies to endopeptidases expressed by genes of the X chromosome (*PHEX*), matrix extracellular phosphoglycoprotein (*MEPE*), dentin matrix protein 1 (*DMP-1*), *FGF-23*, *sclerostin*, and oxygen regulated protein (*ORP143*), are thought to protect osteocytes against hypoxia [156]. These cells were considered as “passive placeholders in bone” in the past. However, they were proven to have numerous functions including bone remodeling - through the activation of both osteoclasts and osteoblasts -, as well as in endocrine cell functioning [156]. The interactions between osteocytes and osteoblasts/osteoclasts are reviewed in Section 5.4. Osteocytes are also responsible for the excretion of proteins such as CD44, galectin 3 and osteocalcin. These proteins have the function to promote cell adhesion and the regulation of mineral exchange in the bone. Osteocytes also produce Runx2 and Osterix, which are required for osteoblast differentiation, and are followed by ALP and collagen, necessary for the formation of the osteoid. Several soluble molecules produced by osteocytes positively interfere with biomineralization (e.g. PHEX, MEPE and DMP-1) [156]. The communication between osteocytes, which occurs mainly by gaps composed of connexin 43 [157, 158], are required for their survival, maturation and correct activity. The diverse roles of osteocytes also encompass their phagocytic activity, during osteolysis, since they have lysosomes in their constitution. One of the most remarkable functions of osteocytes is mechanosensing by translating stress factors into biologic signals [156]. Vazquez *et al.* [159] developed a 3D *in vitro* co-culture systems to assess the effect of mechanical loading on the interaction between osteocytes and osteoblasts. They demonstrated that osteocytes subjected to mechanical appropriate mechanical cues act upon osteoblasts towards bone formation [159].

5.2.3 Osteoclasts—Osteoclasts, generated in the bone marrow from mononuclear monocyte-macrophage precursors derived from the hematopoietic lineage [160], are the only cells capable of resorbing bone and, consequently, play an essential role in bone remodeling. The receptor activator of nuclear factor kappa-β ligand (RANKL) and macrophage colony stimulating factor (M-CSF) are reported to drive osteoclasts' proliferation, differentiation and survival [160, 161]. Bone resorption occurs in the presence of factors secreted by osteoclasts: hydrogen ions that lead the acidification of the resorption compartment dissolving the mineral bone matrix, and cathepsin K, an enzyme responsible for the digestion of the insoluble fraction of the matrix (mainly type I collagen). These cells are bound by the bone matrix through integrins (β1 for collagen, laminin and fibronectin, and αvβ3 for osteopontin and bone sialoprotein) [162]. Such binding polarizes osteoclasts, creating an actin ring that seals the periphery of the ligation of the osteoclasts to the matrix, and a ruffled border in the resorbing surface, which leads to the secretion of H^+ ions, followed by the exocytosis of enzymes from the acidified vesicles [162].

5.3 Heterotypic cell-cell interactions in bone

It is well established that bone cells interact in adult bone to regulate homeostasis process, supporting the balance between bone resorption and formation that allows the maintenance of the tissue's integrity [162]. We review the interactions between the main reported cells that constitute healthy bone - osteoblasts, osteoclasts and osteocytes -, and their crosstalk with the vascular system that irrigates and also constitutes functional bone. Due to their relevance in bone healing, interactions between immune cells and bone resident cells are also reviewed.

5.3.1 Osteocytes-osteoblasts—Bone formation is regulated by several signaling mechanisms, with particular importance for the Wnt/β-catenin pathway [1]. The activation of canonical Wnt signaling in early osteoblasts promotes osteoblast differentiation and bone formation [163], with opposing effects observed when Wnt signaling is disrupted [164]. Osteocytes secrete Wnt antagonists, which include sclerostin and the LRP5/6 inhibitor Dickkopf-related protein 1 (DKK1). Both molecules inhibit osteoblast differentiation and bone formation [165, 166]. Moreover, the *in vivo* loss of secreted frizzled-related protein 1 (SFRP1), which is a competitive antagonist of Wnt ligand, resulted in increased bone mass and mineral density, as well as in *in vitro* enhancement of osteoblast proliferation and differentiation into osteocytes [167]. With their capacity to interfere in canonical Wnt signaling, therefore affecting osteoblasts differentiation, osteocytes play a regulatory role in bone formation.

5.3.2 Osteoblasts-osteoclasts—Signaling between osteoblasts and osteoclasts is crucial for osteoclast maturation [1]. It is known that osteoblasts and stromal cells produce RANKL, M-CSF, and osteoprotegerin (OPG), while early osteoclast precursors produce c-Fms (M-CSF receptor) and receptor activator of nuclear factor κ B (RANK) - a receptor for RANKL. RANKL and M-CSF stimulate osteoclast differentiation, while OPG is an inhibitor of RANKL, and competes with RANKL for RANK [1]. Low levels of OPG lead to accelerated osteoclast development, which culminates in osteoporosis: a disease characterized by the disruption of bone resorption/formation balance, and in which bone

resorption exceeds bone growth [160]. Although osteoblasts were thought to be the main providers of RANKL to osteoclasts, osteocytes have proven to have an extremely relevant role in this mediation [168].

Besides the role of osteoblasts and osteocytes on osteoclastogenesis, the reverse role of osteocytes on osteoblasts' function has also been a matter of interest. In 2011, osteoclasts were reported as being uncapable of stimulating RANKL reverse signalling in osteoblasts through direct cell interactions [169]. However, in 2018, Ikebuchi *et al*. presented a breakthrough mechanism driven by extracellular vesicles, in which osteoclasts modulate osteoblasts function and, therefore, bone formation [170]. In the suggested setting, RANKL receptors in osteoblasts are responsible for the reverse RANK-RANKL modulation. Osteocytes release extracellular vesicles containing RANK on their surface, which bind to RANKL on the surface of osteoblasts (and hypothetically osteocytes), triggering intracellular signalling. The authors proved that mTOR pathway was activated, triggering the production of Runx2, and leading to bone formation.

5.3.3 Osteocytes-osteoclasts—Osteocytes – both healthy and apoptotic at microdamage sites – have been reported to recruit osteoclasts to the bone remodeling sites and were shown to send bone resorption cues to those cells [171]. The expression of the RANKL during the dendritic process associated with osteocytes maturation was associated with the osteocyte-led bone resorption [171]. Upon injury, right after damage, pro-apoptotic molecules are released by osteocytes; contrarily, anti-apoptotic molecules are produced at 1-2 mm distance from the cracks [171]. The promotion of a defective performance of osteocytes in mice through β-catenin depletion led to increased osteoclasts activity [171]. This result demonstrated the dependency of the correct regulation of osteoclasts activity on osteocytes, proving osteocytes' relevant role on the bone remodeling process [171]. Another indication of the close osteocyte/osteoclast interaction was the induced formation of osteocytes, both *in vitro* and *in vivo*, by osteoclasts apoptotic bodies; a similar contact with osteoblast-derived apoptotic bodies, however, did show this ability [171, 172]. Mechanistically, the induction of osteocytes formation by exposure to osteoclasts' apoptotic bodies was not driven by RANKL; instead, it was proved to be a TNF-α-dependent process.

Recent studies have focused on a deeper elucidation of osteocytes-osteoclasts interactions, namely in the understanding of osteoclastogenesis. IL-6 has been reported as a mediator and modulator of this cellular interaction, although the mechanism behind this phenomenon has not been completely unraveled. In 2017, Wu *et al*. performed the characterization of inflammatory factors present in the serum of ten patients who underwent orthognathic surgery [173]. The authors found that both IL-6 and RANKL were stimulated in serum 3 to 7 days after surgery. The characterization of inflammatory cytokines from patients' blood, along with an *in vitro* study in which an osteocyte cells line (MLO-Y4) was stimulated with IL-6 and IL-6 receptor, allowed correlating increased osteocyte-mediated osteoclastogenesis with the presence of IL-6, unraveling its role in the enhanced expression of RANKL.

5.3.4 Vascular cells interactions with bone cells—Bone-associated blood flow controls oxygen and nutrient delivery/exchange in the tissue, and bone formation and resorption are coupled with bone hemodynamics. During endochondral ossification, the

vascularization of hypertrophic cartilage is one of the determinant steps for bone elongation. Moreover, in fracture healing, the generation of an efficient new tissue is also dependent on a successful vascularization. A tight connection between the growth of blood vessels in bone and osteogenesis has been reported [174]. Endothelial and osteoblastic cells have a molecular crosstalk in which angiogenesis and osteogenesis are synergistically promoted. Osteoblasts are known to secrete angiogenic factors, including VEGF [175] and erythropoietin [176], which mediate their crosstalk with endothelial cells. Nonetheless, the mechanisms and molecules involved in this process have not yet been fully unraveled.

Bone vasculature has recently been presented as a unique network with substantial differences from other body vascular systems. Interestingly, vascular growth in bone was proven to be obtained by a tissue specific angiogenesis, in which the Notch pathway is responsible for endothelial cell proliferation and blood vessel growth in post-natal long bone. In a study conducted by Ramasamy *et al*. [177] the authors verified a deficiency on the bone vessel growth and morphology by knocking out the gene responsible for the Notch signaling. In turn, this led to reduced osteogenesis, resulting in the irregularity of bone structure in mice. Huang *et al*. [178] identified chemokine (C-X-C motif) ligand 9 (Cxcl9) as an angiostatic factor secreted by osteoblasts in the bone marrow environment. Mice with constitutive mTORC1 (an Cxcl9 activator) in osteoblasts demonstrated enhanced VEGF secretion; however, this was accompanied by an unexpected decrease in the phosphorylation of its receptor (VEGFR2), as well as downstream signaling in endothelial cells, and reduced vasculature formation in bone.

The structure of bone vasculature was proved as a unique construction by Kusumbe *et al*. [179] who identified a new capillary subtype in the murine skeletal system, presenting distinct molecular, morphological and functional properties. These vessels were shown to be crucial for the correct bone development and maintenance, since they generate a distinct molecular and metabolic microenvironment, linking angiogenesis and osteogenesis, and lastly maintaining perivascular osteoprogenitor cells. The study of human vasculature and the identification of capillary subtypes specific to bone may represent a step ahead on the understanding and development of vascularizing strategies. Also, the scrutinization of the individual interactions between human bone vascular cells and other resident bone cell types besides osteoblasts (including osteoblasts and osteoclasts), as well as the comparison of these results with more complex co-culture systems, is still in high demand for a full mastering of biochemical/biophysical signaling dictating the achievement of fully functional bone tissue.

5.3.5 Immune cells interactions with bone cells—Despite not having been reported to reside permanently in healthy mineralized bone tissue, except for osteal macrophages - OsteoMacs, immune cells residing in the bone marrow are in a close anatomic location with bone. The crosstalk between bone and immune system cells has often been overlooked, and usually focuses on the role of such cells in disease [180]. We here report some of the studied cell crosstalk facts involving bone cells and bone-related/bone-constituent (OsteoMacs) immune cells, connected to the regulation of bone's normal physiology.

OsteoMacs are probably the most studied immune system cells in bone tissue. They reside on the endosteal and periosteal surfaces and compose 10 to 15% of most tissues [94]. *In vivo*, OsteoMacs form a shell over mature matrix-producing osteoblasts at sites of bone modeling [181]. Depletion of macrophages *in vivo* results in complete loss of endosteal OsteoMacs and their associated osteoblasts, suggesting that this population is needed to maintain mature osteoblasts in the bone structure [94, 182]. Furthermore, OsteoMacs can also function as osteoclast precursors [156]. Raggatt *et al.* [183] confirmed that primary OsteoMacs isolated from endosteal bone tissue can differentiate towards the osteoclast lineage *in vitro*, in the presence of both RANKL and CSF-1 factors.

Immune cells, namely macrophages and monocytes are the first cells to interact and react with foreign pathogens or implanted devices [184]. Despite the advances concerning the elucidation of the role of OsteoMacs in bone biology, little is known about their differentiation behavior in the presence of biomaterials used in bone regenerative strategies, as well as about the factors involved in the process. Most strategies employed in bone biology field are adapted from studies focusing on biomaterials integration by soft tissues and many of them play with properties of the surface biomaterials that ultimately enable the modulation of macrophages behavior [184]. Considering the crucial role of OsteoMacs in bone formation and remodeling, further steps should be taken towards the incorporation of this population into 3D *in vitro* bone remodeling models as well as bone regenerative approaches. The independent analysis of OsteoMacs on biological tissues has been associated with some cellular detection limitations, and this must be considered while studying the role of these cells, mainly in *in vivo* settings. Studies targeting *in vivo* depletion of OsteoMacs often cause collateral decrease in osteoclasts [185]. Indeed, these two cell types show major similarity, which are assigned to their shared progenitor myeloid lineage, produced growth factors and other molecules. While osteoclasts are reported to be easily distinguished from resident macrophages with targeting well-established biomolecular techniques [160], the reverse cell depletion process is not free of risks, leading to the possible misinterpretation of the role of these cells on bone biology. Recently, the identification of the CD169 marker as specific for OsteoMacs allowed an unbiased analysis of their role on bone development [185]. Using this osteoclast-bias-free depletion method, the role of bone resident macrophages as pro-anabolic supporters of osteoblasts function during bone homeostasis and repair was elucidated, both on intramembranous and endochondral pathways [185].

Besides macrophages, other immune cells have deserved attention for their role on healthy bone maintenance. The reduction of bone-related B- and T- lymphocytes in mice have led to osteoporotic scenario [186]. Moreover, it is known that mature B-cells produce more than half of bone marrow-derived OPG, which contributes to osteoclastogenesis restriction [186] and T-lymphocytes are also thought to interact with B-cells to enhance OPG production [94]. HSCs-derived megakaryocytes, known to produce platelets, were shown to enhance the *in vitro* osteoblast proliferation and differentiation through the expression of RANKL, OPG and some unknown anti-osteoclastic factors [187]. Despite the evident role of a plethora of inflammatory cells in the maintenance of healthy bone status, most studies concerning their role still focus exclusively on post-injury/healing stadia. Nonetheless, currently available information shows the relevance and active role of these cells on basal bone maintenance,

and that their depletion often culminates in disease scenario. Therefore, an investment on the elucidation of the role of inflammatory cells on the maintenance of healthy bone may drive the discovery of new diseases, and may also benefit the existing know-how targeting pro-regenerative techniques through the exploitation of novel *in vitro*-designed co-culture setups.

5.4 Co-cultures for bone regeneration: modulation of stem cell fate and improvement of tissue integration

Cells are often included as part of regenerative therapies due to their ability to naturally synthesize ECM proteins responsible for tissue reshaping and release biomolecules that dictate the success of bone development and correct function. Crosstalk between bone resident cells, immune cells, endothelial cells and MSCs are key players of a coordinate chain of events that occur during bone healing. Since different cell types cohabit in the *in vivo* environment, modulating each other's response by direct contact or release of molecules, it is essential to study and develop bone engineering strategies that somehow could recreate and take advantage of specific features of this niche. Table 1 is dedicated to an analysis of already reported cell-cell combinations applied in the context of bone tissue regeneration.

We identify four main axis of co-culture setups applied in bone tissue engineering: (i) co-culture of different primary bone cells, (ii) stem cells and resident bone cells, (iii) bone/stem cells and endothelial cells (targeting vascularization) and (iv) bone/stem cells and immune cells (targeting immunomodulation).

Bone resident cells, i.e. osteoblasts, osteoclasts and osteocytes are the pillars of bone. As reviewed in Section 5.3, dedicated to the description of heterotypic cell interactions, these cells are in constant communication with each other and adjacent cells, modulating their response through a complex network of signaling that provides the simultaneous degradation, formation and maintenance of the tissue [188]. Some of these interactions were transposed to tissue engineering, (i) to developed strategies that aim the recreation of the 3D bone environment and processes and (ii) to improve the quality and performance of bone regeneration approaches. Most studies aimed at the development of *in vitro* 3D models that recreate some of the *in vivo* bone events by establishing the interactions between osteoblasts-osteoclasts or osteoblasts-osteocytes into a scaffold/platform or by self-assembling [159, 189–191] (Table 1). Studies focusing on the crosstalk between osteocytes and osteoclasts in 3D biomaterial-based *in vitro* environments are still absent. In fact, although many fundamental studies have directly addressed these interactions, the transition to a tissue engineering 3D perspective needs to be further explored.

MSCs, addressed in Section 5.2.1.1, are well accepted as relevant cells for implantation in regenerative medicine platforms, owing to their clinical potential and easy retrieval. hiPSCs are another attractive stem cell source due to their ability to be reprogrammed from easily accessible tissues [134]. Although the incorporation of stem cells into bone regenerative strategies was an exceptional achievement, the combination of co-cultures comprising stem cells and adult bone resident cells within 3D cultures is seen a highway to achieve higher complexity models, resulting in regenerated tissues of superior quality [192]. Birmingham *et al.* [6] investigated whether soluble factors produced by osteoblasts and osteocytes could

influence bone formation. By establishing an indirect co-culture that allowed the direct contact between those two bone cells, while restricting it to MSCs, the authors proved the need of the biochemical crosstalk between both bone cells and MSCs to direct them towards the osteoblast lineage, in the absence of osteogenic media [6]. Besides better recreating the bone niche, co-cultures can also function as an alternative to conditioned media used in tissue engineering methods. So far, the characterization of these co-cultures in a 3D context has been essentially made through hydrogel-based strategies [193, 194] (Table 1).

The achievement of constructs rich in functional vascular networks is one of the well-known gold standards of bone regeneration targeting effective osseointegration and defect restoration [195, 196]. Approaches based on the presentation of vascularization-inducing biochemical cues through biomaterials are common to induce vascular cells migration and invasion of scaffolding materials. Endothelial cells aimed at promoting tissue vascularization, however, are used in technologies that either compete or complement the delivery or localized presentation of biochemical cues [197]. While endothelial cells cultured alone showed limited potential for vascular growth, studies have shown that their combination with osteoblasts or BMMSCs as well as with biomolecules involved in osteogenesis enhanced their vascularization capacity [197–203]. The exploitation of co-culture systems is a growing trend to establish biomimetic cell-based approaches targeting functional angiogenesis on regenerative constructs and/or full vascularization [204].

Different cell sources have been suggested as effective promoters of scaffold vascularization agents [205]. The use of mature endothelial cells and EPCs on co-culture strategies targeting bone regeneration using tissue engineered constructs was systematically reviewed by Liu *et al.* [204] concerning aspects as type of co-cultured cells (adult primary bone cells, stem cells), application of static and dynamic flow/tension regimes (reported to modulate bone formation and ageing processes [206]), type of used ECM-support, as well as critical aspects such as cell culture media used for *in vitro* experiments, cell seeding methodologies, establishment of direct and indirect co-culture setups, type of animal models, and how these factors affected bone formation and vasculature quality.

Pluripotent stem cells, including ESCs and iPSC are nowadays rising as important sources of endothelial cells [205,207]. Other stem sources, including BMMSCs, have also been suggested as naïve platforms to be differentiated into endothelial/vascular cells [208]. The use of stem cell-derived endothelial cells is nowadays a breakthrough in the generation of high yield *in vitro* tissues. However, their application in bone regeneration is poorly explored, even though the use of autologous sources of stem cells and further endothelial and tissue-specific differentiation may represent a step forward in the design of low-invasive (e.g. using ASCs) technologies. The characterization of the effect of different co-culture setups is another aspect that is still in need of further in-depth study; efforts to perform relevant characterization have relied on micropatterning strategies (mostly targeting 2D culture systems), use of transwell systems, and microfluidics to generate multicompartment structures [209]. Future optimization of such setups will allow further elucidation of the role of distinct culture setups on the synergic role of vasculature formation and bone tissue development on 3D/4D relevant structures, which will be organized in ECM-like structural organization and temporarily-controlled remodeling.

Controlling immune-mediation as a driving force for bone regeneration has also been explored for tissue-engineered systems. The conjugation of immune system cells with either adult bone cells or stem cells is currently a major trend. Primordial advances on immunoregulation for tissue engineering targeted the direct role of biomaterials upon implantation (or upon cytokine stimulation *in vitro*) [210–214], or their use as cytokine-release mediators to induce bone formation and successful vascularization [112]. Macrophages are the most widely studied immune cells in co-cultures for bone regeneration [215–217], and most of these studies target the osteogenic differentiation of adult MSCs under 2D direct and paracrine co-culture setups. Indeed, secreted factors from immune cells are described to affect MSCs [218, 219], and synergic effects are reported and suggested as, for example, means to obtain anti-inflammatory macrophages as therapeutic agents [220]. The effect of macrophages' polarization state has also been correlated with MSCs osteogenic fate promotion [221, 222]. Interestingly, the co-culture of MSCs with M1 macrophage pheonotypes was reported to promote enhanced osteogenic differentiation of BMMSC [221]. Other studies, though, correlate M2 anti-inflammatory phenotypes with higher ALP and mineralization of both bone marrow and adipose tissue stem cells [222, 223]. The discussion over the timely presentation of macrophages with distinctive roles on injury sites on physiological bone healing may hide the clues to design regulated biomaterial and co-culture systems to promote the presence of the most adjuvant immunoregulatory cells on implantation sites.

In a recent approach, the mechanomodulation of macrophages using magnetized superparamagnetic scaffolds allowed driving their phenotype to a M2-like status; media conditioned by these macrophages was later proved to enhance two important bone regeneration features: osteoblasts osteogenesis and endothelial cells angiogenic potential [224]. Indeed, biophysical stimuli (including topographic cues) seem to provide crucial cues to modulate macrophage activity and its subsequent interaction with stem cells [225]. There seems to be a broad room for exploitation of immune cells as bone regeneration adjuvants. While monocytes/macrophages start to see their roles unraveled and potentiated in the field of tissue regeneration, other poorly explored cells with proved potential (e.g. NK cells [226]) may lead to relevant scientific discoveries and technological breakthroughs. The role of lymphocytes as supportive cells for tissue engineering has also been scrutinized [227], mainly taking in consideration their key role in autoimmune diseases as rheumatoid arthritis [228]. Few studies report the characterization of heterotypic cells interactions while cultured on biologically relevant biomaterials, which can be used as *in vitro* tissue models and/or as implantable devices. Moreover, the results obtained from these studies are difficult to correlate as different biophysical and chemical cues are provided by different materials.

Sophisticated biomaterial designs will be necessary to enable co-culture setups with different and relevant cell types driving effective osteogenic differentiation, osseointegration and beneficial immune response upon implantation. Such co-cultures may be performed in direct or indirect setups with highly complex cellular communication occurring, which may be facilitated by multicompartmental and time-regulated biomaterials. Another interesting route to achieve the precise location of homotypic and heterotypic cell-cell contacts in either implantable or *in vivo*-formed microtissues may rely on the localized presentation of cell-recognizable domains in distinct fraction of biomaterials. Surface patterning using specific

cell-targeting antibodies is one of the most common approaches to control cell positioning. These systems have been suggested in the 1990's as "immunospots" for disease diagnosis [229, 230], and antibody-coated cell-specific microbeads (so caller "immunobeads") are routinely used in cell isolation from blood and tissues. This concept was extended for the spatially controlled cellular patterning of in 2D biomaterials [231], as well as in 3D beads targeting the *in situ* formation of robust microtissues through the selective adhesion of specific cells, namely MSCs and HUVECs, to polymeric injectable biodegradable particles [232, 233]. Cell patterning and specific cell-cell contact may be directed not only by biomaterial-based approaches, but also by cell-driven strategies. Cellular surface engineering, i.e. the direct chemical modification of cellular membrane, may be used to spatially control cell-cell assembly of cells during the formation of highly organized tissues [234–238]. The application of these concepts to bone tissue regeneration may lead the way for the achievement of scaffold-free implantable multicellular microtissues with, for example, evenly distributed vascular networks.

5.5 Protein-mediated cell-cell contact in bone

5.5.1 The role of cadherins, connexins and pannexins—

Cells can communicate by two processes, involving indirect and direct contact. Most interactions that occur during bone formation, development and remodeling have been shown to be driven by direct cell contact. Cadherins are the main proteins responsible for cell-cell attachment [252]. These proteins are glycoproteins located at the cell membrane, which promote cell-cell adhesion by a calcium-mediated mechanism. Cadherins (molecular weight around 120 kDa) are constituted by two domains: the extracellular and the transmembrane domain. The calcium-binding site (five repeats; responsible for the ability of cells to bind the same cadherin) is located in the extracellular domain. Cadherins can be classified in the following way: type I and type II. In these two types, cadherins can be divided even more: type I: N-, E, M- and R-; type II: 5 to 12 [252]. The cytoplasmic C-terminal tail of cadherins is responsible for the stabilization of the adhesion. This structure is organized by the binding of cadherin to β-catenin and plakoglobin, which connect cadherins to the actin cytoskeleton, via N-catenin, actinin, ZO-1 and vinculin, in a dynamic process. Adherent junctions, i.e. the junctional structures between two adjoining cells, allow the communication and adhesion between cells [252].

In bone, there are three major cadherins: E-cadherin, N-cadherin and cadherin-11 [253–255]. Cell-cell adhesion mediated by cadherins is essential for the function of bone-forming cells during osteogenesis. The absence of those cadherins was shown to inhibit osteoblasts differentiation [256]. During osteoblast differentiation, cadherin-2 is downregulated over the process, and cadherin-11 becomes the main cadherin for osteoblast functions. For successful osteogenesis, cell-cell contact amongst cells of the osteoblastic lineage and the osteoclasts precursors is necessary. This interaction is mediated by RANK (receptor) and RANKL. RANK is present in osteoclasts precursors, while RANKL is present in the membrane of osteoblastic cells [1, 257, 258].

Connexins are proteins involved in cell-cell contact, allowing the rapid dissemination of molecules (smaller than 1 kDa) and ions by diffusion among cells. They link cells through

gap junction channels, that facilitate electrical and chemical coupling [259]. The most widely reported connexin in bone - produced by osteocytes, osteoblasts and osteoclasts - is Cx43 [260]. In addition, both cells also produce Cx37 [261], and osteoblasts produce Cx45 and Cx46 [158]. When osteochondroprogenitors, as well as committed osteoblast progenitors, lack Cx43 protein, there is a decrease in bone mass and density [260]. Interestingly, the deletion of the gene that codes Cx43 from mature osteoblasts and osteocytes did not lead to any effect on bone mineral density or bone length. This suggests that Cx43 is essential for osteochondral progenitors, but not in committed osteoblasts [260]. Cx37 has recently been proved to regulate bone mass [261]. The lack of this connexin led to increase in bone mass. However, this effect was shown to be gender dependent, with males being more affected than females [261]. The higher bone mass observed in individuals with depleted Cx37 is related with a decrease in osteoclast differentiation, driving impaired bone resorption [261].

Pannexins are proteins with a very similar structural topology to connexins. However, their sequence is not homologous with connexins, and they only function as an unpaired channel [262, 263]. One of the genes encoding pannexins - *Panx1* - is present in murine osteoblastic cells [264], whereas *Panx3* is expressed in various osteoblastic cell lines, primary calvaria cells and in hypertrophic chondrocytes [265–267]. Although some studies have explored the role of pannexins in osteoblast differentiation *in vitro*, *in vivo* studies are still needed [262].

5.5.2 The role of protein-mediated cell-cell contact on bone regeneration: a still unexplored concept in tissue regeneration strategies—The modulation of pro-regenerative niches based on the modification of biomaterials with cell-cell contact-mimetic domains has been suggested as a strategy to locally target the differentiation of stem cells, including pluripotent stem cells and MSCs. Toh *et al.* [268] developed 2D surfaces modified with Matrigel® and E-cadherin micropatterns, to mimic cell-matrix and cell-cell adhesion motifs, respectively. The culture of pluripotent ESCs on the patterns showed that integrin and E-cadherin adhesions were capable of locally promoting distinct cell fates, which culminated in the generation of spatially heterogeneous cell colonies. The role of selectively modified biomaterials on the modulation of clinically relevant iPSCs has also been a matter of debate [269]. Recently, nanoporous and microporous with pore sizes ~5 nm and ~120 μm, respectively, were applied as platforms for the study of the role of cell-cell interactions on the paracrine function of MSCs [270]. The authors hypothesized that cell-cell contact through N-cadherins, as well as cell-matrix interactions, would be increased in biomaterials with higher pore size. Alginate-based hydrogels and porous scaffolds featuring similar mechanical and chemical profiles were synthetized to show different pore size ranges [270]. The secretory profile of MSCs cultured on both biomaterials showed significant differences, and the retrieved conditioned medium from both conditions differently influenced the function of C2C12 myogenic precursors, proving the potential of cadherin-mediating biomaterials as platforms for the modulation of potentially therapeutic cytokines produced by MSCs.

Biomaterials with the ability to present cell-cell contact motifs to cells are still in their early infancy on the bone regeneration field. A study by Cosgrove *et al.* [9] described the use of a biomaterial-based approach to elucidate the role of cell-ECM and cell-cell contact of MSCs

in osteogenesis driven by mechanical transduction phenomenon. The authors modified a hyaluronic acid hydrogel with a HAVDI adhesive motif from N-cadherin (to emulate the cell-cell ligation) and a RGD adhesive motif from fibronectin (to emulate the cell-ECM ligation), for the co-presentation of these motifs. HAVDI ligation decreased the contractible state of the cells (and the nuclear YAP/TAZ location), which led cells to wrongly interpret the ECM stiffness, causing a change in the downstream cell osteogenic differentiation and proliferation. On the same year, Zhu *et al.* [271] also presented a methacrylated hyaluronic acid-modified hydrogel with N-cadherin and the integrin-binding domains and hypothesized that such moieties would allow presenting an "orthotopic" environment to MSCs. Interestingly, N-cadherin-containing hydrogels improved the osteogenic differentiation of encapsulated cells, both *in vitro* and *in vivo*, which seems contradictory with the results presented by Cosgrove *et al.* [9] in the same year.

The low number of studies reported on the effect of cell-cell domains-presenting biomaterials on the regenerative medicine field, in particular for bone tissue engineering strategies, still hamper a profound discussion about the role of these domains on stem cells differentiation and biological tailoring to achieve improved regeneration outcomes. The opportunity for the development of systematically modified materials that combine ECM and cell-cell interaction cues is a current opportunity for the design of more efficient biomaterials capable of contributing to the elucidation of fundamental aspects of bone niche interactions.

5.6 Soluble biomolecules present in bone environment

5.6.1 The primary role of biomolecules in bone formation and regeneration—

Biomolecules play a crucial role in both formation and repair of bone tissue, and are constantly present through all bone formation and repair phases. They are responsible for the recruitment, proliferation, differentiation and migration of the osteoprogenitor cells [2]. As mentioned on Section 4, upon bone injury, immune cells are recruited to the defect site and coordinate actions triggered by the release of cytokines and growth factors, that culminate in the recruitment of MSCs, remodeling and vascularization of the tissue [2]. Bone physiology during development, under healthy conditions and upon regeneration involves the participation of an elevated number of biomolecules. In this Review, we will briefly describe the roles of widely reported BMPs and pro-angiogenic VEGF, as these are the most commonly used cytokines and growth factors on tissue regeneration strategies as externally-provided factors. The delivery of off-the-shelf cellular factors (in cell-free strategies) to regulate bone healing has been recently reviewed [272]. An interesting table that correlates the most important growth factors in bone tissue repair, their function and working period can be found in a recent Review by Wang *et al.* [273].

BMPs are a family of multifunctional growth factors containing over 20 members, generally classified into four categories: BMP-2/4, BMP-5/6/7/8a/8b, BMP-9/10 and BMP-12/13/14 [274]. From those 20 types of identified BMPs, at least 7 have shown osteoinductive potential [275, 276]. Those include protein forms with 30 to 38 kDa molecular weight, composed of two disulfide-linked polypeptide subunits. These proteins are involved in the regulation of cell proliferation, differentiation and matrix biosynthesis during reconstruction

process of human bone, in coordination with other molecules. Moreover, BMPs are unique proteins with the ability to induce bone formation individually. While several homodimer forms of BMPs were reported to be osteoinductive, the heterodimer forms of these molecules including BMP-2/-6, BMP-2/-7 and BMP-4/-7 have shown more potency both *in vitro* and *in vivo* as compared to the respective homodimer mixtures, with 20-fold higher osteoinductive activity for BMP-2/-7 heterodimer in particular [277]. The higher activity of BMP heterodimers has been associated with higher affinity for receptors than homodimer counterparts [278–280].

The detailed role and structure of each BMP family member is reviewed in Reference [260]. These molecules stimulate the differentiation of MSCs into osteoblastic lineage and promote the proliferation of osteoblasts and chondrocytes, being an active member of endochondral ossification and bone healing process (see Section 3.2 and 4.1) [281, 282]. BMPs bind to receptor complexes consisting of type I and type II transmembrane serine/threonine kinases. The role and associated potency of different BMPs receptors can be found in Reference [276]. BMPs target cells by activating mothers against decapentaplegic (Smads) and mitogen activated protein kinase (MAPK) pathways [281] that converge at transcription factors as Runx2 to promote osteoblast and chondrocyte differentiation from MSCs [44]. In tissue regeneration therapies, bone formation induced by growth factors administration results from a combined action of the administered molecules and endogenous produced factors [276]. Along with BMPs, TGF-β - including TGF-β1, TGF-β2, and TGF-β3 - is a family of proteins also involved in skeletal embryonic development and postnatal bone homeostasis through the same pathways [44].

VEGF, a crucial intervenient of vascular growth, is also deeply involved in the correct bone development and regeneration, linking both osteogenesis and angiogenesis [283, 284]. This growth factor is involved in both intramembranous ossification and endochondral bone formation. In the later, VEGF stimulates vessel invasion and the recruitment of chondrocytes into hypertrophic cartilage while in the former it is released by osteoblasts upon hypoxia exposure and induces endothelial migration and proliferation and vessel permeability [283, 284]. In turn, osteogenic factors such as BMP-2, are produced by endothelial cells, leading to osteoblast differentiation and mineralization [284].

Besides the previous mentioned signaling molecules, others with similar relevance for bone tissue development and regeneration include cytokines, PDGF, FGF, among others. A detailed description of the source, function and target of those proteins can be found at Dimitriou *et al.* [100].

5.6.2 Biomolecules in tissue engineering strategies—Biomolecules are essential in the bone regeneration process due to their key role on the recruitment of MSCs, tissue remodeling and vascularization [2] upon bone injury. The understanding of the individual tasks, combined actions or even potentiating effects of growth factors have led the development of biomaterial-based structures targeting bone regeneration. Strategies to modulate cell response based on bioactive agents may rely on the controlled release of these molecules through finely controlled drug delivery systems [285]. Systems designed to

enable the pulsatile or controlled sequential release of different bioactive agents have been applied in tissue regeneration strategies [286, 287].

BMPs are the mostly studied growth factors for bone tissue engineering. BMP-2 and BMP-7 are the most used ones, with application in clinical medicine. However, they have showed limited success, owing to the reported formation of ectopic bone [288]. Besides promoting osteogenesis, these two BMPs, as well as PDGF and VEGF play a critical role in promoting neovascularization of bone tissue [197, 199, 283]. Since bone is a highly vascularized tissue, the performance of a scaffold can be dependent of its ability to induce new vessel formation in the transplantation site [21]. As these growth factors are often costly, Yu *et al.* [201] reported a new peptide, designated bone forming peptide-1 (BFP-1), easy to synthetize at lower cost [289], that derives from the immature region of BMP-7. This biomolecule was shown to enhance vascularization through the up-regulation of VEGF receptor gene in endothelial cells [201]. The incorporation of BFP-1 into beta-tricalcium phosphate (β-TCP) scaffolds containing endothelial cells successfully promoted angiogenic functions of endothelial cells of the construct *in vitro* and enhanced vascularization and bone regeneration *in vivo* [198].

The release of bioactive molecules in a controlled and site-specific manner is also crucial for the success of tissue engineering strategies. Complete reviews addressing the controlled release of bioactive agents for bone regeneration can be found in References [286, 290–292]. The complex design of biomaterials as a route to mimic naturally occurring or much needed therapeutic phenomena, or as a way to allow effective administration of drugs with paramount importance for bone regeneration, has been explored through the establishment of finely controlled degradation profiles of adequate implantable biomaterials. For instance, a biodegradable drug delivery system designed to enable the pulsatile release of PTH – a FDA-approved drug for the treatment of osteoporosis – for 21 days, presenting a promising method to circumvent the daily injectable administrations of this drug [293]. An advanced 3D scaffold design consisting of finely tuned biodegradability profiles allowed the application of a nanofibrous material loaded with PTH in the high-quality regeneration of a mouse critical size (2.3 mm) calvarial defect, as compared to regular injections of PTH or continuous release of the hormone through a releasing biomaterial [294].

As previously mentioned, growth factors have important effects on the regulation of bone formation, remodeling and regeneration. Their clinical application, however, is often hampered by the administration of supraphysiological doses of soluble compounds reported to be rapidly cleared from the body, and that show limited therapeutic effect, elevated costs, and are associated with serious side effects such as the formation of ectopic bone (e.g. for BMP-2 and BMP-7) [295]. The presentation of growth factors immobilized in biomaterials with controlled spatiotemporal release or localized presentation have been suggested to enhance tissue regeneration outputs obtained from the delivery of these molecules to defect sites. Through the entrapment of BMP-2 in alginate/chitosan self-standing membranes produced by the layer-by-layer electrostatic assembly technique, Caridade *et al.* [296] achieved a slow-releasing system, in which only a ~15% fraction of the growth factor mass was released from the biomaterial structure after 30 days of immersion in a physiologic-mimetic solution. Slow BMP-2 releasing materials supported the formation of localized

ectopic bone fragments in a subcutaneous mouse model, while membranes chemically tailored to promote faster release did not achieve a pro-regenerative performance. Other membrane materials based on electrostatic interactions between natural polysaccharides – chitosan and chondroitin sulfate – also showed the ability to uptake and retain high amounts of growth factors, namely TGF-β3 [297]. These materials released only 1% of the total loaded TGF-β3 mass after 15 days of immersion in an aqueous phosphate buffer, which suggests their possible application as growth factor-presenting materials, with extremely low drug release profiles. The majority of growth factors related with bone regeneration and used as therapeutic agents interact with cells through cell membrane-based mechanisms. In the particular case of BMP-2, the initiation of signaling pathways occurs through the binding of plasma membrane receptors, followed by the phosphorylation of the protein, culminating in the Smad signaling activation [298]. The use of biomaterials to present growth factors to cells, independently of release mechanisms, is then considered an advantageous approach to circumvent excessive cargo release and provide highly localized biochemical signals in defect sites. Cell-interacting biomaterials containing covalently immobilized proteins and growth factors have been shown to modulate stem cells response and drive effective bone repair [299]. Indeed, the modification of biomaterials with covalently attached growth factors was suggested as a promising manner of maintaining physiological levels and prolong their life time [300, 301]. For example, methoxy poly(ethylene glycol) injectable hydrogels covalently modified with BMP-2 through a click reaction showed enhanced synergic osteogenic differentiation of human periodontal ligament cells as compared to soluble BMP-2 formulations [302].

The high cost of recombinant growth factors has driven the design of biomaterials with ECM-mimetic properties, in which these molecules are selectively attracted to specific scaffold locations, which then serve as growth factors reservoirs [303]. One of the most well reported examples of ECM proteins-growth factors physiological interactions is the formation of fibronectin-BMP2 complexes [304]. In biomaterials, however, the effectiveness of the interactions between growth factors and ECM proteins is strictly dependent on protein rearrangement and conformational aspects. Salmerón-Sanchez and co-workers [305] developed an approach to control fibronectin conformation and exploit growth factor (BMP-2) recruitment and presentation to cells. The use of poly(ethyl acrylate) materials allowed adsorbing fibronectin in a fibrillar fashion, in opposition to the globular conformations obtained on control polymeric surfaces. The fibrillar arrangement of fibronectin showed a synergistic presentation of integrin-binding sites and bound BMP-2, which drove MSCs osteogenesis and the full regeneration of a nonhealing bone defect. Other bioinspired studies based on the use of heparin-based domains or charged polyelectrolytes for partially selective growth factors sequestering from blood and plasma components have been incorporated into tissue regenerative approaches [306, 307].

The delivery of extracellular vesicles (EVs) as regeneration modulators is an increasingly relevant trend in the field of tissue engineering [308, 309]. These nanosized structures, which include exosomes and shedding vesicles, produced by cells were once seen as “garbage bags” used to excrete waste [309]. Nowadays, they are considered as powerful tools to induce cell response because they are known to carry biologically relevant cargoes that include proteins, lipids, as well as coding and non-coding RNAs [310]. Reports on the

pro-regenerative and immunomodulatory roles of these structures can be found in References [308, 309, 311, 312]. The use of EVs generated *in vitro* after the differentiation of stem cells into the osteogenic lineage has proven to be effective as inducers of the osteogenic differentiation of naïve stem cells. Moreover, stem cell-conditioned medium and isolated EVs were capable of regulating osteoblasts activity and promote the regeneration of bone defects *in vivo*, and osteogenic differentiation *in vitro* [313, 314]. These effects are thought to be mainly derived from the EV-mediated delivery of microRNAs with positive impact on bone formation [315–317]. Human MSCs-derived exosomes retrieved from different stages of stem cells osteogenic differentiation were capable of committing homotypic cells into the same fate; nonetheless, only exosomes from late osteogenic differentiation timepoints were capable of inducing ECM mineralization [318]. microRNA profiling of exosomes from different stages of MSCs osteogenic differentiation showed different patterns, which was partially correlated with the observed findings.

The design of bioinstructive materials capable of surpassing regulatory issues and allow cost-effective and safe drug administration, or even the localized recruitment of endogenous pro-regenerative factors is currently one of the most widely spread trends for bone regeneration therapies development [319]. For example, biomaterial-based synthetic approaches capable of mimicking the microRNA delivery function of EVs are gaining momentum [320, 321]. The integration of the know-how of ECM properties capable of withstanding highly effective growth factor sequestration from the implantation medium with effective mechanisms for delivery of *in vitro*-generated cargo-loaded vesicles may represent an elegant way to mimic biological functions of the regenerating bone tissue.

5.7 Cells-ECM interactions in bone

5.7.1 Cell-ECM natural interaction in native bone—The ECM is a complex network comprising proteins (soluble and insoluble), growth factors and polysaccharides. It provides physical structure and a biochemical context to the cellular microenvironment [322]. In body tissues, the communication amongst cells and the surrounding ECM is mainly made through three types of proteins: integrins, selectins and immunoglobulin [323]. This adhesion contributes to cell biological processes as immune response, metastases, inflammatory process, division and death of cells, tumor progression and cell polarity. An extensive 3D imaging map of the localization of bone cells, ECM proteins and other molecules as well as potential interactions between them in whole mouse femurs is available at Coutu *et al.* [324]. Figure 2b shows a schematic of the various interactions that occur in the ECM. The bone matrix is mainly composed of collagen (85-90%) and other types of proteins. The ECM has two mechanisms by which it affects cellular behavior: (i) by the direct interaction with cells, and (ii) by harboring growth factors for cell proliferation and differentiation; biomimetic biomaterials designed to mimic such ECM features are addressed in Sections 5.5. (approach (i)) and 5.6.2 (approach (ii)).

The connection between cells and ECM - case (i) - is made through proteins existing on cell surface, integrins, which regulate not only the cell-to-physical matrix adhesion but are also responsible for some intracellular signals [325]. These proteins recognize specific peptide sequences and bind to specific peptide domains by the presence of two distinct subunits: α

and β. The binding of the ligand to this intramembranous protein is dependent on the association of these two subunits, making it possible for only one integrin to recognize and connect to specific types of ECM proteins [326]. A Review by Shekaran *et al.* [327] focused on the role of different full proteins and peptide domains, as well as cellular interacting integrins, in bone tissue and repair. Recently, a study reported that in an initial stage, fibroblasts α5β1 integrins connected to fibronectin are able to sense mechanical load and activate adhesion-related pathways in less than a second to reinforce adhesion [328]. It is still unknown whether this phenomenon also occurs for bone cells or even stem cells. The elucidation of this effect on a wider range of cells may give further insight on mechanosensing aspects, which may drive cell fate modulation through rapid-acting cell-biomaterials interactions.

The understanding of the role of ECM on bone regeneration has been addressed through combinatorial studies, in which different features of ECM (e.g. biophysical and biochemical aspects) are varied and studied in different proportions. For example, Huang et al. [329] studied the combined effect of mechanical factors - i.e. ECM stiffness - and the presence of ECM insoluble proteins on the osteogenic differentiation of MSCs, cultured as 2D monolayers, in basal medium. The tested ECM cell-binding proteins - type I collagen, fibronectin, vitronectin and laminin - induced the *in vitro* osteogenic differentiation of MSCs, implying that the right ECM composition is enough to trigger the process. This study also showed that, although type I collagen is the main protein in bone ECM, no difference was observed in its capacity to induce more osteogenic differentiation than the others tested proteins. In fact, fibronectin showed more ability to drive osteogenic differentiation, followed by laminin, type I collagen, and vitronectin. The authors also verified that mechanical stretching of the cells improved differentiation [329]. Another *in vitro* study by Mathews et al. [330] showed that type I collagen and laminin were the most successful ECM proteins in inducing the proliferation and adhesion of MSCs, and a high percentage of MSCs differentiation occurred by contact with fibronectin, vitronectin and type I collagen. Combinations of adhesive ECM proteins – fibronectin, laminin, osteocalcin – mixed with methacrylate gelatin hydrogels were suggested by Dolatshahi-Pirouz et al. [331] as a way to study the combinatorial role of ECM proteins and soluble factors in BMMSCs, using a high-throughput strategy; mixtures of proteins in the presence of bone-inducing cytokines, resembling the close-to-native complexity of ECM, led to higher osteogenic differentiation of MSCs. Gothard et al. [332] studied the *in vivo* effect of adding growth factors and osteoinductive soluble molecules to an alginate/demineralized bone ECM hydrogel. All formulations, even the ones excluding growth factors or soluble factors, induced bone formation in rats. The authors hypothesized that this behavior may be related to reminiscent amounts of cytokines in the demineralized bone ECM used to synthetize the hydrogels. The denaturation and fragmentation of the cytokines through UV-irradiation of hydrogels suggested that, indeed, that could be the explanation for the observed general bone formation. Despite the efforts to correlate different biomaterials processed from proteins of the bone or enamel (as it is the other mineralized tissue in the human body) [333], type I collagen and fibronectin remain the two most commonly reported proteins in tissue engineering and osseointegration approaches. Many other proteins integrate bone structure in smaller fractions than type I collagen, but still present important roles in bone physiology.

Table 2 focuses on the description of the role of several bone proteins, namely the ones presented in smaller percentages, and whether they have been used in tissue regeneration therapies.

Despite the undebatable importance of controlling protein composition for the synthesis of bioinspired biomaterials aimed at regulating cell adhesion and function, the immobilization of full proteins onto biomaterials is not strictly necessary to promote integrin-binding events. Recombinant protein fragments and short polypeptides with protein-specific cell binding domains are valid alternatives to achieve cell membrane exposure to those molecules, while enhancing bioactivity and facilitating their incorporation into biomaterials. Aspects that may hamper protein function and efficient binding, including correct protein rearrangement and subsequent exposure of bioactive domains to reach cell membrane, may be surpassed with the use of fragmented proteins peptide domains. The conformation of full-length proteins and their presentation to cells is dictated by factors as biomaterial substrate chemistry. While it is interesting to verify proteins proneness to re-arrange into different configurations in response to substrate chemistry, and although it can be used as a versatile modulator of cell response, it is challenging to predict the behavior of ECM proteins adsorbed into novel or unstudied biomaterials. Keselowsky *et al.* [334] showed that human fibronectin, at a density of 40 ng/cm^2 adsorbed to alkanethiols self-assembled monolayers with highly controlled chemical features, including CH_3, OH, COOH and NH_2 fixed densities, led to distinct cellular recognition of immature osteoblast-like cells (MC3T3-E1 cell line) through integrin binding mechanisms. The binding of soluble integrins to each substrate after fibronectin adsorption was characterized, and major differences were observed: OH and NH_2 substrates led to high amounts of bound $\alpha 5\nu 1$ integrin; COOH surfaces enhanced $\alpha 5\nu 1$ and $\alpha\nu\beta 3$ integrins binding; and CH_3 did not promote the binding of any of the integrins. These phenomena led to distinctive modulation of focal adhesions composition and respective signaling provided by cells from each substrate, resulting in distinctive osteogenic differentiation potencies [335]. Substrates with OH and NH_2 groups led immature osteoblasts to increased gene expression of osteogenic markers including ALP, bone sialoprotein and osteocalcin, and promoted the deposition of higher amounts of mineralized matrix [335]. The use of anti-fibronectin antibodies to impair integrin/cell binding to fibronectin showed that cell response -in particular mineralization-, was indeed tailored by protein configuration in each chemically distinct substrate.

Although it is well accepted that the incorporation of either full proteins or short peptide sequences into biomaterials as integrin binders are promising strategies to modulate cell response aspects that include osteogenesis [336], a tight control over the spatial distribution of integrin-recognizing ECM domains on biomaterials surfaces has proven to be pivotal to tailor a plethora of cellular phenomena like adhesion, proliferation, migration and multilineage differentiation [337]. Tethering/spacing of ECM proteins on biomaterials surfaces was reported to modulate specific cellular aspects with major influence on bone repair, including control of stem cells fate [338] and endothelial cell spreading and proliferation [339].

Integrin clustering is the key aspect in the determination of cellular adhesion strength to materials and modulation of integrin interaction with plasma membranes proteins, which

include talin and vinculin, reported to dictate actin contractility and focal adhesion stabilization [340, 341]. These are key aspects in cellular mechanosensing and response modulation, which are reviewed in Section 6. A recent and complete review addressing the importance of integrin clustering on biomaterials design and cell response modulation can be found in Reference [337]. Interestingly, dramatic differences are known to occur in integrin clustering on 2D substrates and in ECM-resembling 3D materials [342]. In 2D substrates, integrins organize and gather in > 1 μm clusters identified as focal adhesions [343]; in 3D matrices, these structures are often much smaller and show shorter lifetimes [344]. Despite such disparity, 2D biomaterial models have been the most widely used source to understand fundamental aspects of protein/peptides spacing and their effects on cell response. Nonetheless, and despite the difficulty on controlling bioactive domains precise positioning in 3D matrices, a remarkable insight using 2D vs 3D modelling was provided by Lepzelter *et al.* [342] based on Monte Carlo membrane fluctuation simulations [345].

In tissue regeneration and biomaterial design strategies, ECM composition is often discussed based on direct cell-protein interactions phenomena, or on the ability of ECM structure to bind/recruit organic agents able to interact directly with cells (e.g. growth factors). The role of ECM proteins and specific peptide domains in the formation of inorganic deposits, which are crucial for bone formation, is a field of study that has not received as much attention as direct cells-organic structures contact. Organic components of the ECM are recognized as essential for *in vivo* recruitment and deposition of high quality minerals, as well as for their stabilization, orientation and growth, in processes independent from the direct action of cells [439–441]. A recent study reports the ability of decellularized native ECMs, such as the one from the periosteum, on the promotion of the nucleation of calcium phosphates and bone-like crystals [442]. Despite the raising evidence that decellularized ECM can be used to promote biomineralization upon implantation, few recent studies address this aspect. Moreover, systematic studies focusing on the design of highly controlled protein (or other ECM components)-based compositions and their effect on bone-mimetic apatite deposition are still in need. An extension of arrays/biomaterial libraries developed in a wide range of studies focused on cellular response control could be an interesting approach to perform a rapid assessment of the biomineralization-induction potential of different biomaterial compositions. A detailed description of the formation of mineralized components of bone is out of the scope of this Review. A systematic revision of bioinspired mineralization occurring in organic ECM frameworks can be found in a complete Review publication by Benesch *et al.* [439] from 2008, in which the role of individual ECM proteins and peptide domains was systematically described.

6 Bone mechanobiology: the role of externally applied forces and ECM matrix mechanical/viscoelastic properties

6.1 The native mechanical environment of bone

Mechanical and physical signals that occur through walking, running and other types of movements have a crucial role in the induction of osteogenesis, as well as in the maintenance of healthy bone [443]. Interesting observations have been reported during the last years, which correlate the “Mechanostat Theory” with biochemical signaling occurring

during bone homeostasis. The “Mechanostat Theory”, suggested by Harold Frost in the 1890’s [443], correlates bone growth and loss with local elastic deformation (in the form of compression and elongation), which occurs in a life-long regime, due to peak forces exerted by surrounding muscles [444]. Tyrovola and Odont [445] reviewed several studies, in which compressive/tensile deformations were applied to bone and periodontal ligament tissues, and in which a correlation between the observed behavior and the OPG/RANKL/RANK system was established. An example that shows the correlation of the “Mechanostat Theory” and the OPG/RANKL/RANK bone remodeling system is the one occurring in the tooth/periodontal ligament interface. The compression of tooth, during orthodontic movement, led to the increase of RANKL concentration [446, 447], promoting osteoclast formation. The tensile stretching applied to the periodontal ligament promoted the increase of osteoblasts OPG concentration in a magnitude-dependent manner [447], while inducing a simultaneous RANKL concentration decrease it. The relative concentrations of OPG and RANKL on both tensioned and compressed sides of tooth regulate local bone modeling, remodeling and root resorption.

Other physical and mechanical factors that influence bone health are drag force and shear stress. Shear stress occurs in bone on the unmineralized matrix around the osteocytes that forms canals by which the interstitial fluid passes, creating a force along the surface, on a parallel fashion [448]. A consequence of bone deformation is the generation of interstitial flow on osteocytes, creating a drag phenomenon on the fibers that connect cells [449–452]. Healthy bone remodels in response to mechanical stresses: in the absence of loading, bone resorption is increased, while in the presence of flow perfusion through the movement of extracellular fluid radially toward the bone cortex [453] bone is known to remodel. Subsequent studies focusing on the role of perfusion on primary bone cells and stem cells behavior have shown increased mineralized matrix deposition in a dose-dependent manner [448, 453]. Weinbaum *et al.* [450] suggested a mathematical model to explain how bone cells detect mechanical loading, and how flow behaves through the pericellular matrix surrounding an osteocyte process in its canaliculus. Despite the small deformations predicted by the model, and the small dimensions of the pericellular annulus (typically 0.1 μm), the flow shear stress on the membranes of the osteocyte processes was roughly the same as for the vascular endothelium in capillaries. Still little is known about the role of perfusion shear stress and interstitial flow in bone biology [451, 454]. However, it is known that osteocytes are the main mechanosensing cells in bone and that, upon exposure to fluid flow, they stimulate osteoblasts, thus producing more bone tissue and prostaglandins, which are responsible for the activity of osteoblasts and osteoclasts [171]. Early studies focused on unravelling the effect of hydrostatic pressure and substrate stretching on osteocytes behavior [455]. However, flow-induced shear stress has shown to affect osteocytes in a more relevant manner, as compared to osteoblasts [156]. An extensive list of biological phenomena, that has been increasing in the last years, has shown that osteocytes respond to shear stress by releasing nitric oxide (NO), adenosine triphosphate (ATP) and prostaglandins. Moreover, gap junctions and hemichannels are open, and several signaling pathways (e.g. Wnt/β-catenin, protein kinase A (PKA)) are initiated after shear stress induction. The mechanisms for load sensing in osteocytes are thought to depend on the dendritic process, or bending of cilia [171]. Glycocalyces on the surfaces of dendritic processes have been shown to be

related with osteocytes mechanosensing; however, on the cell body, different mechanosensing mechanism are known to be active [171]. The TGF-β superfamily - which includes BMPs, activins, and growth differentiation factors (GDFs) - has been suggested as one of the most important mediators of cellular response to physical cues via a feedback loop mechanism, reviewed by Wu *et al.* [44].

The role of paralogous transcriptional factors Yes-associated protein (YAP) and PDZ-binding motif (TAZ) in osteogenesis are described since 2004 and 2005, respectively. Overtime, contradictory roles of each factor were reported regarding osteogenic differentiation of stem cells. Recently, their role as combinatorial promoters of bone development was reported [456–458]. Upon deletion of YAP/TAZ from skeletal lineage cells, osteogenesis-imperfecta like-phenotypes were generated, and bone properties were reduced through lower collagen content and organization. Homozygous TAZ deletion led to spontaneous fractures on mice, while dual (YAP/TAZ) deletion caused neonatal lethality. Dual deletion led reduced osteoblast activity and increased osteoclast activity, negatively affecting bone synthesis and remodeling.

YAP/TAZ is known to play a crucial role in mechanotransduction phenomena, working as sensors for mechanical cues, with relevant application on growth factors-free approaches for mesenchymal stem cells differentiation, mainly driven through the modulation of ECM (or biomaterials) rigidity. Their role as nuclear relays of mechanical signaling exerted by ECM stiffness and induced cell shape has been associated with Rho GTPase acitivity and tension of the actomyosin of the cytoskeleton [459]. Besides the direct effect of matrix stiffness on cellular mechanosensing via YAP/TAZ, MSCs were shown to be mechanically regulated by shear stress applied to tissues through shear flow; the application of shear stress to MSCs showed improved osteogenic differentiation in a Rho-ROCK dependent manner. [460–462] Important aspects as bone loss related to microgravity conditions experienced during space flights has also been correlated with poor TAZ nuclear accumulation, leading to low osteogenesis [463].

In 2016, two zinc finger repressors – Snail and Slug -, best known for their participation in epithelial-to-mesenchymal transition mechanisms, were described to perform binding interactions with YAP/TAZ, forming complexes, activating YAP/TAZ/TEAS and *Runx2* downstream targets that control stem cell osteogenesis [464]. Knockout mouse models targeting Snail, Slug or both combined allowed unraveling a biological mechanism in which both transcription factors cooperatively control stem cell self-renewal, osteogenic differentiation and bone formation [465].

6.2 Modulation of regenerative systems: engineering mechanosensing targeting osteogenic differentiation and bone growth

Biomaterials with different mechanical properties have triggered the differentiation of MSCs into different lineages (Figure 3). The directing effect of microenvironments' mechanical features on stem cell differentiation were first reported by Engler *et al.* [466]. Collagen-coated 2D polyacrylamide gels impacted on the fate determination of BMMSCs precisely accordingly to their stiffness. Gels with elastic modulus similar to native adult or developmental tissues directed cells into the neurogenic, myogenic or osteogenic lineages, in

a nonmuscle myosin II-dependent pathway. Type I collagen and collagen-coated 2D gels were also applied to study the adipogenic, chondrogenic and smooth cell differentiation of BMMSCs [467], as well as osteogenic differentiation [468]. MSCs retrieved from alternative sources including adipogenic, cardiac and mammary tissue responded to stiffness cues of flat substrates, altering their phenotype to adipocytes, endothelial cells, and epithelial cells, respectively [469–471]. ESCs have also been differentiated into the pancreatic, mesodermal and osteogenic lineages using hyaluronic acid and type I collagen substrates through single mechanotransduction mechanisms [472–474]. Aspects beyond substrates' modulus, including their presentation to cells and spatial organization were addressed by Yang *et al.* [475], who designed biomaterials with stiff and soft regions, presented in ordered or random designs. BMMSCs showed higher adhesion and spreading on substrates with higher percentages of stiff regions, in a dose-dependent manner. However, when stiff regions were distributed on the surface on a randomized manner, cells showed lower levels of YAP activation, and their phenotype changed to smaller and rounded. The apparent actin disruption caused by random patterns led to lower ALP expression and higher CD105 (stemness marker) expression.

An extremely limited number of physiological phenomena occur in strictly 2D processes, which include, for example, the deposition of osteoblasts on osteoid matrix. However, the emulation of regeneration processes in most human tissues requires the replication of a 3D environment to induce cues presented by native ECM. The establishment of reliable correlations between distinctive mechanical properties of hydrogels and consequent differentiation lineages of cells encapsulated in the 3D environment is a demanding task. Stiffness variation in hydrogels are frequently obtained by varying the extension of crosslinking mechanisms, which often alters physical and chemical aspects of hydrogels that may include (i) exposure to chemically (un)reacted groups; (ii) availability of cell adhesion motifs to surface membrane integrins; (iii) porosity; (iv) pore size; (v) water content; and (vi) viscoelasticity [457–461]. While surface chemistry of hydrogels studied as 2D substrates for stem cell culture could be easily homogenized using, most commonly, type I collagen coatings on the cell-exposed part of the hydrogel [481, 482], the achievement of fully comparable 3D systems that unequivocally allow to isolate the "stiffness" variable are challenging to prepare. Wen *et al.* [480] partially addressed this question by modulating several aspects of hydrogel design hypothesized to function as a source of bias to 2D collagen-coated polyacrylamide systems. Without any alteration of stiffness values, hydrogels with varying porosity did not alter protein tethering on the biomaterials' surface, and both ASCs and BMMSCs fate onto the adipogenic and osteogenic lineages, which remained exclusively dependent on the substrates' elastic modulus. A comprehensive list of 2D and 3D approaches used to study stem cells morphological re-organization and differentiations induced by mechanical properties can be found in Reference [483].

The encapsulation of BMMSCs in alginate hydrogels modified with the integrin-binding peptide RGD showed that hydrogels with elastic modulus ranging from 2.5 to 5 kPa led cells preferentially into the adipogenic lineage, while stiffer hydrogels (11 – 30 kPa) induced osteogenesis [477]. ASCs encapsulated in a bacterial origin polymer – gellan gum (modified with methacrylic groups) – also underwent soluble factors-free osteogenic induction by simple encapsulation in ~50 kPa hydrogels [484]. Interestingly, in both studies the

observation of the osteogenic phenotype was not dependent on cell spreading onto the hydrogels [477, 484]. In contrast to primordial studies in 2D substrates, that reported a correlation between cell fate and morphology, in 3D structures cells are able to go through osteogenesis through integrin binding and nanometric rearrangement of adhesion ligands [477]. Other studies targeting stem cells osteogenesis on 3D biomaterials focused on the maintenance of microstructure with variation of mechanical properties through the incorporation of mineralized structures on the surface of 3D porous scaffolds [485], and on the supplementation of fibrin gels with sodium chloride to enhance hydrogels' mechanical properties and drive osteogenic response [486]. The osteogenic response on poly(ethylene glycol) hydrogels modified with the RGD peptide domain was proved to be integrin dependent [487]. Interestingly, and in contrast with data reported for 2D biomaterials-driven osteogenesis via mechanotransduction pathways, MSCs differentiation did not involve actin filaments and microtubules associated with myosin contractility, neither ROCK activity [487], suggesting that in specific 3D biomaterials formulations osteogenesis mediation may be regulated by different pathways than the ones observed in 2D cultures.

Mechanical transduction targeting bone regeneration has not been limited to materials stiffness. Indeed, the use of 2D-to-3D biomaterials with nanopattern cues has proven to be efficient on driving osteogenesis of stem cells and osteoprogenitor cells in the absence of any soluble cues or substrate chemistry [488–497]. This response is often associated to cytoskeleton organization and generation of large focal adhesions, which has been correlated with possible direct mechanotransduction pathways [498]. A thorough Systematic Review focusing on the osteogenic potential of several nanotopographies, with focus on aspects as size, shape, anisotropy/level of organization of the patterns [499] allowed retrieving some interesting information about osteogenesis-inductive topographic cues: nanopillar features with heights lower than 20 nm and disordered nanopits seem to systematically increase osteogenic differentiation of stem cells. Nonetheless, a careful analysis and studies correlating results obtained with the same materials is necessary to harness the field.

Most studies on mechanotransduction targeting stem cells differentiation focus on the single property of hydrogels' elastic modulus. Often, purely elastic materials are used as substrate, or the viscoelasticity of the matrixes is not considered or discussed. However, an important aspect of physiological ECMs is their viscoelasticity [500], which leads to stress relaxation that stimulate ECM remodeling through cellular forces. In 2011, Cameron *et al.* [501] addressed the effect of biomaterials' creep behavior on MSCs morphology, proliferation and differentiation fate. 2D polyacrylamide materials with constant ~4.7 kPa storage modulus (elastic component) and increasing loss modulus (viscous component) led increased MSCs spread area and proliferation but drove a decrease on focal adhesions size and maturity, which was hypothesized to be related to a creep-mediated loss in cell cytoskeletal tension. ALP activity on MSCs seeded onto higher loss modulus gels was increased, suggesting that the modulation of this factor can trigger osteogenesis, even in soft hydrogels. Chaudhuri *et al.* [502] investigated the role of stress relaxation on cell spreading on substrates with equal elastic modulus. Fibroblast and osteosarcoma cell lines responded to substrates' relaxation properties by spreading onto soft materials with high stress relaxation, on a similar manner to cells cultured on stiff materials. Later on, the same team investigated the role of these properties on MSCs differentiation on 3D hydrogels [503]. Alginate hydrogels modified

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with RGD domains were tuned to show equal elastic modulus, adhesive peptide modification (in two different levels) and degradability, while altering their relaxation times from 70 to 3300 seconds. MSCs adipogenic and osteogenic differentiation were modulated on combined effects of matrix elastic modulus and stress relaxation properties, with 17 kPa hydrogels with fast relaxation properties providing the most advantageous condition for osteogenesis.

The possibility of modulating ECM-cells interactions and consequent cell response through the local modulation of mechanical cues administered to cells is a powerful tool to design highly effective biomaterials independent of recombinant costly growth factors or drugs with possible side effects. While the effect of native ECM and ECM-mimetic biomaterials is well established in the literature, other overlooked aspects of mechanics are now reaching the realms of “biomechanics” and “biophysics”. Stress relaxation is currently under deep scrutinization as a modulator of stem cells differentiation, and aspects as 4D/spatiotemporal modulation of biomaterials properties are now addressed as enablers of ECM remodeling mechanisms [504, 505]. The design of materials with on-demand changing compositions is poorly described. However, their potential as sequential modulators of cell behavior open the possibility of establishing extremely complex cell response patterns and co-cultures with potential high impact in the achievement of biocompatible, bioinstructive and nature-mimetic regenerative systems.

7 Conclusion and future directions

The field of bone regeneration has benefited from the progressive elucidation of anatomical and physiological aspects of native human tissue. Natural occurring bone precursors and also adult tissues’ morphology and composition have inspired therapies targeting bone damage. Some of the most interesting and promising approaches have allowed emulating bone development phenomena by the control of biochemical signaling through the mimicking of endochondral ossification pathway, or by giving biophysical cues to stem cells that resemble the mechanical properties of the unmineralized precursor of bone (the osteoid).

Holistic approaches combining multiscale features and properties have demonstrated high potential for the design of new biomaterials and scaffolds based on bone’s anatomy [506]. Top-down and bottom-up strategies have been integrated, enabling an increase in the complexity of designed materials, and allowing a full-scale control of the scaffolds’ properties. Independently of the strategies used to manufacture implantable biomaterials, well-accepted crucial features must be considered, namely the adequacy of their mechanical properties to the site of implantation, adequate triggering of immune response, and the type and spatial-temporal distribution of constructs’ properties. A strict control over these aspects is a powerful tool to modulate cell fate, guiding cellular behavior and response *in vitro* and *in vivo* (e.g. cell adhesion, proliferation, viability, differentiation, matrix production).

Recently, attention has been directed to other aspects of bone biology and their understanding as a source of valid know-how to design novel regenerative approaches. In particular, control over the insoluble fraction of bone - comprising ECM proteins and glycoproteins, as well as cellular structures, and even immobilized growth factors aimed at

interacting with cellular membranes – has been identified as a strategic way to direct stem cell fate and promote bone regeneration. In particular, and despite their ubiquitous presence in human ECMs, studies targeting the use of glycans in biomaterials as tools to modulate cell behavior are still rare [507].

Although several co-cultures between resident bone cells and stem cells have played an important role on fundamental studies, their application on biomaterials models as *in situ* cell delivery systems to promote bone repair is still scarce. Tailoring of inflammation and regeneration-associated aspects are also under rapid exploitation, mainly using macrophages polarization techniques. Nonetheless, there is a need for the elucidation of such co-cultures and for the design of simple yet effective ways to apply these concepts into the clinics. The role of ECM proteins and their interaction with soluble factors present in the bone injury environment are another branch of biomaterials design that we here identify as still underdeveloped. The identification of the principal components affecting bone regeneration may allow processing cost-effective biomaterials with ECM-mimetic features using advanced biotechnological approaches, independent on the use of costly recombinant technologies.

Certainly, the wider understanding, characterization and application of soluble factors (e.g. growth factors) and nanostructures structures containing soluble cues (e.g. EVs) produced by cells themselves are strong trends that show a high impact as versatile tools to direct cell differentiation. In particular for the case of EVs, their application as off-the-shelf naturally produced nanoparticles integrated into biomaterial matrices may represent a valuable tool in future regeneration techniques.

Externally applied stimulation happens in bone in individuals' everyday life and are known to be crucial for bone remodeling. Recently, normal fetal movement has been highlighted as crucial for the development of high quality newborns' skeletal growth [508], and mechanical forces in utero were suggested as possibly impactful on the long-term. While some works have focused on the use of bioreactors to mimic compression/elongation and interstitial flow that occur in native bone, with improved outcomes concerning MSCs differentiation into the osteogenic lineage [509, 510] (Figure 4), there is still a long way to pave in the emulation of all relevant mechanical physiological stimulation. A recent study focused on the combinatorial assessment of 3D biomaterial matrices stimulation along with different compression pressures [511]. More studies enabling the unraveling of ECM protein composition, cellular co-cultures setups and physiologically-occurring mechanical stimulation are needed to lift this field from a mostly unifactorial trend to a multifactorial platform (Figure 5). High-throughput screening technologies adapted specifically to tissue regeneration needs, along with the creation of directed and effective high-content analysis methods are promising routes to unravel compositional, interactional and external factors-associated with bone biology. A complete emulation of bone structure and function will be a costly, time consuming and difficult road. On its turn, the identification of key factors leading desirable cell response and implant integration in the wounded tissues would allow the "just-enough" complex design of effective tissue regeneration strategies, enabling the easier selection of components to fit the marketplace using safe, easily regulated and cost-effective strategies.

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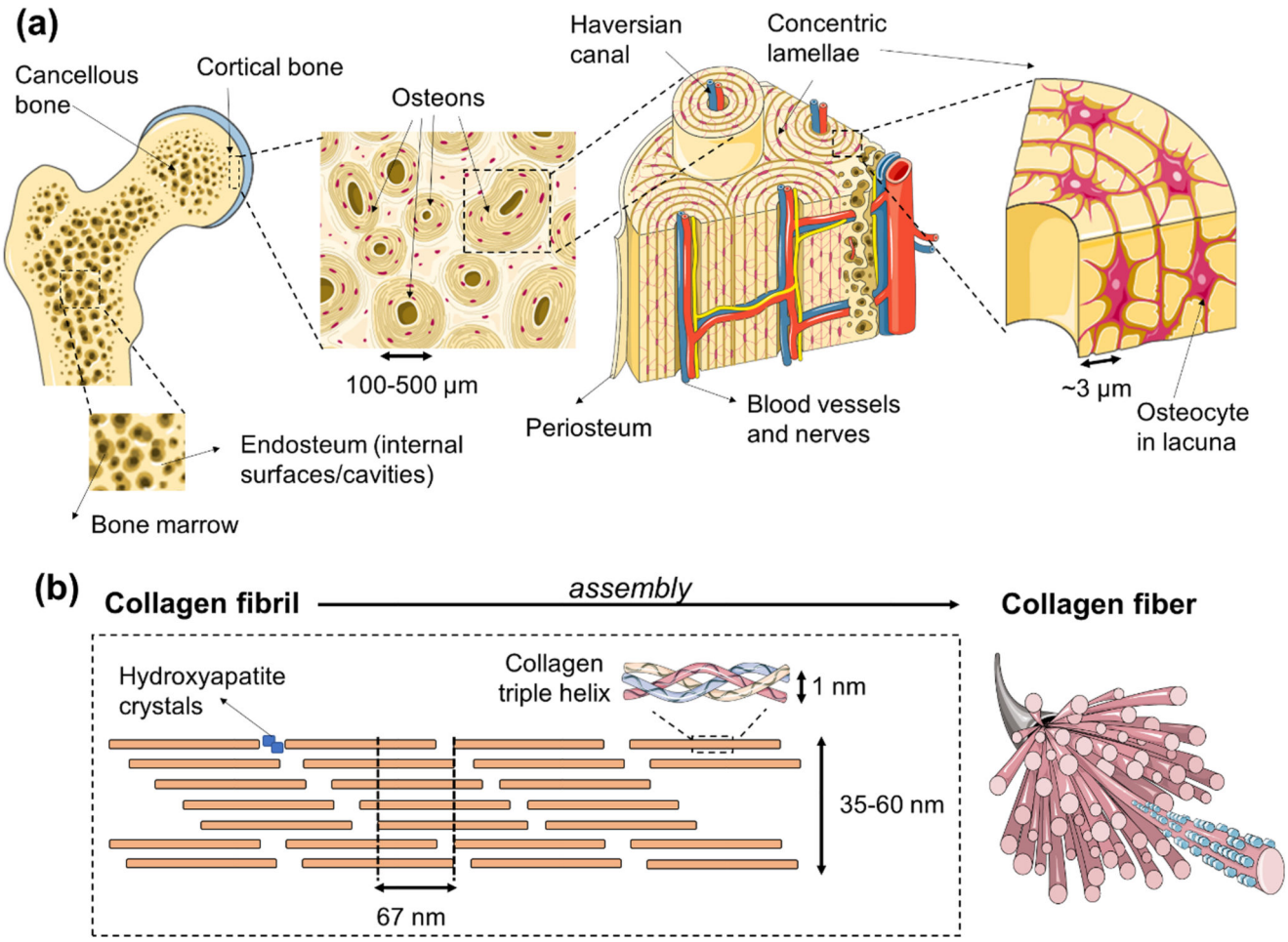

Figure 1.

Interscale representation of bone. **(a)** A macroscopic-to-microscopic view of cancellous and cortical bone. Bone marrow lies in the cavities of cancellous bone, which are lined by the endosteum structure. Tightly packed osteons integrate cortical tissue, which is covered by the periosteum membrane. Osteons are formed by Harvasian canals, which contain blood vessels and nerve tissue, surrounded by concentric lamellae that show thicknesses of circa 3 μm. Osteocytes reside in the osteon inside lacuna structures. **(b)** Bone tissue is constituted at the nanometric scale by collagen fibers that comprise assembled collagen triple helix structures that give rise to the collagen fibril, with a characteristic periodic spacing of 67 nm, and gaps of 40 nm where the mineral component of bone is located.

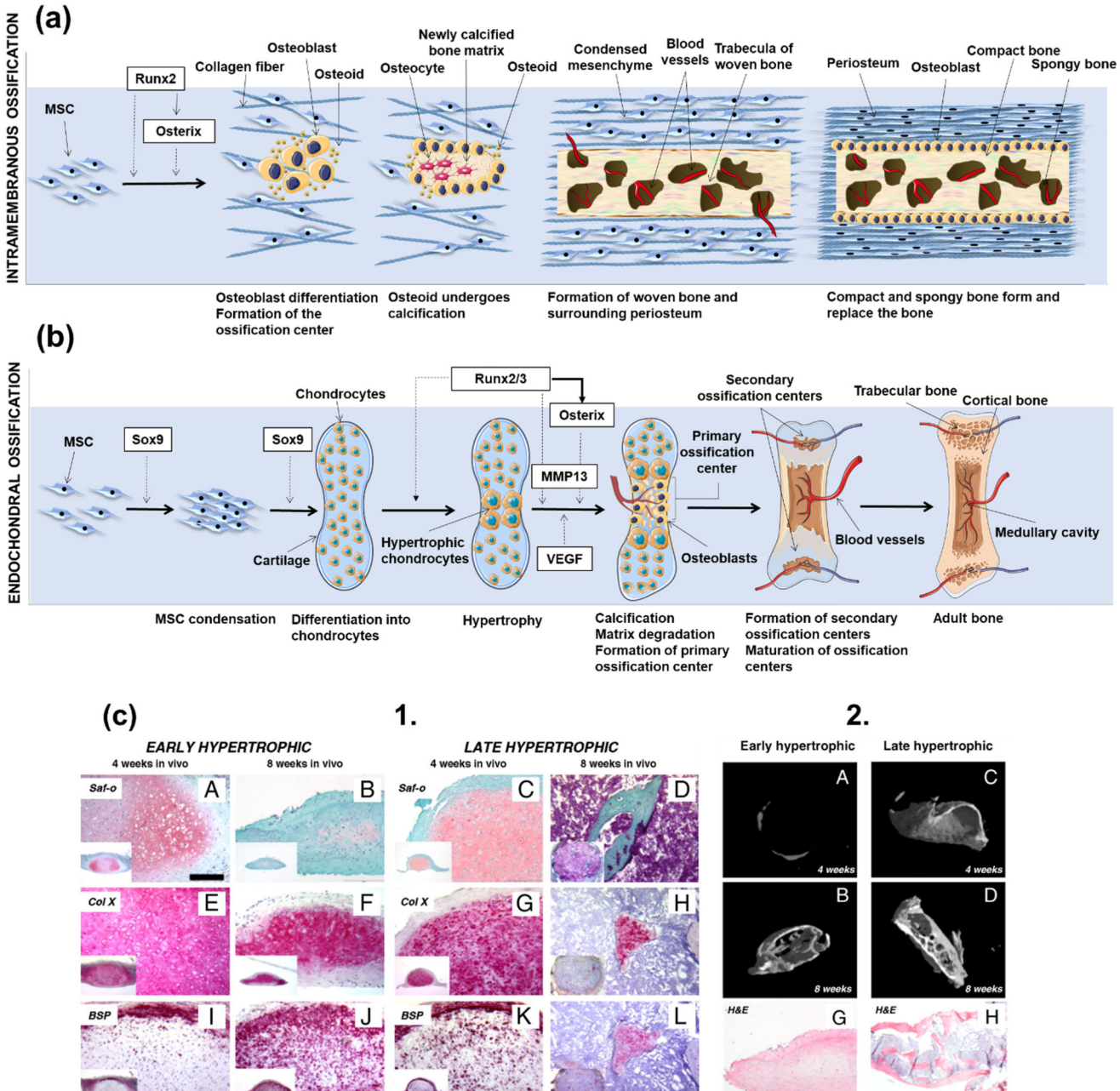

Figure 2.

(a) Schematic representation of intramembranous ossification. At an initial stage, MSCs cluster and differentiate into osteoblasts, forming the ossification center. Runx2 is deeply involved in the regulation of osteogenic differentiation, either directly or by inducing the late expression of Osterix. Osteoblasts start to produce the osteoid, which calcifies in few days. Osteoblasts trapped into the calcified matrix differentiate into osteocytes. Vascularized mesenchyme condenses on the external area of the woven bone, generating the periosteum. The woven bone is produced, with vascularized internal spaces that will form the marrow

cavity. The surface of trabeculae is filled with matrix forming the compact bone. Spongy bone persists at the inner part. **(b)** Schematic representation of endochondral ossification. After condensation, MSCs starts to differentiate into chondrocytes, generating a cartilage template. Chondrocytes in the middle of the cartilage become hypertrophic. Sox9 and Runx2/3 are indispensable transcription factors for the initiation of chondrogenesis and the hypertrophy of chondrocytes, respectively. Hypertrophic chondrocytes induce vascular invasion. At this stage, Osterix functions as both a downstream and transcriptional partner of Runx2/3 during calcification and matrix degradation in cartilage, and cooperate with Runx2/3 to induce MMP13 expression. Osteoblasts differentiate from cells brought into the cartilage template with blood vessels invasion, starting to produce bone at a primary ossification center. Bone formation then spreads along the shaft forming secondary ossification centers. Finally, the adult bone, containing both trabecular and cortical bones and the medullary cavity is formed. **(c) 1.** Scotti et al. [74] induced endochondral bone formation in vitro using human MSCs. Hypertrophic tissue structures were implanted into nude mice to assess their ability to form trabecula bone. Both early (A-J) and later (C-L) hypertrophic samples went towards differentiation after in vivo implantation, although the latter specimen presented a more intense remodeling after 4 weeks (K), with the cartilaginous template almost resorbed after 8 weeks (L). **(c) 2.** Quantitative microtomography (μCT) of explants demonstrated that the deposition of mineralized matrix at the early hypertrophic samples (A-B) was reduced when compared to the late hypertrophic implanted structures (C-D). In fact, late hypertrophic samples displayed an interconnected network of trabeculae throughout the core after 8 weeks of implantation (D) [81]. Histological analysis by hematoxylin/eosin staining (G-H) revealed the presence of trabecular-like structures in the outer collar and inner core of the late, but not of the early, hypertrophic samples [81]. Figure 2(b) was adapted from Reference [81].

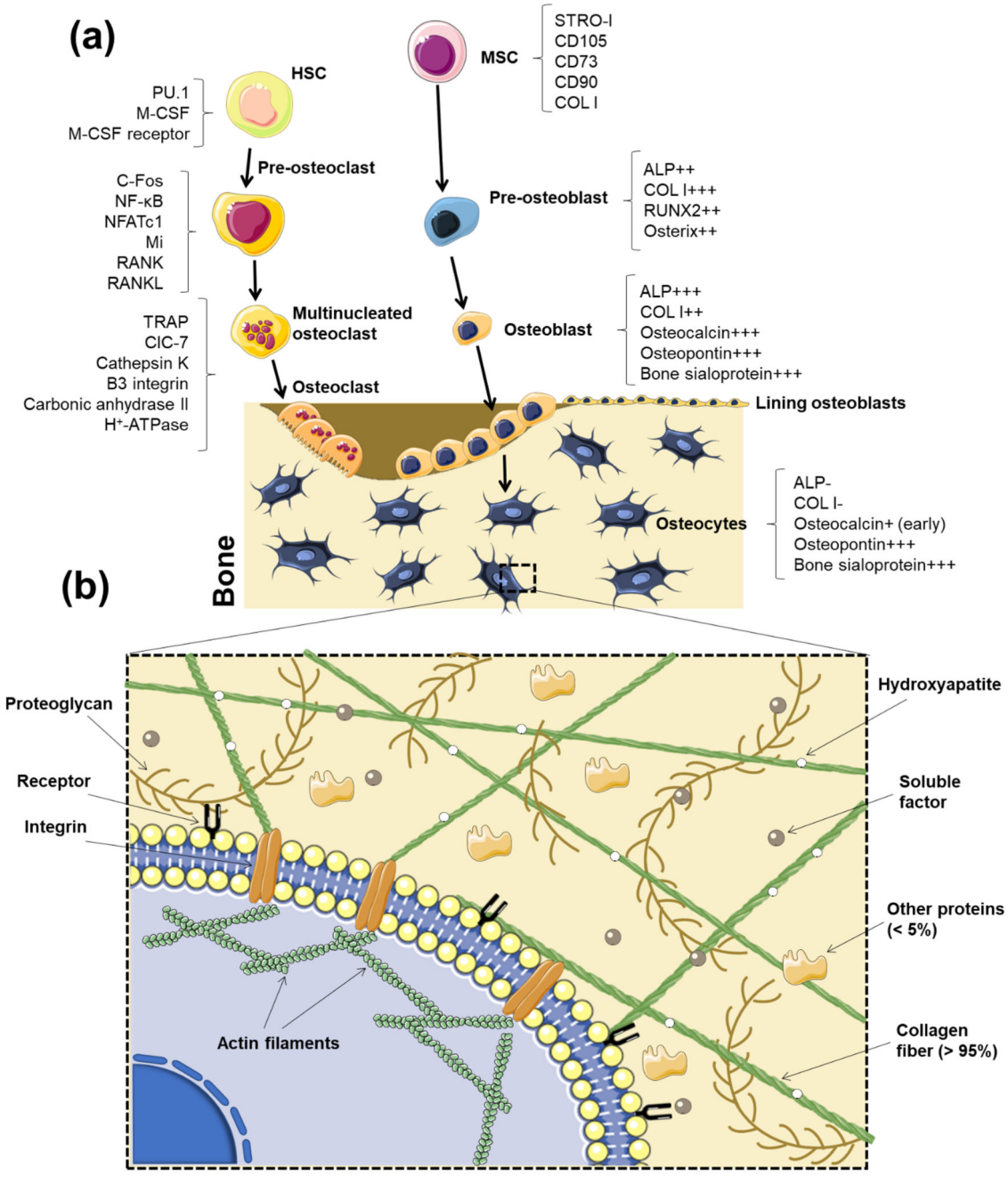

Figure 3.

(a) Schematic representation of the bone cell differentiation process, generating from mesenchymal and hematopoietic stem cells. Osteoblasts descend from MSCs, which firstly differentiate into pre-osteoblasts. Osteoblasts proliferate and align in the surface of the bone while others undergo maturation into the osteocyte phenotype. HSCs differentiate into pre-osteoclasts, which become multinucleated, and finally originate mature osteoclasts responsible for bone resorption. Factors produced or expressed by different cells present in the bone niche are presented aside each cell type schematic representation. **(b)** A variety of

factors constitute the bone extracellular environment. Biological, physical and topographical features compose a specific microenvironment capable of guiding cells into predetermined phenotypes and function. Cells interact with ECM through receptors and other proteins localized at the surface.

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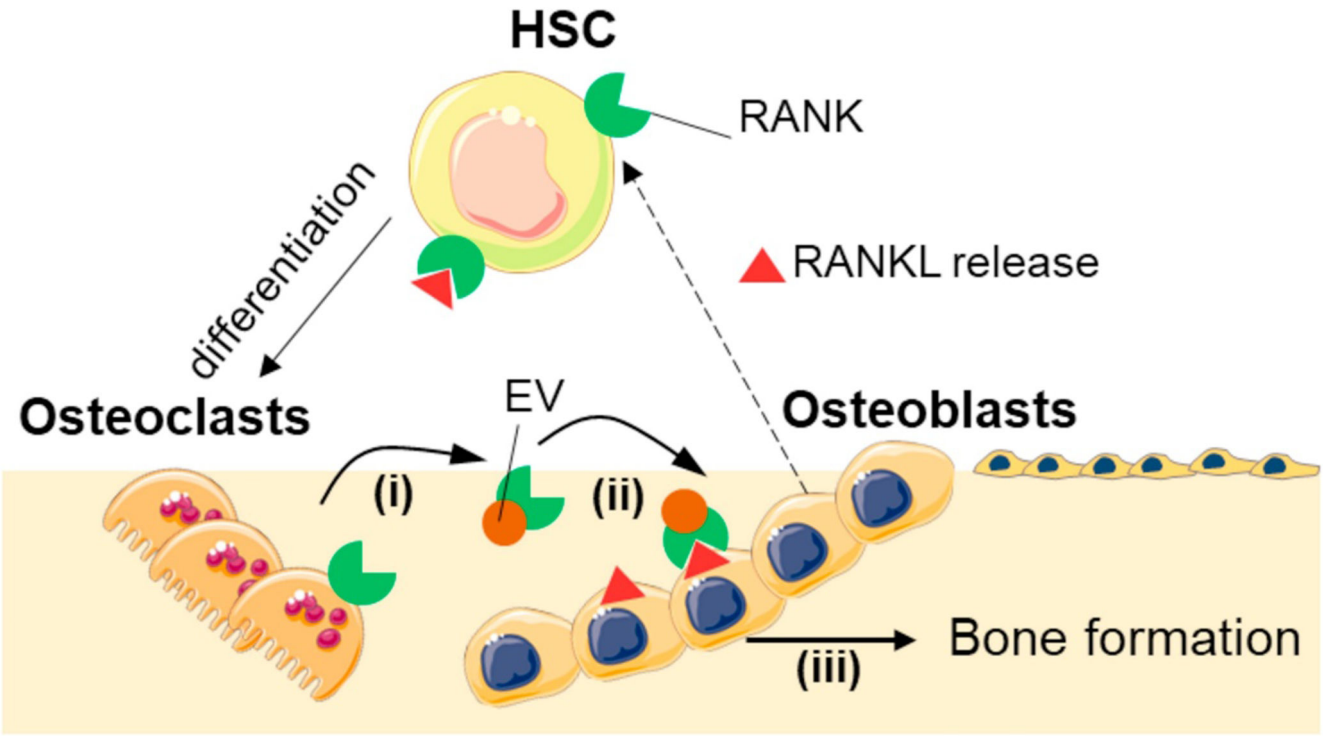

Figure 4.

Representation of reciprocal interactions between osteoblasts and osteocytes. Osteoblasts (and osteocytes) release RANKL, which binds to hematopoietic stem cells, giving rise to their differentiation into osteoclasts. Ikebuchi et al. [170] proved that osteoclasts are capable of modulating osteoblasts' ability to form new bone through the release of extracellular vesicles (EVs) that contain RANK on their surface (i). The vesicles migrate to osteoblasts' surface (ii) leading to the binding of vesicular RANK to RANKL present at osteoblasts' surface, and directing osteoblast to form new bone (iii). The image was adapted from an original scheme by Zaidi et al. [515].

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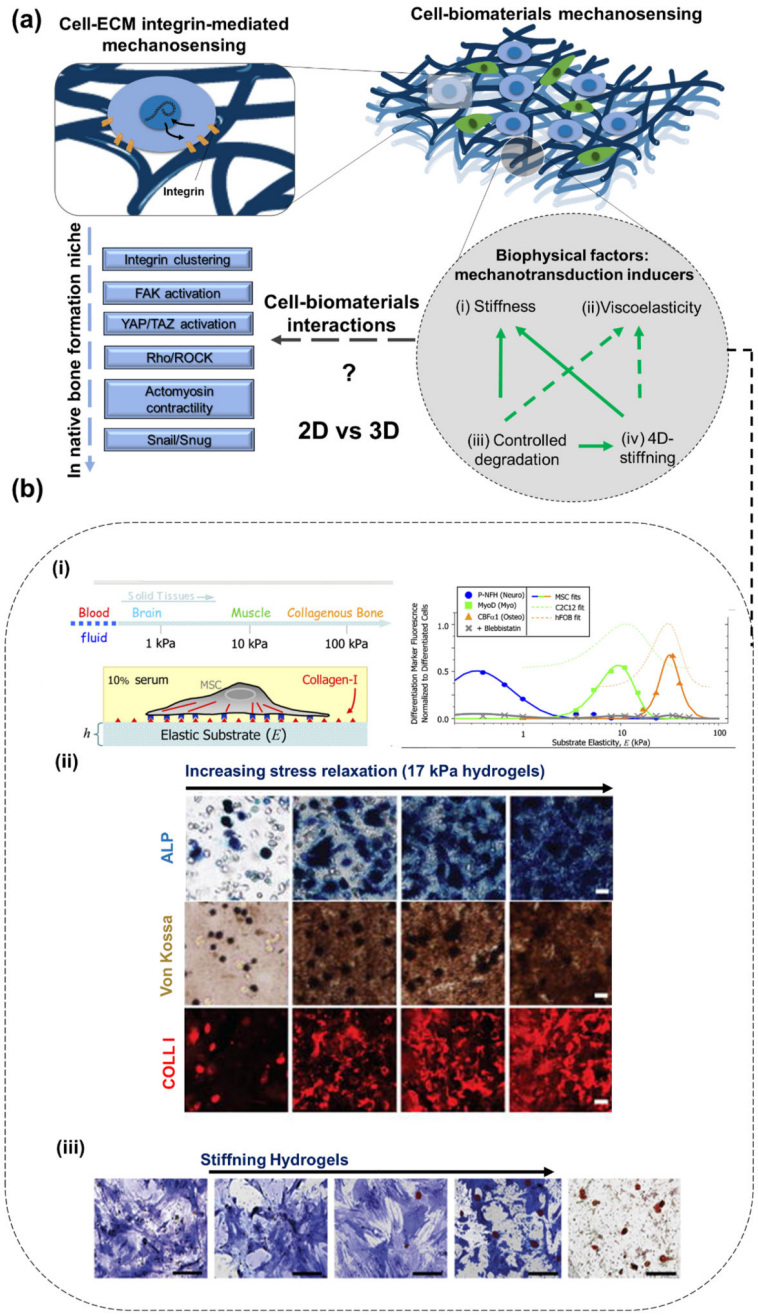

Figure 5.

(a) Bone mechanical microenvironment is mediated by integrin-mediated binding of bone cells to the ECM. Several pathways are described in the bone healing and homeostasis process, which include integrin clustering in the presence of stiff substrates, leading the activation of focal adhesion kinases (FAKs), which later drives the activation of the YAP/TAZ pathway. Focal adhesions also activate the RHO GTPases, which favor F-actin polymerization through the activation of RHOassociated protein kinase (ROCK) [458]. The Snail/Slug pathway is also known to occur during bone formation [464]. On biomaterials,

most of these pathways (with exception to Snail/Slug) have been reported to occur in, for example, MSCs culture on 2D substrates. However, stem cells osteogenic differentiation on specific 3D setups were independent from these well-known mechanisms [487]. So far, the mechanisms driving osteogenic differentiation in 3D matrices in vitro require further exploitation towards full understanding. Interestingly, not only stiffness has been addressed as a modulator of cell response towards the osteogenic commitment. Other properties including viscoelasticity (e.g. stress relaxation) and 4D spatiotemporal degradation or stiffening have been suggested as modulators of stem cells commitment [512]. The variation of physical aspects may impact the measured properties of biomaterials overtime. In the grey circle, continuous green arrow indicates the direct impact of the variation of one factor on other overtime; discontinuous green arrows show properties that will probably influence others. **(b)** In 2006, the ability of 2D hydrogels' stiffness to solely direct BMMSCs multilineage differentiation was proven for the first time [466] - example (i); stress relaxation on biomaterials with constant elastic modulus is another factor capable of directing higher production of osteogenesis-related markers by MSCs, including ALP, type I collagen and phosphate deposition (stained by von Kossa) [503] - example (ii); hydrogels with varying properties overtime showed the ability to increase the production of ALP by MSCs - example (iii) [513]. Figure 4(b) was adapted from References [466, 503, 513].

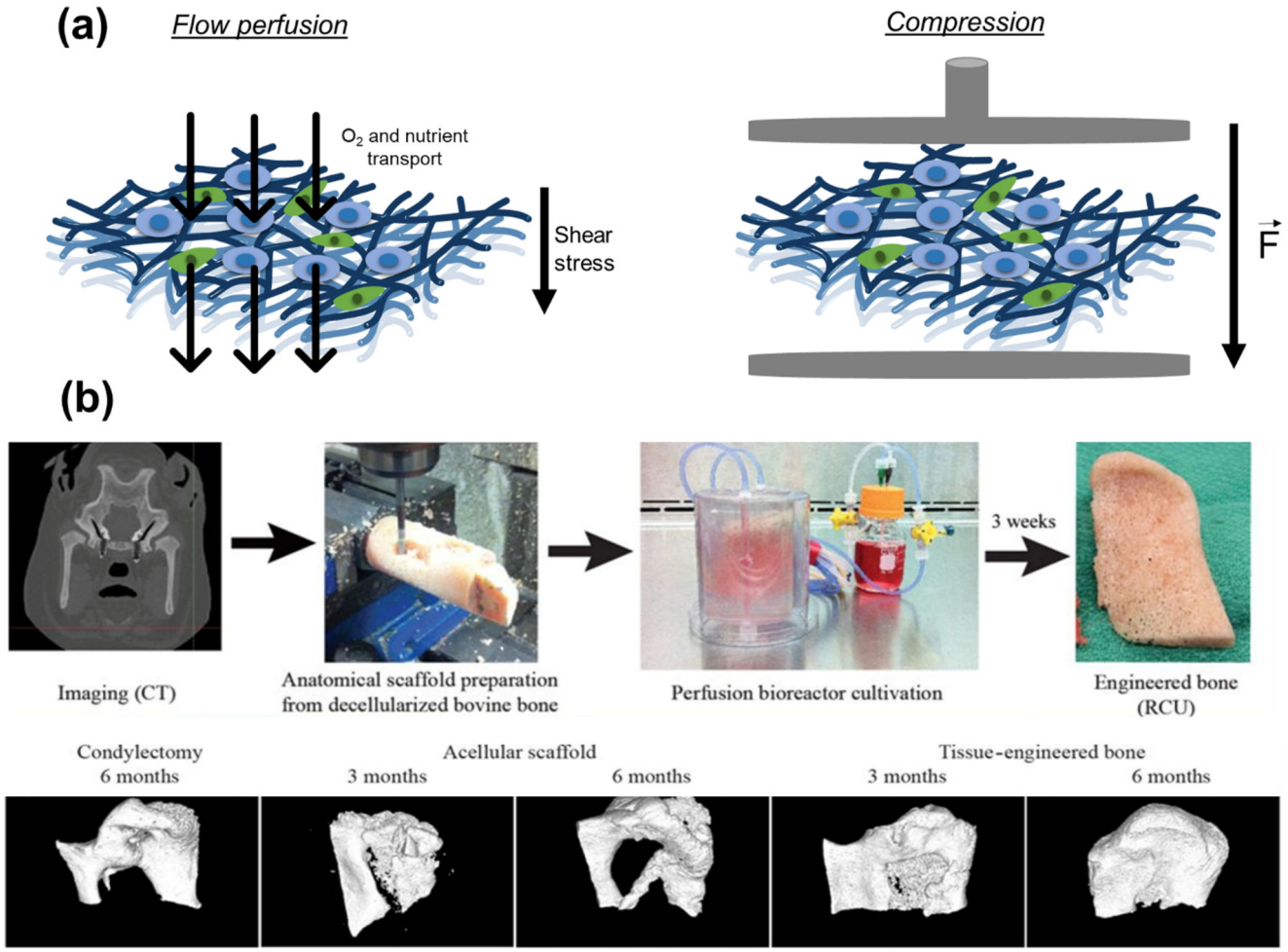

Figure 6.

(a) Engineered devices, combining biomaterials and external stimulus allow mimicking the in vivo-occurring stimuli. Different types of bioreactors allow stimulating cells in distinct manners by mimicking the fluid shear stress through a perfusion flow method and the strain caused by compression. Figure 5(a) was produced using Servier Medical Art. **(b)** Perfusion flow was successfully applied for the re-cellularization of 3D scaffolds targeting facial bone reconstruction in a porcine model [514]. Indeed, such perfusion flow has proven to be effective on the homogeneous (re)population of large biomaterial and/or decellularized ECMs structures with cells of interest. The acquisition of bone defect morphology and dimensions was performed by microcomputerized tomography (μCT). Decellularized bovine bone was machined to present the exact shape of the defect, and later filled with autologous porcine ASCs, which were cultured in the 3D scaffold under perfusion flow, and later implanted in the bone defect, rendering full wound regeneration. Figure 5(b) is adapted from Reference [514].

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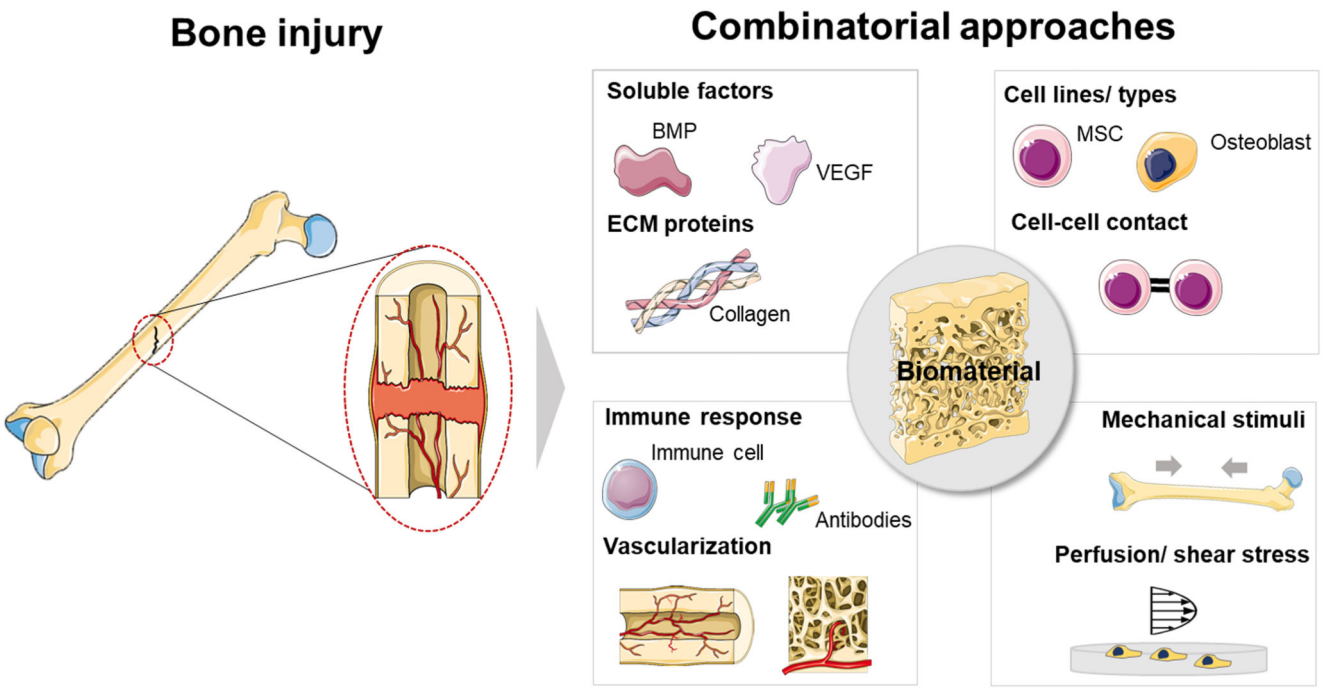

Figure 7.

The treatment of bone injuries may benefit from the deconstruction of the native tissue niche and on the application of concepts learnt from basic physiology to the design of efficient regenerative therapies. Although simplistic approaches based on the variation of single factors may be easier to regulate and produce with high fidelity as industrialized systems, bone's intricate multicellular healing and homeostasis processes – characterized by fine immunological spatiotemporal coordination and unique vascular and mechanical environment – suggest that the combination of specific transversal aspects of bone physiology may hide the cue for more effective, rapid and high-quality bone formation.

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Table 1

Cell-cell interactions applied in tissue engineering strategies.

Type of cell interaction	Tissue engineering approach	Main achievements	References
MSCs-(pre)osteoblasts	• Co-culture of pre-osteoblastic cells and MSCs on a perfusion cell mixer microbioreactor	• Pre-osteoblastic cells guided MSCs towards osteogenic differentiation. Development and validation of a perfusion cell mixer microbioreactor array for the establishment of cell co-culture models in a high-throughput manner	• Occhetta *et al.* [239]
	• Tri-culture of MSCs, osteoblasts and adipocytes in poly(ethylene glycol)-based hydrogels	• Development of a photopatternable 3D culture system that enabled observation of distinct gene expression dynamics resulting from dynamic paracrine interactions between MSCs, osteoblasts, and adipocytes	• Hammoudi *et al.* [194]
MSCs-vascular cells	• Development of injectable and porous nano calcium sulfate/alginate with BMP-2 gene-modified MSCs and endothelial progenitor cells	• BMP-2 gene -modified MSCs and endothelial progenitor cells in nano calcium sulfate/alginate scaffolds increased the new bone and vascular formation	• He *et al.* [197]
	• HUVECs culture with human ASCs inside permeable hollow capsules containing adhesive microparticles. Subcutaneous implantation in nude mice and assessment for osteogenic proteins and mineralization	• Osteogenic constructs containing HUVECs led to a faster and robust formation of mineralized tissue. The establishment of the co-culture, without pre-incubation *in vitro*, was enough to promote ectopic formation of a mineralized structure.	• Correia *et al.* [240]
	• 3D printed polycaprolactone/hydroxyapatite scaffold coated with hydrogel containing HUVECs and ASCs	• Prevascularized 3D printed scaffold for the healing of large bone defects	• Kuss *et al.* [241]
	• Co-culture of hiPSC-MSCs and HUVECs on a calcium phosphate cement scaffold	• HUVECs co-cultured with hiPSC-MSCs promoted bone regeneration. The construct has potential to enhance bone and vascular regeneration in orthopedic applications	• Liu *et al.* [242]
	• Co-culture of HUVECs and hBMMSCs as feeder cells on a bio-derived bone as scaffold	• Interaction between hBMMSCs and HUVECs provided support for the long-term *in vitro* culture of bone hematopoietic stem/progenitor cells in the bone scaffold	• Huang *et al.* [243]
Osteoblasts-osteocytes	• Culture of osteoblasts and osteocytes on a 3D *in vitro* co-culture system	• Development/validation of an osteocyte-osteoblast co-culture model for the study of mechanically induced osteocyte control of osteoblast bone formation	• Vazquez *et al.* [159]
Osteoblasts-osteoclasts	• Rotational co-culture of human primary osteoblasts and osteoclasts precursor cells	• 3D self-assembled mineralized tissue constructs for *in vitro* study of bone-related stimuli	• Clarke *et al.* [189]

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Type of cell interaction	Tissue engineering approach	Main achievements	References
	without exogenous osteoconductive scaffolding material		
	• Effect of MSC-conditioned medium and human osteoblasts cultured on different titanium implant surfaces	• MSCs and osteoblasts cultures suppressed osteoclast activity in a surface dependent manner	• Lotz *et al.* [191]
	• Co-culture of osteoblasts and osteoclasts on a preosteoblastic ECM-derived surface (effect on osteoclast-osteoblast behavior)	• ECM crosslinking density is an underlying force in the coupling between osteoblast and osteoclast behavior	• Hwang *et al.* [244]
	• Co-culture of hiPSC-derived osteoblasts and osteoclasts on 3D hydroxyapatite-coated poly(lactic-co-glycolic acid)/poly(L-lactic acid) scaffolds	• Co-culture of both cell types on 3D hydroxyapatite-coated poly(lactic-co-glycolic acid)/poly(L-lactic acid) scaffolds recapitulate the remodeling process of human bone	• Jeon *et al.* [7]
	• Co-culture of osteoblasts and osteoclast on silk films	• Films remodeled (mineralized) in co-culture demonstrated increased roughness parameters, fractal organization, and mineral clustering compared to osteoblasts culture alone	• Hayden *et al.* [190]
Osteocytes-osteoclasts	• Co-culture of osteocytes and osteoclasts with addition of different molecular weight collagens	• Type II collagen stimulated osteogenesis and suppresses osteoclastogenesis in a dose-dependent manner	• Elango *et al.* [245]
	• Co-culture of osteocytes and osteoclasts under flow perfusion conditions using a microfluidics device	• Osteoclast precursors (RAW264.7 cells) showed increased cell density and osteoclast differentiation in co-culture with osteocytes that did not undergo mechanical stimulation	• Middleton *et al.* [246]
Vascular-bone cells	• Co-culture of human dermal microvascular endothelial cells and human osteoblasts on a 3D polycaprolactone-starch scaffold	• Creation of a co-culture strategy to form vascular-like structures *in vitro* on a 3D scaffold, through the heterotypic cell crosstalk and in the absence of external pro-angiogenic stimuli	• Santos *et al.* [247]
	• Co-culture of HUVEC, at different ratios, and human osteoblasts on copolymer scaffolds, in dynamic culture system	• A low ratio of HUVEC resulted in capillary-like structure formation and influenced the expression of osteogenic markers	• Xing *et al.* [248]
	• Co-culture of HUVEC and human osteoblasts on a macroporous calcium phosphate cement	• Creation of a macroporous calcium phosphate cement-microvasculature construct for a wide range of orthopedic applications with enhanced angiogenic and osteogenic capabilities	• Thein-Han *et al.* [249]

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Type of cell interaction	Tissue engineering approach	Main achievements	References
Immune-MSCs/bone cells	• Osteoblast/macrophage co-culture on calcium silicate cement	• Osteogenic differentiation of osteoblasts is stimulated by macrophage on calcium silicate cement	• Tu *et al.* [250]
	• Co-culture of MSCs and monocytes towards osteoblast and osteoclast differentiation in biphasic calcium phosphate granules	• Development of a 3D miniaturized model of bone tissue for drug screening	• Gamblin *et al.* [251]
Multi-cultures	• Tetra-culture of osteoblasts, osteoclasts, endothelial cells and BMMSCs	• Creation of an *in vitro* 3D bone remodeling model for studying the molecular basis of the cross-talk between cells	• Bongio *et al.* [193]

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Table 2

Examples of ECM proteins involved in bone and other mineralized tissues (e.g. enamel) formation and regeneration, and their use in tissue engineering systems. Locations in mineralized organs - bone and teeth - are indicated in bold.

Protein	Physiological localization	Biological function	Ligation site	Bone tissue engineering systems and osseointegration
Ameloblastin	•Pulp [346] •**Enamel** [346, 347] •**Hertwig's epithelial root sheath** [348] •**Periodontal ligament** [346] •Calvarial development [346]	•Regulation of enamel crystal growth [347, 349] •Cell adhesion molecules for ameloblasts [347] •Cell signaling [346] •Dentin and bone repair induction [346, 350] •Bone healing stimulation *in vivo* [346] •Enhancement differentiation of stem cells, osteoblasts and osteoclast precursor cells (*in vitro*) [349]	•Fibronectin interaction site [351] •Heparin-binding domains [351] •CD63-interaction domains [351, 352] •Calcium binding sites [346]	•Not found
Fibronectin	•**Bone** and other connective tissues [353, 354] •Body fluids [355]	•Cells adhesion, growth, migration, and differentiation [355] •Involved in bone formation [353] •Osteoblast differentiation [356]	•Integrin (α4β1, α5β1, αVβ3, αIIbβ3, αVβ6, αVβ5) [355, 357, 358] •Extracellular matrix components (collagen, fibrin and heparan sulfate proteoglycans) [355, 358]	•Fibronectin/fibronectin-derived peptides coatings improved osseointegration and bone formation *in vivo* and osteoblast adhesion and proliferation and MSC osteogenic differentiation *in vitro* [359–363] •Biocompatible artificial matrix composed by fibronectin and graphene oxide improved *in vitro* osteogenic differentiation of preosteoblasts [364] •Recombinant fibronectin/cadherin bio-inspired ceramic surface improved functionality in adhesion and proliferation of MSCs and osteoblastic differentiation [365] •Multi-layered recombinant fibronectin/cadherin composite exhibited strong MSC-recruiting capacity and provided favorable

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Protein	Physiological localization	Biological function	Ligation site	Bone tissue engineering systems and osseointegration
				microenvironments for the proliferation and osteogenic differentiation of these cells [366]
Laminin	•Basal lamina (**bone**) [330] •Endothelia [367]	•Cell survival, adhesion, proliferation, differentiation and specialized functions [367] •Although laminin is not effective at promoting osteoblast differentiation, it had some proliferative and adhesive activity on stem cells [330]	•Integrin (αVβ3, α2β1, α1β1, α3β1) [358]	•Laminin coatings on implant surfaces promoted osseointegration *in vivo* [368–370] •Laminin coating on blasted titanium discs promoted calcium phosphate precipitation *in vitro* [371] •Development of laminin-111 functionalized poly(ethylene glycol) hydrogels as cell delivery system to intervertebral disc regeneration [372, 373]
Vitronectin	•**Bone** [374] •Liver [374] •Brain [374] •Fat [374] •Heart [374] •Skeletal muscle [374] •Lung [374] •Uterus [374] •Testis [374] •Thymus [374]	•Adhesion of endothelial cells, fibroblasts and bone-derived cells [375, 376] •Regulation of the fibrinolytic, complement and coagulation systems [376]	•Integrin (αVβ3, αVβ5) [339]	•Complexes of vitronectin, insulin-like growth factor-1 and insulin growth factor-binding protein-5 enhanced the attachment and migration of human osteoblasts in 3D culture [377]
Type I collagen	•**Bone**, tendon, skin, ligaments, cornea [378] •Interstitial tissues (with the exception of hyaline cartilage, brain and vitrous body) [378]	•Regulation of bone cell phenotypes [379] •Defines bone biomechanical properties concerning load bearing, tensile strength and torsional stiffness [378]	•Integrin (α2β1, αVβ3) [358]	•Collagen type I coating promoted bone regeneration and implant osseointegration [380] •Dense collagen gel scaffolds containing mesenchymal dental pulp stem cells improved craniofacial bone healing [381] •Silk-collagen scaffold stimulated trabecular bone

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Protein	Physiological localization	Biological function	Ligation site	Bone tissue engineering systems and osseointegration
				growth in tendon-bone healing [382] •Chitosan-collagen films promoted osteoblast differentiation and matrix mineralization in MC3T3-E1 cells [383] •Shape-memory alginate porous scaffold containing type I collagen enhance cell migration and proliferation [384] •Constructs containing a collagen matrix provided better results in terms of vascularization and tissue formation than fibrin matrix [385]
Type IV collagen	•Capillary basement membranes during bone formation [386] •Endothelial lining of cavernous sinusoids [386]	•Unknown	Integrin (α2β1) [358] CD44 [358] TGF-β1 [387] BMP-2B [388]	•Composite bone substitute composed of tricalcium phosphate cylinder, bone morphogenetic protein, and type IV collagen successfully repaired segmental bone defect in the tibia of sheep [389]
Type X collagen	•**Hypertrophic and calcified cartilage** [358, 378]	•Support of forming bone tissue [390] •Aids in the removal of type II collagen fibrils [390] •Mineralization [390] •Influences vascular invasion of the cartilage matrix [390]	•Ca^{2+} [391]	•Not found
Osteonectin (SPARC)	•**Bone** [392] •**Pericellular matrix surrounding osteoblasts and osteocytes** [393] •Kidney [392]	•Osteoblasts differentiation and survival [393, 394] •Adipogenesis inhibition [394] •Regulates collagen fibril diameter [395] •Cell spreading [392, 392]	•Hydroxyapatite [392] •Ca^{2+} [392, 393] •Collagens [393] •PDGF [393] •TGF-β1 [393] •VEGF [393]	•Osteonectin involved in the formation of mineralized fibers and fabrication of nano-hydroxyapatite/ collagen/osteonectin composites for bone graft applications [378] •Supplementation of type I collagen scaffolds with SPARC increased the binding

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Protein	Physiological localization	Biological function	Ligation site	Bone tissue engineering systems and osseointegration
		•Collagen fibrillogenesis [396] •Ca^{2+} and hydroxyapatite binding [392]	•MMP2 [393] •bFGF [393] •IGF [393]	of hydroxyapatite nanoparticles to the scaffold [398]
Osteocalcin	•Extracellular matrix of areas of newly formed **bone**, **i.e. in the subperiosteal region** [392] •**Osteoid of forming bone** [392] •Kidney [399]	•Bone turnover [1, 392] •Regulates osteoclasts [1] •Inhibits bone formation [1] •Calcium binding [392]	•Ca^{2+} [392] •Hydroxyapatite [392]	•Osteocalcin incorporated into Biocement D (Merck Biomaterial GmbH) containing mineralized collagen improved the initial adherence of osteoblast-like cells [400]
Biglycan	•Bone, [392] •Articular cartilage [392] •Endothelial cells of dermal blood vessels [392] •Prickle cell layer [373]	•Cell spreading [392] •Decrease the availability of active TGF-β [396] •Collagen fibrillogenesis promotion [396] •Bone mineralization [401] •Cell-cell and/or cell-protein interactions [392]	•Collagen [402] •TGF-β [403] •BMP-4 [403]	•Titanium surfaces coated with collagen fibrils bounded to biglycan influenced proliferation and collagen synthesis by osteoblasts [404,405]
Bone sialoprotein (BSP)	•**Bone** [393]	•Matrix mineralization [396] •Osteoblast and osteoclasts differentiation, adhesion and function [406] •Stimulates osteoclast-induced bone resorption [393] •Angiogenesis promotion [407] •Mediates cell attachment [408]	•Integrins [1] •Collagen [393] •Ca^{2+} [393] •Hydroxyapatite [408] •MMP2 [393] •Complement factor H [393]	•Microgroove titanium surface with immobilized bone sialoprotein II promotes osteoblastic differentiation in human BMMSCs [409] •Surface functionalization of orthopedic titanium implants with bone sialoprotein enhanced human primary osteoblasts differentiation [410] •Bone sialoprotein coating of printed calcium phosphate scaffolds enhanced differentiation of osteoblasts [411] •Modification of PCL/ pHEMA surfaces with bone sialoprotein showed an improvement on attachment

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Protein	Physiological localization	Biological function	Ligation site	Bone tissue engineering systems and osseointegration
				and spreading of osteoblastic cells [412]
Osteopontin (Secreted phosphoprotein 1; OPN)	•**Bone** [392] •Kidney [392] •Endometrial glans of a nonpregnant secretory-phase uterus [392]	•Cell attachment [392] Hydroxyapatite binding [399] •Supports adhesion of bone cells to the mineralized matrix [413] •Inhibition of crystal growth [414] •Inhibits mineralization [414] •Promotes bone resorption [414]	•Integrins [393] •CD44 [393] •Fibronectin [393] •Hydroxyapatite [393] •Ca^{2+} [393] •Collagens [393] •MMP3 [303] •Complement factor H [393] •EGF [393] •PTH [374]	•Hydroxyapatite nanoparticles functionalized with osteopontin in a matrix of poly-d, l-lactic-acid stimulated new bone formation *in vivo* [415] •Osteopontin and the osteopontin-derived synthetic peptide, OC-1016, enhanced osseointegration *in vitro* and *in vivo* [416] •Osteopontin and BMP peptides incorporated on hydrogel scaffolds improved osteogenic differentiation of BMMSCs [417] •Osteopontin coating of hydroxyapatite surfaces enhanced MSCs proliferation [418]
Decorin	•**Bone** [392] •Non-articular resting cartilage [392] •Dermal collagenous matrix [392]	•Collagen binding [392] •Decreases the availability of active TGF-β [396] •Promotes collagen fibrillogenesis [396]	•TGF-β [392] •Collagen [396]	•Collageneous matrix coatings on titanium implants modified with decorin accelerated and enhanced adhesion of osteoblast cells [419] •Collagen fibrils-bound decorin coatings influence osteoblasts behavior [405]
Thrombospondin (type I and type II)	•Muscular and tendinous parts of the myotendinous junction (type I) [420] •Muscle (type I) [420] •**Bone marrow** (type I) [420] •**Mineralized matrix of bone** [392] •Articular cartilage (type I) [420]	•Bone cell attachment [392] •Osteoclast function regulation (type I) [422] •Inflammation regulation (type I) [393] •TGF-β activation (type I) [396] •TGF-β sequestration and collagen fibrillogenesis (type II) [396]	•Collagens [393] •Heparan sulfated proteoglycans (type I) [393] •Fibrinogen (type I) [393] •Laminin (type I) [393] •Ca^{2+} (type I) [393] •Fibronectin (type I) [393] •Integrins [393] •HSPG [393]	•Not found

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Protein	Physiological localization	Biological function	Ligation site	Bone tissue engineering systems and osseointegration
	•Connective tissues (type II) [421]	•MSCs proliferation inhibition (type II) [396, 423] •Osteoblast differentiation promotion (type II) [423] •Adipogenesis inhibition (type II) [423]	•CD47 [393] •CD36 [393] •LRP [393] •Syndecan (type I) [393] •Thy-1 (type I) [393] •Calreticulin (type I) [393] •TGF-β (type I) [393] •Cathepsin (type I) [393] •Elastase (type I) [393] •PDGF [393] •bFGF (type I) [393] •MMP2 [393] •IGF-1 [393] •IGF-BP (type I) [393] •Chondroitin sulfate (type II) [393] •Proteoglycans (type II) [393]	
Tenascin C	•Tendons [424] •**Bone** [424] •Articular cartilage [425]	•Osteoblast differentiation [393] •Fibronectin deposition [393]	•Fibronectin [424] •Integrins [393] •Contactin/F11 [393] •Annexin II [393] •Heparan sulfated proteoglycan [393] •Collagen [424] •Periostin [425]	•Not found
Periostin	•**Bone** periosteum [333] •Periodontal ligament and tendons [333]	•Cortical bone thickness control [333] •Negative regulator of matrix mineralization [333] •Crosslinking of collagen fibrils [333] •ECM organization (especially fibronectin and tenascin C) [396]	•Type I collagen [333] Fibronectin [333] •Tenascin-C [333]	•Not found

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Protein	Physiological localization	Biological function	Ligation site	Bone tissue engineering systems and osseointegration
		•SOST regulation [396]		
Dentin matrix acidic phosphoprotein 1 (DMP1)	•**Bone** and **dentin** [333]	•Ca^{2+} binding [426] •Initiation of nucleation of crystalline hydroxyapatite [426] •Assembly of dentin matrix [427] •Robust osteocyte marker [396] •Regulates phosphate metabolism [396] •Involved in osteocyte function [396]	•Ca^{2+} [426] •DSPP promoter [427]	•Not found
Type III collagen	•**Bone** [428] •Blood vessels [429] •Skin [429] •Lung [429] •Tendons [429]	•Promotion of bone formation [396] •Regulate collagen fibril diameter [429]	•Integrins α1β1 and α2β1 [430]] •vWF [431]	•Coating of hydroxyapatite scaffolds with type III collagen enhanced periodontal ligament-derived cells [432] •Porcine-based non-cross-linked collagen I-III membrane integrated into the subcutaneous connective tissue with mild peripheral vascularization [433]
Versican	•**Woven bone matrix** [434] •**Periodontal ligaments** [434] •Breast [435] •Brain [435] •Ovary [435] •Prostate [435] •Gastrointestinal tract [435]	•Cell adhesion, proliferation and migration [435] •Bone matrix formation during remodeling [436]	•Glycosaminoglycan hyaluronan [434]	•Not found
TGF-β receptor interacting protein-1 (TRIP-1)	•**Bone** [437]	•Regulation of translation of specific proteins [437]	•Cytoplasmic domain of TGFβR2 [438]	• •Not found

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Protein	Physiological localization	Biological function	Ligation site	Bone tissue engineering systems and osseointegration
		•Inhibits signal transduction by the activated TGF-β receptor complex [438] •Osteoblast proliferation and differentiation [437]		