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Review article

Co–culture systems of osteoblasts and osteoclasts: Simulating in vitro bone remodeling in regenerative approaches

Giorgia Borciani[a,c], Giorgia Montalbano[c], Nicola Baldini[a,b], Giorgia Cerqueni[d], Chiara Vitale-Brovarone[c,*], Gabriela Ciapetti[a]

[a] Laboratory for Orthopaedic Pathophysiology and Regenerative Medicine, IRCCS Istituto Ortopedico Rizzoli, Via di Barbiano 1/10, 40136 Bologna, Italy
[b] Department of Biomedical and Neuromotor Sciences, University of Bologna, Italy
[c] Department of Applied Science and Technology, Politecnico di Torino, Corso Duca degli Abruzzi 24, Torino 10129, Italy
[d] Department of Clinical and Molecular Sciences (DISCLIMO), Università Politecnica delle Marche, Via Tronto 10/a, Ancona 60020, Italy

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ABSTRACT

Bone is an extremely dynamic tissue, undergoing continuous remodeling for its whole lifetime, but its regeneration or augmentation due to bone loss or defects are not always easy to obtain. Bone tissue engineering (BTE) is a promising approach, and its success often relies on a "smart" scaffold, as a support to host and guide bone formation through bone cell precursors. Bone homeostasis is maintained by osteoblasts (OBs) and osteoclasts (OCs) within the basic multicellular unit, in a consecutive cycle of resorption and formation. Therefore, a functional scaffold should allow the best possible OB/OC cooperation for bone remodeling, as happens within the bone extracellular matrix in the body. In the present work OB/OC co-culture models, with and without scaffolds, are reviewed. These experimental systems are intended for different targets, including bone remodeling simulation, drug testing and the assessment of biomaterials and 3D scaffolds for BTE. As a consequence, several parameters, such as cell type, cell ratio, culture medium and inducers, culture times and setpoints, assay methods, etc. vary greatly. This review identifies and systematically reports the *in vitro* methods explored up to now, which, as they allow cellular communication, more closely resemble bone remodeling and/or the regeneration process in the framework of BTE.

Statement of significance

Bone is a dynamic tissue under continuous remodeling, but spontaneous healing may fail in the case of excessive bone loss which often requires valid alternatives to conventional treatments to restore bone integrity, like bone tissue engineering (BTE). Pre-clinical evaluation of scaffolds for BTE requires *in vitro* testing where co-cultures combining innovative materials with osteoblasts (OBs) and osteoclasts (OCs) closely mimic the *in vivo* repair process. This review considers the direct and indirect OB/OC co-cultures relevant to BTE, from the early mouse-cell models to the recent bone regenerative systems. The co-culture modeling of bone microenvironment provides reliable information on bone cell cross-talk. Starting from improved knowledge on bone remodeling, bone disease mechanisms may be understood and new BTE solutions are designed.

1. Introduction

In the case of significant loss of bone tissue following trauma, tumor resection or orthopedic disease, spontaneous bone healing may be compromised resulting in patient morbidity and significant health care costs [1].

* Corresponding author.
E-mail address: chiara.vitale@polito.it (C. Vitale-Brovarone).

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Conventional bone grafting procedures are the preferred surgical treatments, with bone autografts representing the gold standard for bone augmentation, and allografts or synthetic bone substitutes as suitable alternatives in reconstructive orthopedic surgery [2]. Despite the quite satisfactory clinical results of the current bone regeneration/augmentation methods, innovative clinical approaches are required: tissue engineering (TE), defined as "the creation of new tissue by the deliberate and controlled stimulation of selected target cells through a systematic combination of molecular and mechanical signals", may be a valid alternative [3]. Even if TE may be carried out in the absence of biomaterials, scaffolds behave as a template during tissue deposition, facilitating cell proliferation, migration and organization in a 3D-environment similar to the niche where cells usually live [4].

The current approach to bone regeneration relies on the bone tissue engineering (BTE) strategy, based on 3D scaffolds in association with cells and bioactive molecules to create an "osteogenic" substitute for bone diseases or defects [5,6]. A number of scaffolds mimicking the structural, mechanical, and biological properties of natural tissues have been developed in order to support new tissue formation when combined with cells [7,8]. Indeed, the development of an artificial ECM to be colonized by autologous or allogenic cells may allow to recreate a "patient-specific biological substitute" as close as possible to natural bone tissue [9].

In bioengineering and regenerative medicine, mesenchymal stromal cells (MSCs) are the favorite cell source, as adult MSCs can be harvested from a variety of anatomical sites, are not-limited in supply, have a robust clonal self-renewal and a mesenchymal multilineage differentiation potential, including the desired bone-forming cells [10,11]. Human MSCs (hMSCs) were firstly isolated from bone, and to date this source is the best known and most commonly utilized, since the harvesting of marrow from the iliac crest is considered quite simple with minimal discomfort for the patient [12–14]. In addition to bone marrow, another source of MSCs for BTE applications is the adipose tissue, which usually grants a large amount of cells: adipose stem cells (ASCs) and bone marrow derived-mesenchymal stromal cells (BM-MSCs) share several features, but display a different phenotype, as well as differences in transcriptome and proteome [15]. ASCs hold a great promise as cell source for autologous bone replacement or regeneration, but no consensus has been reached on their application, since, according to some authors, they have less osteogenic potential compared to BM-MSCs. Likewise, Brennan et al. demonstrated a better angiogenesis but an inferior osteogenesis induced by ASCs when implanted in nude mice [16,17]. A wide variety of other tissues have been proposed as a source of hMSC, including peripheral blood, periosteum, synovial fluid, dental tissues, skin and foreskin among adult tissues, as well as amniotic fluid and membrane, endometrium, limb bud, placenta and fetal membrane, umbilical cord, and Wharton's jelly among foetal and perinatal tissues. For a detailed summary of hMSC sources with the respective cell surface markers and proper culture conditions, see the review by Ullah et al. [18]. The positive effect of MSCs in bone regeneration is known since 1980s, with the first use of MSCs in tissue regeneration dated back to 1993, and the differentiation of MSCs towards osteoblasts (OBs) with production of a mineralized matrix was one of the earliest properties observed [19]. Several *in vitro* studies confirmed that pluripotent MSCs can give rise to colony forming units (CFUs) and generate colonies of osteoblasts, fibroblasts, chondrocytes, adipocytes and myocytes [20].

MSCs exhibit functional differences depending on their tissue source, resulting in a site-specific phenotype, and the MSC fate is influenced by the "past mechanical memory". Apparently, this memory has a crucial role and can be mantained especially when they are initially cultured on stiff substrates and are subsequently transferred on softer ones. For this reason, the use of bone marrow-derived MSCs, which live in the bone niche, is suggested to trigger bone regeneration [21].

The use of autologous MSCs is continuosly expanding, with nearly 1000 registered clinical trials [22], as MSCs exert a positive effect on injured tissues through their paracrine activity and the modulation of the immune response [23,24]. However, critical aspects of the MSC-based therapy are the stringent criteria for patients enrolment, production costs, expansion and insertion, and the safety testing to exclude the risk of exogenous contamination.

The MSC therapeutic effect, the outcomes of the MSC-based cell therapies and a comparison between the use of autologous and xenogeneic or allogenic MSCs have been summarized in some recent reviews [25,26].

In the process of bone formation or repair, OBs arise from multipotent MSCs and secrete the organic part of bone - the osteoid matrix - that will be mineralized, while osteoclasts (OCs) originate from hematopoietic cells of the mononuclear lineage and are responsible for bone matrix resorption. These two cell types are the two main players of bone remodeling, a lifelong process continuously affecting our skeleton.

The remodeling cycle is composed of consequential phases: resorption, reversal and formation [27]. OBs and OCs, together with blood supply and associated connective tissue, assemble in the basic multicellular unit (BMU), a temporary anatomical structure active during the whole lifetime, localized both in cortical and trabecular bone with little morphological differences. Considering their reduced life-span, cells forming this structural unit are continuously replaced to guarantee the right cycle execution [28]. In the last years a great attention has been paid to the role of osteocytes in the remodeling process, as they indirectly modulate bone resorption by controlling OC activity or directly through perilacunar remodeling, while regulating bone formation through different signaling pathways [29]. The balanced activities of OC-mediated resorption and OB-mediated bone matrix formation are part of a complex process identified as "coupling". Briefly, during bone matrix resorption by OCs, the release of different factors, such as growth factors or structural proteins, induces OBs to deposit new bone. This mechanism results in bone deposition under physiological conditions such as skeleton growth during childhood, but the disregulation of this process may cause pathological conditions, including osteopetrosis or osteoporosis [30,31]. These "units" and their complex mechanisms have been investigated and partially understood thanks to *in vitro/in vivo* research and experimental systems, basically using cell cultures. This review aims at summarizing recent co-culture systems using bone cells, with or without the presence of scaffolds for BTE purpose, to mimic *in vitro* the OB/OC coupling mechanism.

2. Bone cells

Osteocytes are the most abundant cells in bone tissue, accounting for about 95% of the bone cell population, a proportion that can increase with age and size of the bone [32]. They reside in lacunae within the mineralized bone matrix and show 40 up to 100 dendritic processes per cell that extend along the canaliculi of the lacunocanalicular network connecting cells, vasculature and bone surface [33]. Osteocytes derive from OBs and represent one of the three possible end-stages of aging OBs, which may alternatively undergo apoptosis or become bone-lining cells (see underneath). For a long time, osteocytes were considered only as "buried" OBs within the bone matrix, while recently their key role as mechanosensors of the skeleton as well as regulators of OB and OC functions has emerged [34]. The regulation of bone formation and resorption may be influenced by the osteocyte-derived sclerostin (SOST) and DKK-1/2 that negatively regulate Wnt signaling: in quiescent bone SOST and DKK-1/2 prevent further bone forma-

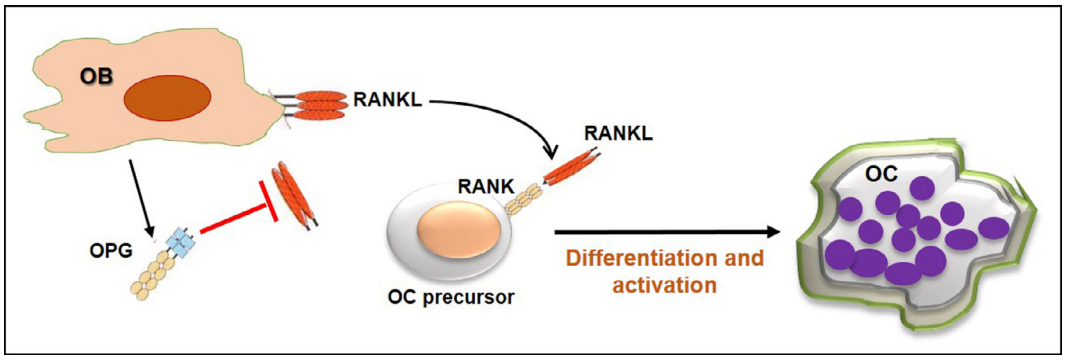

Fig. 1. The paracrine mechanism of osteoclast induction by osteoblasts. The differentiation of RANK-expressing osteoclast precursors to mature OCs is promoted by interaction with osteoblast-secreted RANKL. OBs also produce a decoy RANKL receptor, osteoprotegerin (OPG), which inhibits RANK signaling by masking RANKL.

tion, while during bone remodeling the expression of these factors decreases allowing OB-bone formation [35–37]. Osteocytes can also regulate bone resorption, both indirectly by producing the receptor activator of nuclear factor kappa-B ligand (RANKL) that stimulates osteoclastogenesis, and directly by local osteolysis, especially under pathological conditions [38].

OBs account for about 4–6% of the bone cell population arising from the mesenchymal lineage: they are mature cells of cuboidal shape localized on bone surfaces, with a life-span from few days to about 100 days. The differentiation of MSCs towards OBs initially requires the stimulation by two growth factors, *i.e.* WNT-protein and BMP glycoproteins, both crucial inducers of the commitment. Subsequently, Runt-related transcription factors 2 (Runx2), Distal-less homeobox 5 (Dlx5) and Osterix (Osx) genes are expressed, with Runx2 in turn regulating the expression of collagen type I alpha 1 (Colla1), alkaline phosphatase (ALP), bone sialoprotein (BSP) and osteocalcin (OCN) genes [39,40].

OBs are polarized cells able to produce osteoid onto the pre-existing bone matrix at a rate of 2–3 μm per day, while mineralization of the osteoid starts 10 day after its deposition [41]. Mature OBs are characterized by production and secretion of specific proteins constituting the bone matrix structure, such as type I collagen (~20wt%), osteocalcin and osteonectin (40% - 50% of noncollagenous proteins), osteopontin, bone sialoprotein II, vitronectin, fibronectin, thrombospondin, and proteoglycans like biglycan and decorin [42]. Two subgroups of OBs are identified and in particular the plump cuboidal OBs, that surround active bone mineralizing areas, and the flat epithelial-like OBs that can be found in non-mineralizing regions [43]. As a final stage, OBs turn into a quiescent status, becoming either osteocytes surrounded by a mineralized matrix or quiescent bone-lining cells at the bone surface, or undergoing apoptosis [44].

OCs are multinucleated bone-resorbing cells originated from mononuclear cells of the hematopoietic stem cell lineage. OCs carry out a fundamental role for the bone homeostasis since they are responsible for resorbing both the mineral phase of skeleton and the organic matrix, mainly type I collagen. Their hyperactivation leads to bone-degenerative diseases, including osteoporosis and osteolytic bone metastases, whereas their hypoactivation contributes to osteopetrosis [45]. OCs differentiate from PBMCs under the influence of two main growth factors: the macrophage colony-stimulating factor (M-CSF) which is produced by osteoprogenitor mesenchymal cells and OBs, and RANKL, expressed and secreted by OBs, osteocytes, lymphocytes and stromal cells (Fig. 1). These cells in turn are stimulated by parathyroid hormone (PTH), vitamin D, interleukin-1 (IL-1), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), interferon-gamma (IFN-γ) and other inflammatory cytokines [40,41].

The complex crosstalk between OBs and OCs is based on the mutual influence of OCs and OBs regulated by specific secreted factors: OC-derived factors are able to attract OB precursors to the resorption site where OBs, once acquired a mature phenotype, become able to deposit new bone tissue [46].

The OB lineage-derived RANKL acts binding the RANK receptor on the surface of haematopoietic cells and OC precursors. This mechanism is counterbalanced by the decoy receptor Osteoprotegerin (OPG), produced by OBs, osteocytes and bone marrow stromal cells, responsible for the paracrine inhibition of OC maturation through RANKL sequestration [31]. Indeed, OPG, a member of tumor necrosis factor receptor family, acts as soluble decoy receptor for RANKL: by preventing the binding of the OC–transmembrane RANK to the RANKL ligand, the activation of RANK signaling pathways is blocked with subsequent inhibition of osteoclastogenesis. OPG expression is induced by Trasforming Growth Factor- β (TGF-β), interleukin-1 (IL-1), Tumor Necrosis Factor (TNF), estrogens and Wnt ligands, while it is inhibited by prostaglandin E2 (PGE2) and glucocorticoids [47]. OBs are also able to inhibit and negatively influence OC formation through their derived Ephrin type B receptor 4 (EphB4) [48], whereas other OB-derived factors, such as Sema3B, Wnt5a and TGF-β can promote OC formation [48–50].

During the resorption phase, OCs control the OB commitment through secreted inducers called clastokines, acting with a positive and negative stimulation [51]. The clastokines positively influencing OB differentiation include: sphingosine-1-phosphate (S1P), which also stimulates mineralization, Bone Morphogenetic Protein (BMP-6), wingless-type MMTV integration site family member 10B (Wnt10b), collagen triple helix repeat containing 1 (CTHRC1), complement component 3a (C3a) and EphrinB2 (EphB2), an osteoclast ligand which bind Ephrin type-B receptor 4 (EphB4) on OBs [52–54]. On the other hand, the Semaphorin4D (Sema4D) clastokine has an inhibitory effect on OB differentiation [55].

Another relevant source of OB inducers are the growth factors encased within the bone matrix, such as the TGF-β family, including Bone Morphogenetic Protein 2 (BMP-2), as well as Platelet-Derived Growth Factor (PDGF), and Insulin-like Growth Factors (IGFs) (Fig. 2).

These factors are released during OC resorption: thanks to the activity of plasminogen activators and matrix-metalloproteinases (MMPs) [56] OB precursors and BMU cells are stimulated. As final step, osteocytes produce positive signals to OBs, inducing new matrix deposition [31].

The comprehensive and detailed study of cross-talk mechanisms existing between OBs and OCs during the remodeling process is a crucial aspect, since a better understanding could improve therapies and drugs for bone diseases as well as lead to the development of functional scaffolds for bone regeneration.

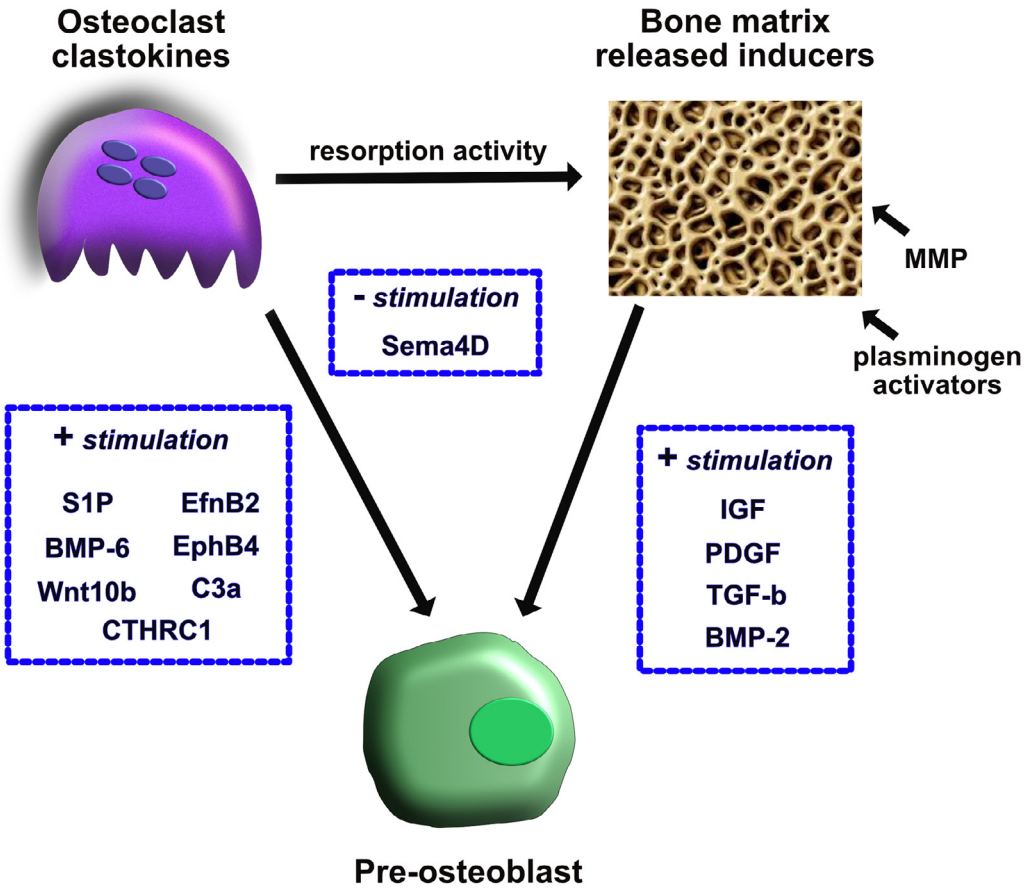

Fig. 2. Schematic representation of the regulation of OB maturation by OC-released clastokines and matrix-released growth factors following resorption by OCs.

3. Bone remodeling process

Bone remodeling, a continuous process that lasts throughout life, is performed by specialized cells: bone resorbing OCs and bone synthesizing OBs, which may assemble in the BMU together with osteocytes and bone-lining cells. Multiple bone remodeling events occur at the same time at different sites of the body, leading to a complete functional renewal of the skeleton every ten years [40,48]. Considering the essential role recently attributed to osteocytes as mechanosensors detecting the need for matrix repair or removal [38,57], and the demonstrated presence of "reversal cells" in the resorption lacuna [58,59], the pivotal role of OBs as initiator of bone remodeling has been currently reconsidered.

Accordingly, two "bridging structures" between OCs and OBs have been described: the reversal zone, *i.e.* an OC-eroded surface containing the reversal cells, pre-osteoblasts or osteoprogenitors, as well as OBs and the canopy, an envelope around the bone marrow, made of cells of the OB lineage and rich in capillaries. This structure is suggested to be the source of OB progenitors that are delivered to reversal surfaces where they differentiate to mature OBs [60]. Consequently, even if OCs and OBs are not placed in direct contact in the BMU, their activities are coordinated, as proved by the physical connection existing between resorption and new bone formation through the reversal phase [61].

The existence of a mixed "reversal-resorption phase" where the anabolic signals of OCs are transferred to osteoprogenitors, inducing their differentiation to mature OBs, while an OB-derived metalloproteinase is contributing to the OC-mediated bone resorption, has been recently suggested. Moreover, the number of osteoprogenitors recruited to the resorption/reversal surface of the human haversian BMUs plays a key role in the switch from resorption to formation process [62].

The BMU has a different morphology and activity in trabecular and cortical bone: in the trabecular bone, the BMU is localized on the surface and is covered by the canopy, that is a kind of "roof" above the remodeling site [63] (Fig. 3), while in the cortical bone, bone resorbing-OCs, immediately followed by differentiating OBs, create a "cutting zone", and the resorbed space is filled by blood vessels, nerves and connective tissue [48].

Generally, in adults, the turnover rate of cortical bone is quite low about 2% up to 3% per year, enough for maintaining adequate biomechanical properties. On the contrary, the rate for trabecular bone is higher and counted to be about 25%, underlining the importance of its contribute to the mineral balance [64,65].

The remodeling cycle consists of consecutive events which take place whithin the BMU, as schematically illustrated in Fig. 4. Initially, OCs generated from hematopoietic precursors (OC precursors) residing in blood and bone marrow are attracted to the bone site by different stimuli, to become mature multinucleated OCs [44,64]. Successively, activated OCs start to resorb mineralized bone and organic matrix for 2–4 weeks before undergoing apoptosis. The intermediate period between OCs resorption and the start of matrix deposition by OBs is called the reversal phase, where the signals derived from matrix resorption induce new bone formation.The last stage is the formation phase, where OBs form new bone. Eventually, a limited number of OBs surrounded by mineral matrix undergo terminal differentiation and become osteocytes. In approximately 4–6 months the last phase of the process is concluded and about 50–70% of OBs undergo apoptosis while the others become bone-lining cells or osteocytes.

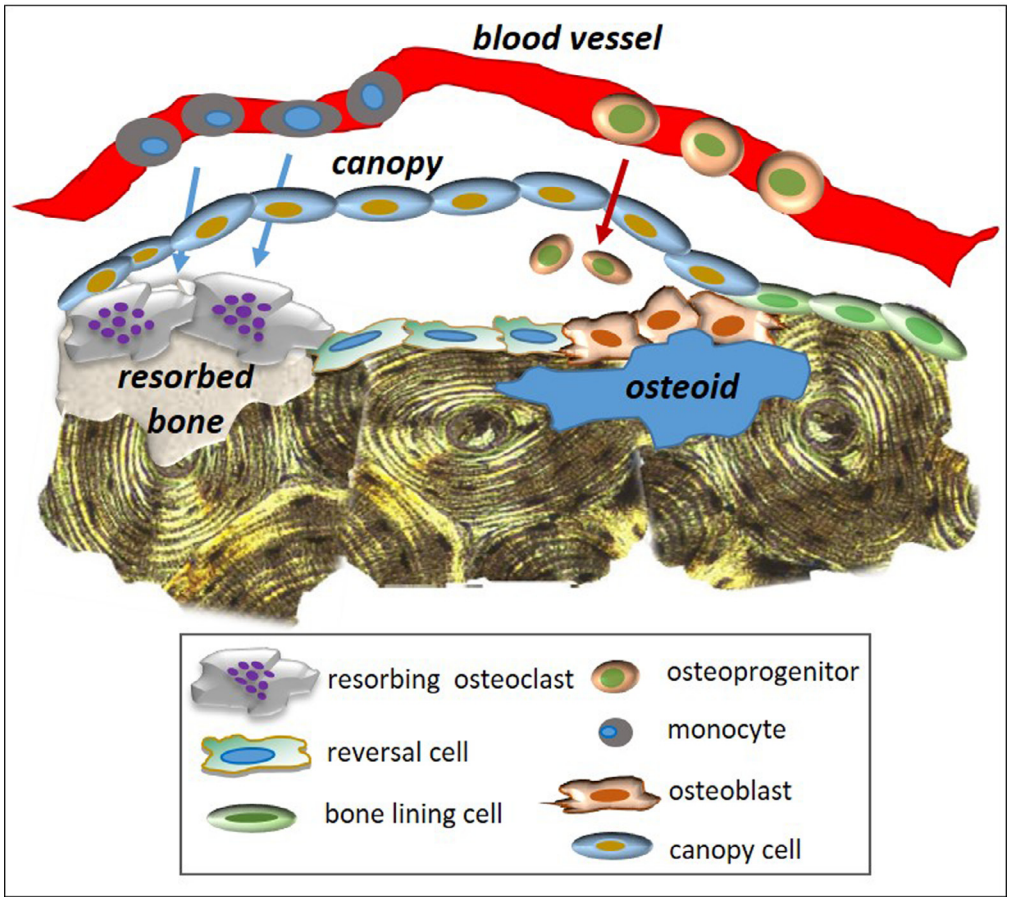

Fig. 3. The Basic Multicellular Unit structure, with the different types of cells participating to the remodeling process.

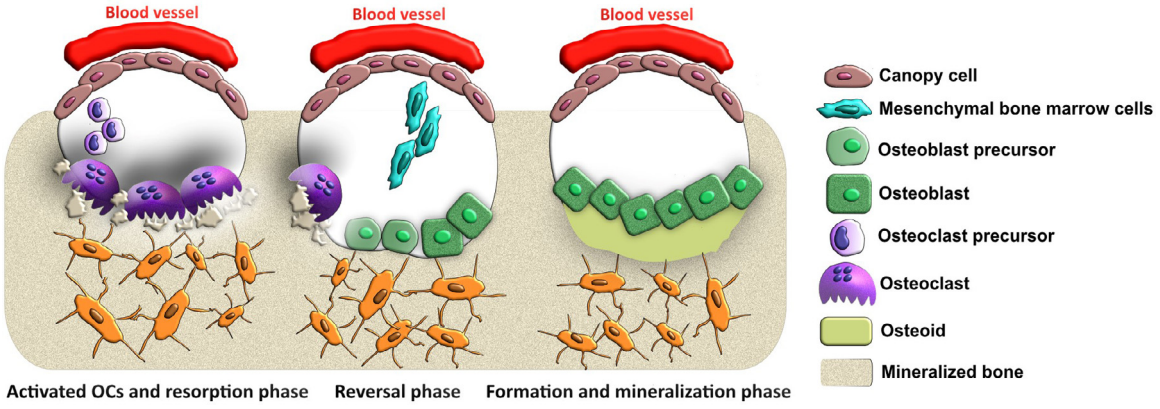

Fig. 4. Cartoon depicting the sequential phases of resorption and formation of bone by cells active in the basic multicellular unit (BMU).

This dynamic and complex process of bone remodeling is strictly regulated by two pathways acting as local and systemic regulation.

The local factors M-CSF and RANKL have a positive effect on OC differentiation, whereas Wnt family growth factors positively affect OB differentiation.

Concerning the systemic regulation of bone remodeling, parathyroid hormone (PTH) and estrogens act to maintain skeletal homeostasis. Leptin, a small polypeptidic hormone secreted primarily by adipocytes, emerged as a pivotal regulator inhibiting bone formation by OBs: even if its role remains controversial, this activity hints at a direct connection between the brain and bone [66,67].

During the fracture healing or remodeling process, as well as bone formation during the skeletal development, another important role is covered by vascularization and oxygen contribution. Bone is a highly vascularized tissue with a large number of vessels and capillaries directly participating to the osteogenic generation of new bone, as demonstrated in bone fractures, where the growth of blood vessels and the recruitment of osteoprogenitors are coupled. Endothelial cells and osteolineage cells are often juxtaposed, with skeletal cells secreting angiogenic factors, whereas endothelial cells (ECs) produce angiocrine factors such as Vascular Endothelial Factor (VEGF) and chemokines that regulate skeletal cell behavior [68]. In adults, VEGF is abundantly expressed by OBs and regulated by the hypoxia inducing factor (HIF) signaling

pathway: preclinical studies have shown that an increased HIF activity in OBs or ECs promotes angiogenesis and bone formation [69,70]. Interestingly, VEGF derived from OBs or released from the resorbed matrix can also stimulate OC formation. This is related to the fact that VEGF has been proved to substitute for M-CSF and cooperate with RANKL to support OC differentiation of non-adherent bone marrow-derived cells *in vitro* [71]. Furthermore, VEGF can directly enhance OC bone resorption and survival of mature OCs via VEGFR-2 signaling [72]. The ability of pre-OC-secreted platelet-derived growth factor-BB (PDGF-BB) to induce type H vessel formation, thereby stimulating bone formation during the coupling step, confirms the link of OCs with endothelial cells and angiogenesis [73]. The evidence that alterations of the complex biochemical interactions between vasculature and bone cells may lead to various clinical manifestations further proves the essential role of vascularization in osteoinduction [74].

In addition to vascular network, another external factor that takes part in the complex coordination of bone cells is the local micro-mechanics.

Bone cells are constantly exposed to mechanical stimuli and the bone mass is preserved thanks to the mechanical loading. The mechanical stimuli are primarily captured by osteocytes and further transmitted, mainly through IGF-1 signaling, to OBs and bone-specific MSCs which cooperate in inducing bone formation [75]. According to a recent study performed on mice, periosteal progenitor cells are also considered mechanosensitive thus reacting to physical loading: the mechanism is based on the sensing of fluid shear stresses by means of their primary cilium and the further expression of osteogenic markers acting on OB differentiation. Moreover, since this progenitor population persists in the adult skeleton, it may significantly contribute to the adult skeletal maintenance [76]. Similarly to what observed for OBs, OCs can react to mechanical stimuli, primarily sensed by osteocytes, by responding to cytokine gradients as reported by Middleton and coworkers, who registered an increase in OC precursor (RAW 264.7 cells) density and OC differentiation when co-cultured with osteocyte-like MLO-Y4 cells in a microfluidic perfusion system [77].

In the frame of a study conducted on the bisphosphonate-related osteonecrosis of the jaw, the mechanotransduction ability of osteocytes has been investigated. In particular the death of the osteocytes following acute mechanical trauma has been proven to induce the release of chemoattractant molecules that triggers OC precursor cells to resorb the damaged bone [78].

4. *In vitro* culture of bone cells

Single cell type cultures are a widely used technique to study cell morphology, molecular pathways and differentiation patterns. However, despite their value, signals traded between different types of cells cannot be recognised in such systems. With the aim to realize *in vitro* systems able to mimic the bone turnover, the simultaneous presence of OBs and OCs is required to reproduce the proper cross-talk and the mechanisms of molecular cooperation.

Despite several OB/OC co-culture systems have been proposed and explored to allow a detailed analysis of interactions and mutual signals, up to now a commonly accepted and optimised model is still lacking.

The set up of a co-culture system using bone primary cells can use different cell sources. In particular, OBs can be provided considering several anatomical sources and different isolation techniques, such as enzymatic procedure or spontaneous cells outgrowth; however all the potential alternatives can influence the final cell culture features [79]. Explanted bone tissue is an important source cointaining early OBs with bone-forming capability, able to differentiate *in vitro* towards a mature phenotype. In this context, the number of OBs isolated from the tissue is highly dependent on the donor site, as well as the size of the bone sample. Moreover, human OBs can be obtained from patients suffering from age-related bone diseases in order to evaluate the cellular changes involved in the disease. Despite the specific focus of this review on human co-cultures of OBs and OCs, the use of animal sources such as rodent-derived OBs for cell culture has been reported and explored. Among them, OBs derived from calvaria of neonatal rats are widely used, thanks to the opportunity to isolate a significant number of faster growing cells [80].

In the case of human source, bone marrow-derived mesenchymal stromal cells (BM-MSCs) are the most extensively studied in the field of regenerative medicine, even if MSCs can be isolated from many other tissues, as previously reported [81,82]. In these *in vitro* models, BM-MSCs are induced to differentiate to OBs using an osteogenic medium containing dexamethasone or vitamin D3, β–glycero-phosphate and ascorbic acid [83,84].

For a BTE approach, the advantages related to the use of BM-MSCs include the easy cell isolation, high proliferative activity and osteogenic differentiation with production of mineralized matrix [85–87]. BM-MSCs harvested from the iliac crest are widely used in experimental systems and clinical trials due to the proven potential for autologous transplantation [84]. However, the poor presence of cells in the bone marrow aspirate, ranging from about 0.001 to 0.01%, normally requires *in vitro* expansion to reach a suitable amount for clinical applications, although some properties of MSCs may be negatively influenced by a prolonged *in vitro* culture [88,89]. The study reported by Fickert et al. conducted on MSCs derived from iliac crest bone marrow aspirates of 15 healthy patients undergoing hip replacement, showed that the long-term cultivation of MSCs may cause a reduced osteogenic differentiation regardless of the donor age [90]. However, *in vitro* expansion of MSCs for clinical use is usually limited to 2–3 passages [91]. Other important aspects to consider in case of MSC cultures concern the MSC heterogeneity and the high variability among different donors. In addition, even if the number of bone precursors seems to be influenced by the donor age, this aspect apparently does not affect their osteogenic potential [86,90].

Another important source of MSCs is represented by the peripheral blood (PB): a consistent amount of PB-derived MSCs (PB-MSCs), with a differentiation ability similar to BM-MSCs, can be harvested from a few milliliters of blood, as shown by Ab Kadir et al. After separation of mononuclear cells from peripheral blood, adherent and suspension cells positive for mesenchymal and hematopoietic stem cell markers, can be induced to differentiate into “specialized” OBs and OCs respectively using proper inducers [92]. However, further studies are required to establish the potential advantages of PB-MSCs for clinical applications.

Adipose-derived mesenchymal stromal cells (AD-MSCs), which can be obtained with a 1–5% frequency of isolated cells through the lipoaspirate technique, present an osteogenic differentiation potential both *in vivo* and *in vitro* [86,93,94]. However, since the difference in the osteogenic potential between AD-MSCs and BM-MSCs *in vivo* is still not completely understood and only few clinical reports testify the use of AD-MSCs for bone regeneration, further studies are required to better explore the bone-forming potential of these cells for clinical applications [95].

Mature OCs can be obtained by culture of buffy coat-derived human monocytes using a proper and well-characterized protocol, accepted starting from 1970s. Monocytes can be also harvested from bone marrow [48,96].

Mature OCs obtained from BM- and PB-derived monocytes differ in several aspects such as resorption mechanism (pits/trenches), number of nuclei, expression of tartrate-resistant acid phosphatase (TRACP) 5a and 5b and OC specific gene expression [97,98].

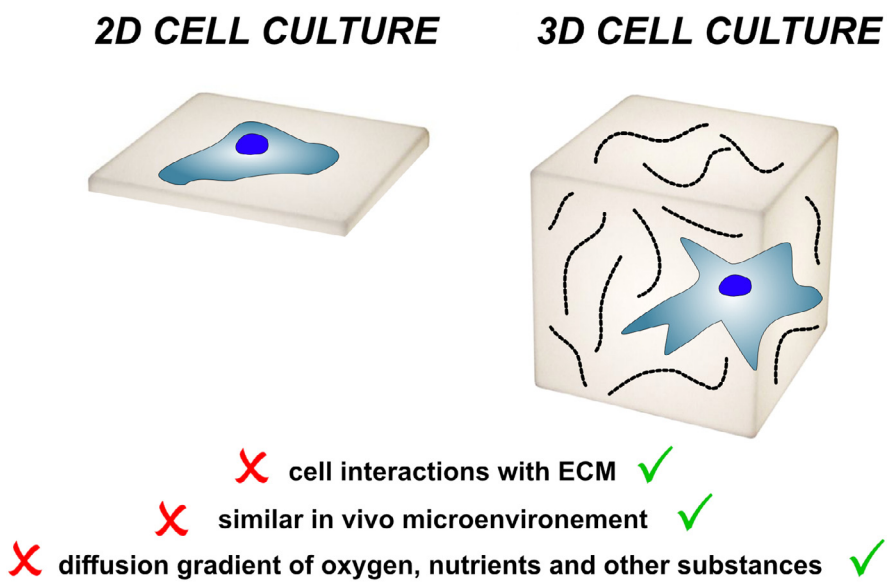

Fig. 5. Cartoon summarizing the properties of 2D- and 3D-cell culture systems.

As already reported, essential factors for the commitment of monocytes towards OCs are M-CSF and RANKL, which promote the fusion of monocytes *in vitro* and their differentiation to mature OCs, with typical markers such as multinuclearity, peripheral actin ring, $\alpha_v\beta_3$ integrin, etc. [99,100].

In conclusion, both OBs and OCs may be cultured *in vitro* and induced to differentiation using standard techniques.

Standard cell culture experiments usually consider a two-dimensional (2D) environment, where cells grow on a flat, solid 2D substrate made of tissue culture polystyrene, that can be treated to foster cell adhesion or untreated, and in presence of a specifically defined nutrient medium. However, in the human body, cells live in a well-organized three-dimensional (3D) microenvironment, receiving multiple signals from other cells and the surrounding matrix.

Surprisingly, even if about 50 years ago a 3D fibrous collagen-based network was described as the proper matrix to grow fibroblasts, the passage from 2D to 3D cell culture systems is rather recent. Indeed, even if the 2D cell culture has proved its predominance up to now, recent research works have recognized the importance for cells of living in a tissue-like 3D microenvironment. In a 3D culture system the cell can indeed retain a proper morphology and phenotype, accomplishing its functions thanks to a more physiological biochemical and biomechanical microenvironment (Fig. 5) [101,102]. Whereas in the 2D cell monolayer the growth factors, soluble molecules, nutrients and oxygen are freely exchanged because of their homogenous distribution in the medium, cell behavior, including signal transduction and gene expression, greatly changes in the spatial and physical constraints of a 3D culture system. In addition, even if the 2D culture system is attractive to biologists for its simplicity and efficiency, most of the 2D models do not provide control of the cell shape, which in turn influences cell activities [103,104].

Alongside the advantages provided by 3D culture systems, many technical challenges remain to overcome, such as the spatio-temporal distributions of oxygen, nutrients, and metabolic wastes.

In this frame, the review edited by Edmondson et al. [103] presents a detailed comparison between 2D and 3D culture addressing the most important aspects also in the field of new drug discovery, while the review reported by Dhaliwal et al. [105] browses through several 3D cell culture systems exploiting the use of different scaffolds.

3D cultures of MSCs are set up for different purposes, such as the successful expansion to achieve an increased cell number without negatively affecting MSC therapeutic potential, or the production of spheroids with enhanced paracrine, angiogenic and anti-inflammatory properties. In particular, spheroids have gained increasing attention for their remarkable regenerative properties, mainly due to the enhanced osteogenic, as well as chondrogenic, adipogenic, neurogenic, and hepatogenic lineage differentiation compared to the 2D model. Since vascularization is a prerequisite for the survival of implanted tissue constructs, the high angiogenic and vasculogenic potential of spheroids is another interesting aspect. Recently, experimental spheroids combining different cell types for TE showed enhanced regenerative capability, due to the reproduction of a more physiological environment, with proper cell morphology and heterotypic cell–cell signaling [106,107].

Both 3D culture and spheroid systems may rely on the use of scaffolds to recreate a more accurate 3D microenvironment. Nevertheless, the cellular self-assembly does not necessarily require the support of 3D scaffolds, as cells can lay down their own ECM in a 3D system, able to further join cells together in a physiological-like arrangement with positive effect on their functionality. For instance, MSCs were shown to be able to secrete fibrous collagen when cultured in a 3D microenvironment *in vitro*, irrespective of the medium composition, as well as maintaining their “self organizing” potential. MSCs were additionally observed to direct endothelial cell organization exploiting intrinsic signaling activity when arranged in a 3D co-culture of heterotypic cell spheroids combining endothelial cells (ECs) with MSCs or their differentiated progeny [108,109].

To approach the 3D cell microenvironment by using an ECM-mimicking support, both synthetic (Matrigel^{TM}, hydrogels, fibrous polymers, etc.) and “natural” substrates (collagen, gelatin, decellularized ECM, etc.) are available: the proper mimicking of ECM using artificial polymers may lead to *in vitro* systems supporting even a triple-co-culture (epithelial, endothelial, and immune cells), as shown by Dohle et al. [110]. In this way, new 3D *in vitro* culture systems may provide clues for developing an effective MSC-niche mimicking scaffold [111,112].

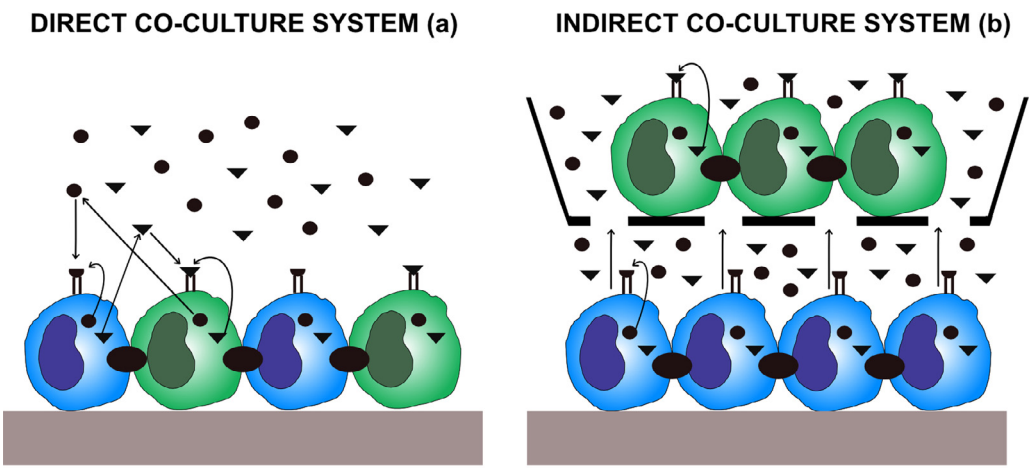

Fig. 6. Schematic representation of the interactions in the co-culture systems. In the direct co-culture model (a) cell-cell communication occurs through direct cell contact as well as autocrine and paracrine signaling (black arrows), while in the indirect co-culture system (b) there is no cell-cell contact and the communication is by autocrine and paracrine ways.

5. Co–culture

The advantages of co-culture *vs* mono-culture models lie in the closer *in vivo* mimicry, which may grant cells with additional functions due to intercellular signal transmission through junctions, exosomes and paracrine activities among the different cell types. Furthermore, the opportunity to reproduce cell-cell interactions may lead to a better comprehension of some phenomena occurring *in vivo*.

Potential difficulties of co-culture systems are mainly linked to the proper selection of the parameters for the co-existence of two or more different cell types: cell ratio, shared medium and timepoints, imaging, cell functions, instruments, labor-time, and adequate tools able to discriminate the different cell contribution.

Based on this, the creation of a multicellular system exploiting the *in vitro* co-culture approach can be seen as a powerful tool to enhance our knowledge about cell-cell communication investigated by means of a close cell interaction through physical contact and/or soluble molecules.

As evidenced by Im et al., the co-culture model can be set up both in 2D and 3D arrangement, with or without a direct physical contact among different cell types [113]. As already remarked, 2D cultures do not allow the recreation of an *in vivo*-like microenvironment and cell functions are less reliable compared to 3D systems. According to that, MSCs were found to lose surface markers, acquire the spindle morphology and change the migratory ability when routinely expanded in 2D [114]. As reviewed by Paschos et al. co-cultures involving the use of stem cells could be a promising approach in TE with the aim to reproduce complex tissues or organoids, especially when supported by innovative scaffolds and bioreactors. In this scenario, significant advances have already been made by combining stem cells together with terminally differentiated cells in a co-culture system [115].

A 2D co-culture model can be realised exploiting two different strategies and particularly considering a direct or an indirect physical contact system as schematically illustrated in Fig. 6.

In the direct contact co-culture, physical interactions and autocrine/paracrine signals can be analyzed, but the inability to understand the different contribution of the diverse cell types, mixed up in the same environment, is a clear disadvantage.

In the indirect co-culture cells are physically separated by a transwell or a porous membrane, with the culture medium and other molecules crossing the pores: since proteins, extracellular vesicles and soluble factors released by one cell type influence the other cell type(s) through paracrine signaling, the sharing of information produces substantial effects even if the physical receptor-mediated cell-cell interactions are hindered [113].

Regarding the type of cells, the design of a co-culture system with primary cells is considered a challenging target: primary cell populations are heterogeneous, often consisting of different subpopulations, and are subjected to donor-to-donor variations. In addition, the multiple cell types in the co-culture system could demand for different nutrients or signals for proliferation and/or differentiation.

In summary, the behavior of co-cultured cells is influenced by key design benchmarks, including (i) the type of cells (ii) the spatial and temporal seeding parameters, (iii) the ratio between the seeding density of the different cell types, (iv) the composition of the culture medium, including serum and supplements, and (v) the static or dynamic system of culture.

The work presented by Battiston et al. comprehensively reviewed all the challenging and critical aspects involved in the setting up of co-culture systems in presence of biomaterials, linked to the wide range of parameters, with a special focus on the role of biomaterials in the modulation of cell responses [116].

The controversal issue related to the use of static *vs* dynamic systems has gained increasing attention in the last years. Bone cells are in fact known to be mechanosensitive and respond to mechanostimulation through the activation of specific molecular signaling pathways [117]. It is widely recognized that dynamic cultures bring advantages over static systems in preserving cell functional properties, as shown in microfluidic bioreactors that allow a tight control of the 3D micro-environment [118]; however, the dynamic co-culture technique is not discussed in this work, due to the large variability of instruments, applied parameters and data analysis.

Moreover, the behavior of bone cells is profoundly affected by the oxygen level, largely via transcriptional changes driven by hypoxia-inducible factor (HIF). In particular, MSCs and OBs live in hypoxic niches, such as bone marrow or bone; since *in vitro* hypoxia may trigger the osteogenic differentiation of precursor cells, strategies considering the pre-conditioning of MSCs with hypoxia for improved bone regeneration or bone healing are currently explored [119,120].

This review provides a description of recent models of OB-OC co-cultures developed *in vitro*, with and without the support of bone engineering materials, aimed at mimicking the bone remodeling system. The reliable *in vitro* reproduction of the complex physiological system could provide fundamental cues to design an artificial scaffold able to guide bone cells to the correct interaction.

6. Bone cell co-cultures

It is now definitely accepted that the bone physiological status is maintained by the OB/OC cooperation, which is disrupted in several bone diseases or following bone injury.

The importance of the cross-talk between OBs and OCs and the advantages of studying both of them in a "all in one system" were already theorised by Rodan and Martin in 1981, who underlined the contribute of OBs in favouring the differentiation and activation of OCs through the release of factors able to bind to OC precursors [121].

The significance of setting up a co-culture of OBs and OCs is to provide an *ex vivo* system for an in-depth examination of the cross-talk between these bone cells and of their signaling pathways during the remodeling process.

Mouse-derived cells or cell lines were employed in most of the early OB/OC co-culture studies, due to their ease of access and repeatability of experimental results.

The group of Suda et al. was probably the first to point out the need of OB cooperation to get functional OCs, as it was recognized that the use of osteoblastic cells or other inducers is essential to obtain tartrate-resistant acid phosphatase (TRAP)-positive mononuclear cells [122]. Also in a previous work, Takahashi et al. found TRAP-positive multinucleated dentine-resorbing cells after 8 days of direct co-culture of mouse-derived OC precursors and OBs [123]. Udagawa et al. observed that OC-like cell differentiation was induced by two bone marrow-derived stromal cell lines: MC3T3-G2 (osteoblastic cell line from mouse)/PA6 (stromal cell line) and ST2 (stromal cell line) were both able to induce the formation of TRAP-positive osteoclast-like multinucleated cells from spleen cell-derived MNCs in co-culture. Moreover, when spleen cells and either MC3T3-G2/PA6 or ST2 were co-cultured on dentine slices with 1 alpha,25-(OH)2D3 and dexamethasone, several resorption lacunae were observed [124].

The pivotal role of bone marrow-derived osteoblastic stromal cells in OC generation either by the soluble factor production, such as M-CSF, or by the physical cell-cell contact, was further confirmed by other studies, and intercellular contact or cell-to-matrix interaction proved to be essential to potentiate the OC resorptive function [99]. Indeed, the absence of M-CSF or the abundance of M-CSF inhibitors cause the paucity of macrophages *in vivo* and consequently of OCs: even if M-CSF is not so strictly necessary for monocyte-macrophage differentiation, it is crucial in supplying survival and maturation stimuli to macrophages [125].

As reviewed by Katagiri et al. about 20 years ago and definitely confirmed by a number of further studies, the RANKL expression by OBs as signaling molecule involved in RANKL-RANK interaction for OC differentiation is the main regulatory mechanism of OB/OC coupling [126].

Several experimental OB/OC co-culture systems with human cells were then designed to simulate *in vitro* the skeletal remodeling process: a few examples of such systems can be found in Table 1.

Nicolin et al. co-cultured murine OBs (type CRL-12,257) and murine mononuclear monocytes (RAW 264.7) without exogenous cytokines and stimulating factors, first to verify the role of RANK-RANKL signaling on OC formation, then to study the mechanism of action of bisphosphonates such as neridronic acid: they found that this co-culture was an interesting alternative to the RANKL/M-CSF cytokine cocktail to investigate the OC formation in a more physiological environment, as well as to study anti-resorption drugs for osteoporosis treatment [127,128]. Applying an indirect co-cultivation system (Boyden chamber/transwell) of Saos-2 cells with RAW 264.7 cells, it was shown that 10 μM silicate was able to upregulate the expression of OPG of Saos-2 cells and to promote mineralization, while the TRAP staining of the RAW 264.7 decreased: based on these results, silicate was suggested as an additive to the human diet, as well as a potential component of medical therapy in osteoporosis [129].

Recently the rat OB/OC co-culture system has been used to evaluate if the interleukin secretion from OBs may enhance OC proliferation and resorption through the indirect action on RANK, RANKL and OPG, to find that OC activity on dentine slices is regulated by IL23 [130]. Another crucial "player" able to influence the cross-talk between OBs and OC-precursors is Akt1, an OB-produced kinase, acting as a mediator of OB-coupled osteoclastogenesis [131].

Even if the use of human cells implies a large variance of donor sex, age and health, primary cells from donors or patients have been frequently employed, giving results which generally confirm the data from rat/mouse cell protocols.

Using a direct *in vitro* co-culture of FLG 29.1, a human clonal cell line of OC-precursors, and Saos-2 osteoblastic cells Orlandini et al. observed the typical ultrastructural features of mature OCs, with a TRAP-positive staining in FLG 29.1 cells and the release of the granulocyte-macrophage colony stimulating factor [132].

In 2005 Atkins et al. tested *in vitro* the ability of phenotypically differentiated "normal human trabecular bone-derived cells" (NHBCs) to support the generation of functional human OCs from precursors in human peripheral blood or bone marrow cells, in order to understand the role of bone osteoblasts in OC formation. In this model the NHBC were used as a "stromal layer", where OC precursors were seeded onto a pre-formed layer of OBs attached to dentine or bone slices. They found that serum-free medium (SDM), *i.e.* α-MEM supplemented with 1% bovine serum albumin, a number of growth factors, ascorbate and dexamethasone (DEX), supported OC formation in the co-culture of NHBCs with CD14+ PBMC (or BMMC or CD34+ BMMC precursors) after the addition of 1,25–dihydroxyvitamin D (1,25-D) and DEX, while the only addition of PTH was not sufficient for OC generation. This medium formulation was consequently considered permissive for OC generation, possibly mediated by rapidly acquired differentiated phenotype of NHBCs in SDM +dexamethasone +1,25-D medium, while fetal calf serum (FCS) was found to contain negative factors for OC generation in a co-culture system [133].

To date, OB-OC co-culture systems have been adopted for several targets, such as the set up of the experimental model *in vitro*, a better understanding of the bone remodeling process, the efficacy of bone drugs or treatments and the development of regenerative strategies. As a consequence, different experimental protocols have been adopted by different authors.

Using human cells, Guihard et al. stated the importance of RANKL to obtain OCs from CD14+ monocytes, which in turn act on mineralization of MSCs, by co-culturing bone marrow derived-MSCs with human circulating CD14+ monocytes (1:10 ratio) in OB differentiation medium with the addition of RANKL (or LPS) [134].

In co-cultures of buffy coat-derived monocytes with human bone aspirate-derived mesenchymal cells on tissue culture polystyrene (TCPS) and cortical bone slices, Schmid et al. have confirmed the key role of M-CSF and RANKL to obtain OCs with effective bone-resorbing capability [135]. More recently, a transwell co-culture system was also applied to investigate the ability of MSCs derived from osteoarthritic subjects to induce the formation of active osteoclasts, possibly through cytokine secretion [136].

According to other authors, the presence of RANKL-producing OBs in the co-culture is *per se* promoting the OC maturation independently from the addition of exogenous inducers. Indeed, Teti et al. found an enhanced OC activity in a co-culture model thanks to the presence of OBs, and, recently, the presence at 7 days of a high percentage of TRAP- and cathepsin K-positive multinucleated OCs, similar to what found with osteoclastogenic inducers, has been reported in a human OB/monocyte indirect co-culture

Table 1
–Bone cell co-cultures.

OB source	OC source	Cell number OB:OC ratio	Direct/indirect contact	Static/dynamic	Conclusions	Author/year/ref no.
Murine OB type CRL 12257 (ATCC)	RAW 264.7 type CRL 2278 murine monocytic/ macrophagic cell line (ATCC)	CRL-2278 & CRL-12,257 at 10^5 cells/ml for each cell line	Direct	Static	TRAP-positive multinucleated cells formation & OC differentiation induced by cell-cell contact of OB and hematopoietic cells	V. Nicolin, 2006 & V. Nicolin, 2007 [127,128]
Saos-2 cells	RAW 264.7 murine monocyte/macrophage cell line	1×10^5/well Saos-2 & 5×10^4 /well RAW 264.7	Indirect	Static	Inhibition of osteoclastogenesis of RAW 264.7 cells	H.C. Schröder, 2012 [129]
OB from skull of Sprague-Dawley rat	OC from femoral bone of Sprague-Dawley rat	OB seeded in the OB-wells & OC seeded in the OC-wells	Indirect (2 wells with 0.45-μm filter in OB-OC connection)	Static	Increased proliferation rate, enhanced TRAP-positive signals and increased lacunar resorption of dentine by OC under IL-23 administration	Y.K. Kang, 2014 [130]
Saos-2 cells	OC-precursors (FLG 29.1)	/	Direct	Static	Mature OC exhibiting a TRAP-positive staining and typical ultrastructural features among OC-precursor population	S. Orlandini, 1995 [132]
Primary OB from mouse calvaria MC3T3-E1 & other cell lines	Bone marrow cells from rat tibiae	/	Direct (collagen-coated dishes)	Static	OB and OC cell-cell contact needed for OC formation and function. Only cell lines of bone origin induce OC function	E. Jimi, 1996 [223]
Normal human trabecular bone-derived OB (NHBC)	CD34+ cells from bone marrow mononuclear cell (CD 34+ BMMC) & CD14+ BMMC & CD14+ peripheral blood mononuclear cells (CD14+ PBMC)	$2–4 \times 10^5$ cells/ml NHBC (0.1 ml) & 6×10^5 cells/ml CD14+ BMMC or 6×10^5 cells/ml CD14+ PBMC or 1×10^5 cells/ml CD34+ BMMC	Direct	Static	Fully functional human OC formed by co-culture of normal peripheral blood- and BM-derived OC-precursors with normal human trabecular bone-derived OB as a stromal layer, in serum free conditions	G.J. Atkins, 2005 [133]
hMSC from bone marrow	Human CD14+ monocytes (hMC) from peripheral blood mononuclear cells CD11b1 monocytes/ macrophages from bone marrow	1 :10 ratio	Direct & Indirect (conditioned medium from CD14++ hMC)	Static	Activated monocytes/ macrophages secrete coupling factors to induce mineralization by MSC, overwhelming the bone resorption signal mediated by RANKL	P. Guihard, 2012 [134]
hMSC from bone aspirates (Lonza) osteogenic- differentiated hMSC	hMC from peripheral blood-buffy coat	8×10^3 MSC & 1×10^5 hMC	Direct	Static	OC induction and resorption using osteogenic-differentiated hMSC	F. V Schmid, 2018 [135]
Synovium-Derived Stromal Cells (SDSC) from healthy and osteoarthritic (OA) patients	PBMC from peripheral blood	$2 \times 10^{6/well}$ PBMC & 2×10^5/transwell SDSC (1:10 ratio)	Indirect (0.4 μm transwell)	Static	Only SDSC from OA subjects are able to generate active osteoclasts from healthy donor PBMC	M. Dicarlo, 2019 [136]
hOB from nasal septum or jawbone	hMC from peripheral blood	2:1 hMC/hOB (direct) & 3:1 hMC/hOB (indirect)	Direct & Indirect (0.45 μm transwell)	Static (direct & indirect) & dynamic (direct)	OC induction by OB and *in vitro* 3D hOB/hOC co-culture model requiring a minimal amount of cells	L. Penolazzi, 2016 [138]

[137,138]. The results of an *in vivo* study employing intravital two-photon imaging to detect mouse OB–OC contact in bone, suggested that soluble RANKL secreted from mature OBs may also strongly induce mature OCs *in vivo* [139].

Moreover, the OB-OC co-culture system has been reported as bone mimicking *in vitro* model to screen molecules/drugs for bone disorders or to measure their activity: some examples are displayed in Table 2.

Among the *in vitro* models for testing bone diseases-related drugs, Hayden et al. evaluated a co-culture system composed of OBs from human bone marrow-derived mesenchymal stem cells and OCs derived from THP-1 monocyte-like cells cultured on silk–hydroxyapatite films. The proposed co-culture aimed at creating a bio-mimetic *in vitro* model as a useful screen to detect the effects of osteoporosis-related therapeutics, such as bisphosphonates or other therapeutic molecules generally used for bone diseases [140].

Bone cell response to calcium phosphate cements (CPC) loaded with solid lipid microparticles (MPs) containing two different Alendronate concentrations (10 or 20% w/w) has been investigated *in vitro* using a OB/OC co-culture model. Following MG-63 osteosarcoma cells seeding on C10- and C20-CPC and the further co-culture with OCs, a significant enhancement of OB proliferation on both C-20- and C-10-CPC was observed in parallel with an important reduction in OC viability when compared to the controls. In addition, results collected on C-20- and C-10-CPC after 7 days proved an enhanced ALP and lower collagen expression, while osteocalcin did not present any evident variation. For what concerns OCs, OPG/RANKL ratio was found to be higher in C-20- and C-10-CPC groups compared to the OC control. The authors concluded that the MPs cements can be considered a good delivery system for Alendronate to exert its beneficial role of inhibition of excessive bone resorption and promotion of bone formation [141].

Another co-culture model of OBs (commercial) and OCs (patient-derived) has been proposed, along with the clinical trial, with the aim to check *in vitro* the efficacy of a micronutrients combination for the prevention or treatment of postmenopausal osteopenic women. The use of both the transwell system or a layered co-culture system led to the increase of osteoblastogenesis and osteoprotegerin, while osteoclastogenesis and RANKL levels decreased following the micronutrient addition [142].

6.1. Co-culture for bone regenerative medicine

With the progression of the regenerative medicine strategies and applications to replace or repair the injured tissues, the cell co-culture system was confirmed to be a useful *in vitro* tool to unravel tissue mechanisms.

The requirement for OCs in the field of research *in vitro* for BTE was underlined in 2006 by Han and Zhang, who indicated the absence of OCs from the current bone formation models as the major responsible of the failure of such systems to reproduce *in vitro* the mechanism of bone formation. As a consequence, they suggested the use of an OB-OC co-culture, potentially in combination with a bioreactor, as a better approach to the real bone environment, which could in the end bring the engineered bone closer to clinical applications [143]. As a matter of fact, in 2004, Vacanti and coworkers already showed the successful differentiation of osteoclast-like cells and osteoblasts derived from a single sample of porcine bone marrow on a mineralized biodegradable polymer, remarking that most of the studies for BTE applications were primarily focused on the role of OBs in the bone formation process in presence of various scaffolds, neglecting the precious contribution of OCs in bone remodeling [144].

The need to foster bone engineering techniques through a better understanding of the coupling mechanism acting in bone remodeling was also underlined by Jones et al., who tested the abil-

Table 2
Bone cell co-cultures to screen therapeutics/molecules.

OB source	OC source	Cell number OB:OC ratio	Substance tested	Direct/indirect contact	Static/dynamic	Conclusions	Author/year/ref no.
OB derived from bone marrow aspirate hMSC	THP-1 cell line-derived OC (ATCC)	15×10^3/cm^2 hMSC & 15×10^3/cm^2 THP-1 (1:1 ratio)	Alendronate or clodronate pre-loaded onto silk-hydroxyapatite films	Direct	Static	Well-defined tissue engineered model of bone remodeling to study long-term (12 weeks) effects of drugs for bone-related diseases on human cells	R.S. Hayden, 2014 [140]
Human osteoblast-like MG-63 cells (Istituto Zooprofilattico Sperimentale IZSBS, Brescia, Italy)	hOC-precursor 2T- 110 (Poietics™ Osteoclast Precursor Cell System, Lonza)	2×10^4 OB on CPC & 3×10^4 hOC-precursor in the bottom well	Two different Alendronate amounts introduced into solid lipid microparticles (MPs) added to calcium phosphate cements (CPC)	Direct (*indirect for controls*)	Static	Following OB:OC co-culture MPs-added cements are a good delivery system for Alendronate to display its beneficial role of inhibition of excessive bone resorption and promotion of bone formation	L.S. Dolci, 2019 [141]
Human adult mesenchymal stem cells (hMSC) (Lonza)	Peripheral blood monocytes	3×10^3 /cm^2 hMSC & 5×10^3 /cm^2 monocytes	Melatonin, strontium citrate, vitamin D3, vitamin K2 (MSDK)	Direct Indirect	Static	MSDK combination induced osteoblastogenesis more than melatonin or strontium citrate alone. MSDK combination favoured bone remodeling to proceed towards equilibrium by allowing some osteoclastogenesis	S. Maria, 2017 [142]

ity of 2D films composed of silk fibroin, chitosan and poly-lactic acid to support growth and differentiation of murine OB cell line and primary OCs in single- and co-culture system [145].

The experimental parameters selected for the development of optimised *in vitro* protocols to recreate the OB/OC reciprocal influence in a co-culture system, such as cell type, cell-cell ratio, shared medium and additives, time-points, etc., have been the main focus of several papers, as summarized in Table 3.

A direct co-culture method using human bone marrow stromal cells and human monocytes, introducing four combinations of inductive agents along the 28 days of cultivation, was set up by Heinemann et al. with the aim to provide an *in vitro* model "for biomaterial research". The reported results showed a significant influence of the medium composition and the temporal sequence of inductive agent addition, and confirmed the ability of osteogenically differentiated human bone marrow stromal cells to induce osteoclastic differentiation of human monocytes in absence of M-CSF and RANKL. Once identified the most promising experimental combination able to form mature OBs-OCs, the "modification" was tested on a composite xerogel of silica, collagen and calcium phosphate as bone substituting material, where OBs and OCs were found to be adherent next to each other directly on the surface of the biomaterial [100].

In order to find the easiest combination of OB-OC able to promote cell aggregation and differentiation, mimicking a bone microenvironment in a 3D static or dynamic co-culture system, Penolazzi et al. [138] employed human normal OBs (hOBs) obtained from bone fragments of nasal septum and human monocytes (hMCs) derived from the peripheral blood of healthy volunteers. Indirect co-cultures were set up in polystyrene 24-well plates using 0.45 μm cell culture inserts seeded with hMCs and hOBs attached at the bottom, cultured in DMEM high-glucose with 10% foetal calf serum without any osteoclastogenic inducer. In these conditions they observed a high proportion of mature TRAP- and cathepsin K-positive multinucleated osteoclasts (hOCs), similar to those found with osteoclastogenic inducers at 7 days, using a 1:3 hMC/hOB ratio, while no mature hOCs were observed when hOBs were absent. Moreover, this indirect co-culture system also supported hOB maturation, as demonstrated by OPN, OSX, and Runx2 expression, as well as the ALP activity and the deposition of mineralized matrix at day 21 of culture in osteogenic medium [138].

The optimization of the OB/OC static co-culture system has been also considered by Jolly and coworkers. Starting from the combination of cryopreserved primary human fetal osteoblastic cells (hFOB 1.19) with human peripheral blood mononuclear cells (hPBMNCs), the authors explored different OB/OC combinations, further defining the ratio of 1 OC:2 OB as the most promising condition. Following addition of hPBMNCs to the differentiated hFOBs on day 3, TRAP-positive OCs were detected after 2 weeks, without any exogenous addition of M-CSF and RANKL [146].

Schulze et al. have recently described a supplement-free co-culture system using human bone marrow-derived mesenchymal stromal cells (hBMSCs) and human peripheral blood mononuclear cells (hPBMCs) as precursor cells, and a native Saos-2-derived extracellular matrix (ECM) as an OC-resorbable substrate [147]. For the direct co-culture, hPBMC were first seeded on the Saos-2-derived substrate, while hBMSCs were added two hours later. On the contrary, the indirect co-culture was performed using two different strategies: one concerning a transwell system, where hPBMCs were plated on Saos-2 matrix in the bottom compartment and hBMSCs on the 3 μm pore-membrane of transwell insert, and a second alternative exploiting supernatants of monocultures. They finally concluded that in the direct co-culture system, hBMSCs promoted osteoclastogenesis of OC precursors in a RANKL-like manner. In contrast, hPBMCs co-cultured indirectly with hBMSCs exhibited some positive OC markers but significantly reduced matrix resorption. Therefore, according to the results reported, the direct cell–cell contact between OC precursors and hBMSCs can be evaluated as the strongest inducer of osteoclastogenesis [147].

An additional important observation was reported by Schmid et al., who remarked that considering *in vitro* co-culture systems, the clear evidence of active bone resorbing OCs is often incomplete or totally lacking [135]. To address this issue, the authors compared different co-culture conditions on bone slices with the aim to detect functional human OCs (hOCs) presenting the characteristic actin ring and expression of cathepsin K and CD51/61 (a_vb_3 integrin). For this study, human bone aspirate-derived MSCs (hMSCs) were primarily seeded onto bone slices or tissue culture polystyrene, while human monocytes from buffy coats (hMCs) were subsequently cultured onto the hMSCs. In these conditions, the detection of a_vb_3-positive hOCs and cathepsin K intense staining was possible only in presence of osteogenic medium. Moreover, typical lacunae were evidenced on bone slices, which confirmed the formation of functional hOCs.

To investigate the effects of perfusion co-culture on bone tissue regeneration *in vitro*, another interesting study reported the dynamic co-culture of HMSC-derived OBs and THP-1 (human acute monocytic leukemia cell line)-derived OCs on a chitosan-hydroxyapatite (chitosan-HA) superporous hydrogel using a spinner flask, where the outcomes were subsequently compared to static cultures [148].

In details, a two step cell seeding strategy was applied: first, the adhesion of a high number of hMSCs to the scaffold was achieved thanks to a eight-day static pre-culture period, then the THP-1 cells were seeded onto the OB-layer. The two types of cells were statically co-cultured for 4 days to ensure THP-1 cell attachment on hMSCs-seeded constructs prior to shift to perfusion conditions. Thanks to the dynamic system, the cells adhered to the scaffold, with minimal cell loss, mechanically stimulating OB and OC differentiation in a one step approach. The developed protocol that considers a cell pre-seeding in static conditions followed by a dynamic culture, proved to induce an enhanced response of cells co-cultured in scaffolds, as confirmed by the detected higher cell density and morphological changes compared to static cultures, where cell adhesion was limited to the scaffold surface.

An interesting "tissue construct", without any exogenous scaffolding materials, was generated by Clarke et al. by seeding commercial primary normal OBs and normal human OC precursors as a cell mixture in a rotating vessel using OB basal medium (supplemented with 10% fetal calf serum (FCS), 5 μM sodium ascorbate and 100 $\mu g/ml^{-1}$ penicillin/100 IU ml^{-1} streptomycin) [149]. By promoting a direct cell-cell interaction and aggregation, and following addition of OC differentiation factors and OB mineralization agents, the proposed 3D co-culture system (up to 4 mm in diameter) was classified as a "unique *in vitro* analog of human bone" with histological and biochemical properties similar to native *in vivo* remodeling bone. According to the authors, all three main types of bone cells (OBs, OCs and osteocytes) were detected in the 21 day-old mineralized construct, as confirmed by the expression of mRNA transcripts for specific proteins markers.

The potential modification of OC functional activity induced by OBs extracted from a different tissue source has been recently suggested. A supporting evidence is provided by a co-culture system where mouse OC-precursors were seeded in presence of OBs isolated from calvaria or long bones of mice. A high number of TRAP-positive multinucleated cells, as well as a higher RANKL-OPG ratio was recorded for co-cultures containing calvaria-derived OB compared to the long-bone-derived OBs. These observations thus suggest that the heterogeneity of OCs may derive from a different induction triggered by OBs residing in different sites [150].

According to Costa-Rodriguez et al. the influence of OCs on the behavior of bone-like cells have received limited attention. In

Table 3
Bone cell co–cultures without scaffolds.

OB source	OC source	Cell number OB:OC ratio	Direct/indirect contact	Static/dynamic	Conclusions	Author/year/ref no.
hMSC from bone marrow	Human monocytes (hMC) from peripheral blood-buffy coat	8×10^3/well hMSC & 3×10^5/well hMC	Direct	Static	OC induction by osteogenic inducers addition, also on silica, collagen, and calcium phosphate xerogel	C. Heinemann, 2011 [100]
hOB cell line hFOB 1.19 (ATCC CRL-11372)	hPBMC from peripheral blood	hPBMC:hOB co-cultured at 1:1, 1:4, 2:1, 1:2 ratio	Direct	Static	1 OC: 2 OB ratio chosen: the TRAP-positive cells were evenly distributed as compared to the other experimental groups	J. Jolly, 2018 [146]
hMSC from bone marrow	hMC from peripheral blood-buffy coat	$2.5 \times 10^3/cm^2$ hMSC & $1.5 \times 10^5/cm^2$ hMC	Direct & indirect (transwell & CM)	Static	OC induction by hMSC under direct, with TRAP positivity, resorption and metabolic activity of PBMC enhanced in comparison to indirect co-culture. Partial induction but no resorption under indirect contact	S. Schulze, 2018 [147]
Commercial human primary OB (hOB) (Lonza or PromoCell)	hOC precursors (hOCP cells) either from Lonza or freshly isolated from peripheral blood-buffy coat	hOB:hOCP cells cultured at 2:1, 4:1, 10:1 ratio	Direct	Dynamic (rotating vessel)	Primary adult hOB and hOCP combined in rotational culture aggregate to form a '"tissue construct"	M.S.F. Clarke, 2013 [149]
OB isolated from calvaria or long bones (tibiae) of mice	Mice OC-precursors (OCP) from different sources (bone marrow from calvaria, long bones, spleen, peripheral blood)	8×10^3/well OB & 2×10^5/well OCP	Direct	Static	High number of TRAP-positive multinucleated cells, higher RANKL-OPG ratio recorded for calvaria-derived OB *vs* long-bone-derived OB. Different ability of OB from calvaria and long bone to induce osteoclastogenesis	Q. Wan, 2016 [150]
Human osteoblast-like MG-63 cells [human bone marrow cells (hBMC) used as control]	hPBMC from peripheral blood	$1 \times 10^2/cm^2$ MG-63 (I) or $1 \times 10^3/cm^2$ MG-63 (II) & $1.5 \times 10^6/cm^2$ PBMC	Direct	Static	MG63 (I and II) induced osteoclastogenic response of hPBMC, in turn promoting MG-63 proliferation and expression of osteogenic markers. hBMC most parallel to MG63 II results. Reciprocal modulation of osteoblastic and osteoclastic behavior	J. Costa-Rodrigues, 2011 [151]
Mesenchymal stem cells (hMSC) differentiated to osteoblast-lineage (Lonza)	RAW 264.7 murine monocyte cell line	1×10^5 /well RAW 264.7 prior to 7×10^4/well hMSC	Direct	Static	OC influence positively the development of hMSC towards OB when cultured *in vitro*. The same onto 3D bone graft granules	S.S. Sinclair, 2011 [152]
Mononuclear cells from human bone marrow (hBMSC) in osteogenic medium	hPBMNC from buffy coat	3×10^4/well hBMSC in osteogenic medium & 5×10^4 /well hPBMNC	Indirect (0.4 μm transwell)	Static	hPBMC positively influence hBMSC proliferation and deeply influence hBMSC metabolism by BMP-2 production (in combination with osteogenic medium)	R.P. Pirraco, 2013 [154]
hMSCs (Lonza) or isolated from bone marrow in osteogenic medium	hPBMC from buffy coat	Increasing ratio of PBMC:MSC, 1:10 hMSC:depleted hPBMC	Direct & Indirect (0.4 μm transwell)	Static	hPBMC potently induce hMSC differentiation towards OB: increased ALP at 7 days and mineralized bone nodules at 21 days. Monocyte-induced osteogenic effect requires cell contact.	V. Nicolaidou, 2012 [155]
hMSC from femoral heads	Programmable cells of monocytic origin (PCMO)	2×10^5/well hMSC & 2×10^5/well PCMO (1:1 ratio)	Direct	Static	PCMO positively influenced ALP expression/activity and mineralization by hMSC under osteogenic culture conditions. PCMO promote osteogenic differentiation of MSC *in vitro* but they are not able differentiate towards OB-like cells	C. Zachos, 2014 [156]
hMSC from bone marrow	Mononuclear cells (MNC) from peripheral blood	$4 \times 10^3/cm^2$ hMSC & $1 \times 10^5/cm^2$ MNC	Direct & indirect (transwell)	Static	The presence of MNC enhanced hMSC ALP activity, especially when in direct contact, and mineralization. VEGF addition has a stimulatory effect on hMSC osteoblastic differentiation	K. Joensuu, 2015 [159]

their study, MG-63 and human peripheral blood mononuclear cells (hPBMCs) were studied in monocultures and co-cultures observing that MG-63 plated at two different densities greatly induced the osteoclastogenic response of hPBMCs independently from their RANKL production. At the same time, the presence of hPBMCs favoured MG-63 proliferation and the expression of osteogenic markers, leading to the conclusion that a reciprocal modulation of the OB and OC behavior occurs in case of OB/OC co-cultures [151]. Similarly, the influence of OCs on the differentiation of human mesenchymal stem cells (hMSCs) toward the OB lineage was confirmed by a study conducted on OCs and hMSCs simultaneously seeded on 3D bone graft granules [152].

A different mechanism to explain the regulation of OB activity mediated by OCs has been recently described by Zhang et al., who suggested that the binding of soluble RANK to transmembrane RANKL on OBs may provide a novel potential mechanism of reverse signaling able to promote osteoblastogenesis [153].

The influence of human peripheral blood monocytes/macrophages (hPBMCs) over the early osteogenic differentiation of human bone marrow stromal cells (hBMSCs) in the presence of dexamethasone-supplemented medium has been assessed in the study presented by Pirraco et al., where a porous transwell allowed the interaction between the two cell types through paracrine factors. The higher proliferation rate and ALP activity, as well as osteocalcin and osteopontin transcripts overexpression of hBMSCs in co-culture compared to the mono-culture, were ascribed to the effect of BMP-2 produced by monocytes/macrophages [154].

With the aim to investigate monocyte/MSC signaling to better understand the reciprocal interaction, Nicolaidou et al. designed a direct co-culture of monocytes/macrophages in contact with hBM-MSCs, observing that monocytes/macrophages are critical regulators of osteogenic differentiation via production of oncostatin M (OSM) and induction of STAT3 signaling in hBM-MSCs. The authors concluded that an increased bone formation may be obtained by activation of STAT3 in bone cells in case of osteoporosis and arthritis, as well as during the repair of fractures [155]. A similar behavior has been remarked also for the so called programmable cells of monocytic origin (PCMO), which demonstrated a positive effect when tested in direct co-culture with MSCs under osteogenic culture conditions [156]. According to the studies previously mentioned, some authors state that “monocytes and macrophages directly regulate osteogenic differentiation of MSCs through a mechanism that involves cell contact” as strict as in 3D cell spheroids [157], while others mainly support the idea that paracrine factors, such as BMP-2, are apparently enough to induce MSC to osteogenesis, as reported by Pirraco et al. [154]. We can conclude that, as discussed in several reports, both mechanisms can be accepted and may act at the same time [139,158].

To evaluate the effect of monocytes/macrophages on the osteogenic differentiation of MSCs in 3D-co-cultures a protocol, where THP-1 monocytes, M1 macrophages or M2 macrophages were co-cultured with adipose-derived mesenchymal stromal cells on 3D poly (lactic-co-glycolic) acid (PLGA)/polycaprolactone (PCL) scaffolds, was designed. Using osteogenic medium for up to 42 days, the authors showed that osteogenic differentiation of such mesenchymal stromal cells was inhibited by monocytes and both macrophage subtypes in 3D scaffolds [159]. In contrast, Joensuu et al. showed that monocyte/macrophage lineage cells were needed for the effective OB differentiation of MSCs in co-cultures with physical contact between MSCs and MNCs [160].

6.2. OB/OC cooperation on materials/scaffolds

As remarked by Scheinpflug et al., the use of scaffolds provides the mechanical support and the right biological cues to bone cells beneficial to the deposition of new ECM while better reproducing the remodeling process; this allows to establish a co-culture system more closely resembling the *in vivo* regenerative situation [161].

Accordingly, various nanoscale or nanoporous scaffolds mimicking the native ECM, including electrospun fibers, hydrogels, microfluidics and patterned surfaces, have been reviewed by Kook et al. and described as promising approaches to develop functional co-culture systems.

Despite the positive implementation of the co-culture system with functional materials providing a more reliable 3D environment, the authors evidenced the difficulty in imitating the complex microstructure of the actual ECM. However, the use of 3D printing technologies as well as decellularized ECM are proposed as advanced and promising tools to design well-organized scaffolds with a high degree of complexity and precision [162].

According to Goubko et al., the self-assembly of multiple cell types into functional tissues should be encouraged by patterning two or more types of cells on scaffolds. The authors reviewed a number of techniques developed since the 1960s, such as photolithography, soft lithography and printing techniques, which were applied with the goal of improving control over the cell microenvironment *in vitro* through the spatial localization of cells on a designed substrate [163].

A list of recent studies where OB:OC co-cultures were developed to simulate bone cell interactions into bone replacing scaffolds is reported in Table 4.

The design of degradable scaffolds able to be remodeled and replaced by autologous bone tissue as key strategy for BTE applications was highlighted in 2006 by Domaschke et al. After seeding buffy coat-derived monocytes (Mc) and mouse ST-2 osteoblastic cells (OB) onto mineralized collagen scaffolds, the differentiation and subsequent mineralization was induced by co-culturing the cells in the respective differentiation medium, where RANKL-L and M-CSF are used for Mc, and dexamethasone, vitamin D3, b-glycerophosphate for OBs. Considering the degradation of the mineralized collagen matrix after 4 weeks carried on by mature OCs and the subsequent formation of new ECM by OBs, the authors concluded that the co-culture of OBs and OC-like cells on the collagen scaffold can be defined a useful *in vitro* model for bone remodeling and BTE applications [164].

A long-term study (8–32 weeks) compared co-cultures of human mesenchymal stem cell-derived OBs and THP-1-derived OCs onto silk films with OB- or OC-single cultures, particularly investigating the remodeling process of silk films triggered by OBs and OCs by means of Scanning Electron Microscope-based surface reconstructions, confocal reflectance microscopy, and microtomography (micro-CT) techniques. Results demonstrated as increased ECM deposition, as well as increased roughness parameters and mineral clustering throughout the 32 weeks of culture were detected in case of OB/OC co-cultures compared to the mono-culture of OBs [165].

Another research study, aiming at monitoring *in vitro* scaffold remodeling while exploring the potential of OB/OC co-cultures to increase bone tissue healing process, assessed the seeding of OBs from mouse calvaria and OC-precursors from mouse bone marrow on Skelite disks at a 1:1 ratio in a 3D co-culture. X-ray computed micro-CT was performed on empty ceramic disks before cell seeding as a reference, and after 8 weeks of co-culture in osteogenic medium. Under these conditions, an organized bone tissue with oriented lacunae and clear separation between non-mineralized osteoid and mineralized bone was observed for the OB/OC constructs, while an immature bone tissue was formed in constructs presenting OB mono-culture. The authors suggested the combination of the histological analysis with the non-invasive X-ray computed micro-CT as a valid approach to evaluate and quantify ECM

Table 4
Bone cell co–cultures with scaffolds.

OB source	OC source	Cell number OB:OC ratio	SCAFFOLD	Direct/indirect contact	Static/dynamic	Conclusions	Author/year/ref no.
Mouse ST-2 osteoblastic cells (OB)	Human primary monocytes (MC) from buffy coats	$5 \times 10^5/cm^2$ MC & $2 \times 10^4/cm^2$ OB	Membrane-like scaffold made of mineralized collagen I fibrils	Direct	Static	*In vitro* model of remodeling process. Human osteoclast-like cells differentiate on biomimetic mineralized collagen I scaffolds & degrade scaffold in co-culture with OB which build new extracellular matrix	H. Domaschke, 2006 [163]
OB derived from bone marrow hMSC (Lonza)	THP-1 derived osteoclasts (ATCC)	hMSC & THP-1 total number $15 \times 10^3/cm^2$ (1:1 ratio)	Mineralized silk protein biomaterial films	Direct	Static	Long term co-culture: 8, 16, 24, 32 weeks. Increased surface remodeling, mineral clustering, extracellular matrix deposition in co-cultures in comparison to mono-cultures	R.S. Hayden, 2014 [164]
OB from mouse calvaria (C57BL/J6 mice)	OC-precursors from mouse bone marrow (C57BL/J6 mice)	OB:OC precursors 1:1 ratio	Skelite disks (67% Si-TCP / 33%HA)	Direct	Static	Significant increase in fibrous and mineralized osteoid tissue, scaffold biodegradation, highly organized ECM in co-cultures in comparison to OB cultures	A. Ruggiu, 2014 [165]
Human MSC from bone marrow aspirate (BMSC)	Human bone marrow–derived haematopoietic cells (BMHC)	3×10^4 BMSC on ZTA & 1.2×10^5 BMHC	Micropatterned zirconia-toughened alumina substrates (ZTA)	Direct	Static	Adhesion of both types of cells. Differentiation of OB-like cells (BMSC with bone forming potential) with nodular clusters & "discouraged" osteoclastogenesis	M. Halai, 2014 [166]
Human bone marrow stromal cells (BMSC)	Human bone marrow hematopoietic cells (BMHC)	1×10^4 BMSC onto TiO_2 substrates & 1.5×10^5 BMHC	Polished titania & titania patterned with 15 nm-high disordered nanopillars	Direct	Static	Increased osteogenesis without increasing osteoclastogenesis in co-culture. Increased *in vivo* osseointegration in rabbit femora (bone to implant contact)	R.K. Silverwood, 2016 [167]
hOB from femoral head or shoulder	PBMC from human peripheral blood	8×10^4 OB onto PCL-CaP & 5×10^5 PBMC	filamentous polycaprolactone with calcium phosphate surface coating (PCL-CaP)	Direct	Static	OB proliferation & ECM secretion facilitated in presence of PBMC. Unstimulated, growth factor-free co-culture (OB+PBMC) system as a platform to evaluate scaffolds intended for bone regeneration	A. Hammerl, 2019 [168]
hiPSC-derived MSC differentiated towards adipo-, chondro- & osteo-genic lineage	hiPSC-derived osteoclastogenic monocytes-macrophages	1.5×10^6 hiPSC-MSC & 1.5×10^6 hiPSC-macrophages	PLGA/PLLA (1:1) scaffolds with hydroxyapatite (HA) particles (0, 1%, 5%)	Direct	Static (and ectopically implanted in mice)	5% HA-added scaffold induce hiPSC-macrophages to OC & hiPSC-MSC to mature osteogenic OB. *In vivo* mature lamellar bone & increased bone matrix deposition with hiPSC MSC/-macrophage co-culture on high-HA scaffolds	O.H. Jeon, 2016 [169]
MC3T3-E1 pre-OB from mice calvaria	C7 mouse bone marrow macrophages	MC3T3-E1:C7 1:100 ratio	Bioactive glass foam scaffolds 70S30C (70 mol% SiO_2, 30 mol% CaO)	Direct	Static	Sustained growth and viability of MC3T3-E1 and C7 in co-culture. Formation of thick extracellular matrix. Tubule-like structures formed after bovine aortic endothelial cells (BAEC) separate seeding on scaffold	S. Midha, 2013 [170]

deposition and scaffold biodegradation in a 3D co-culture *in vitro* [166].

Following their isolation from human bone marrow, Halai et al. co-cultured MSCs and OC-precursors on micro-patterned zirconia-toughened alumina ceramic substrates to detect their bioactive potential. The results showed that osteogenesis was partially induced, while really few OC-like cells were observed on the ceramic surface. Since no induction factors were exploited, the authors estimated the developed method as a reliable representation of the osseous micro-environment found around orthopedic or dental materials [167].

The influence of the material surface on cell behavior, as well as the positive effect of nano-topography on bone cell adhesion is already well known. In this scenario, Silverwood and coworkers investigated the outcome derived from a human bone marrow-derived co-culture of osteoprogenitors and OC-progenitors on a 15 nm disordered nanopillar-structured titania surface. At 28 days of co-culture ALP, osteopontin and mineralization assays evidenced an increased osteogenesis, while qRT-PCR for OC specific genes and TRAP staining showed the absence of enhanced osteoclastogenesis suggesting a potential improved osteointegration of the nano-patterned material [168].

The positive contribution provided by ECM-mimicking components, such as calcium phosphate (CaP), has recently been underlined by Hammerl et al., who tested a medical grade polycaprolactone (PCL) microfilamentous scaffold coated with a thin layer of CaP. Both single culture and co-culture of OBs isolated from the femur head or shoulder during surgical implantation and PBMCs derived from healthy donors were tested up to 63 days in an unstimulated and growth factor-free culture system. The results showed that the PCL-CaP was superior to PCL scaffold in terms of OB and PBMC colonization. In addition, OB proliferation and ECM deposition was favoured in the presence of PBMCs, while the presence of OBs apparently suppressed the capability of PMBCs to form multinucleated cells on PCL/CaP, and none was found on PCL scaffolds. Overall, the authors concluded highlighting the relevance of the unstimulated and growth factor-free co-culture (OB/PBMC), considered as a cost-effective *in vitro* platform for the screening of scaffolds intended for bone regeneration applications [169].

The role of hydroxyapatite (HA) in inducing the formation of functional OBs and OCs from human induced pluripotent stem cells (hiPSCs) has been reported by Jeon et al. The study reported the development of a new engineered 3D human bone model by co-culturing hiPSC-derived MSCs and hiPSC-derived macrophages on 1:1 PLGA/PLLA scaffolds added with 0, 1% and 5% HA. The collected results showed as *in vitro* the composite scaffolds containing the highest percentage of HA (5%) promoted not only the OC-differentiation of hiPSC-macrophages as confirmed by NFATC1, CATK, CTR, and TRAP5b markers, but also a stronger osteogenic induction of hiPSC-MSCs compared to lower HA concentrations or PLLA/PLGA alone. Moreover, when the 5% HA containing scaffolds with co-culture of hiPSC-derived MSC/monocytes-macrophages were ectopically implanted in mice, mature lamellar bone and a greater amount of bone matrix were deposited. The authors remarked that 1) monocytes and macrophages, as OC-progenitors, play a pivotal and non-immunological regulatory role in bone formation, regeneration, and homeostasis *in vivo*; 2) local cues provided by the HA can guide intercellular signaling between hiPSC-MSCs and monocytes-macrophages to more accurately mimic bone physiology [170].

Similarly to HA, bioactive glasses are classified as materials able to provide an ECM-mimicking surface, thanks to the ability of promoting the deposition of a hydroxyl–carbonate apatite (HCA) layers on their surface when dipped in physiological solutions. According to that, 70S30C glass foam scaffolds with open interconnected macropores pre-treated with cell culture medium, induced the deposition of a layer of calcite or HCA detected by XRD analysis at 3 weeks. Clear evidence of a sustained growth and viability of MC3T3-E1 osteoblasts and C7 osteoclasts co-cultured on the HCA layer was recorded after 1 and 3 weeks. Moreover, media containing the dissolution ionic products from the cell-free 70S30C scaffold induced a consistent increase of MC3T3-E1 proliferation in 2D culture, and promoted the C7 differentiation toward mature OCs, confirming the bioactivity of the bioglass and its potential as bone substitute [171].

6.3. Angiogenesis-promoting OB:OC co-cultures

As remarked by Cenni et al., since the formation of new vessels is critical for a successful engineering of bone tissue, scaffolds should be tested for their ability to favor endothelial cell adhesion, proliferation and function, while morphological and functional relationships between endothelial cells and OBs should be evaluated with co-cultures [172].

Regarding the vascularization potential of bone substitute biomaterials, a number of studies about human 2D and 3D co-culture models, and the production of proangiogenic factors, have been reported by the group of Kirkpatrick [173,174].

In a recent study human umbilical vascular endothelial cells (HUVECs) were co-cultured with human bone marrow mesenchymal stromal cells (hBM-MSCs), and the potential of clonally derived hBM-MSCsto simultaneously support angio-/vasculo-genesis and osteogenesis has been analysed. Interestingly, the majority of hBM-MSC clones, which supported increased blood vessel formation *in vitro,* were found amongst those CFU-F-derived hBM-MSCs which supported tri-lineage (adipose-osteogenic-chondrogenic), and, to a lesser extent, bi-lineage (osteogenic-chondrogenic) differentiation, with the strength of this association being donor dependent [175]. Moreover, according to a recent meta-analysis, the co-transplantation of endothelial progenitor cells and MSCs significantly promotes angiogenesis and bone regeneration [176].

Some recent experimental models for BTE combining three-four types of cells are summarized in Table 5 (tri & quadri-culture, Table 5).

In order to analyze the interactions among circulating cells, endothelium and organ-specific microenvironments, a tri-culture system combining HUVECs plus MSCs plus osteogenic-driven MSCs (OD-MSC) was recently proposed. By applying a “Design of Experiment” statistical approach to identify key differences among experimental conditions, the authors found synergic correlations among critical parameters, such as hydrogel type, HUVEC absolute density, hydrogel thickness, HUVEC/MSC/ODMSC ratio and culture medium. They concluded that the combination of multiple parameters can affect EC self-assembly into physiological microvascular networks within a bone-mimicking environment and this could be translated to any vascularized tissue [177].

Using the indirect method based on transwell separation, Pagani et al. set up separate cultures, as well as bi-cultures and tri-cultures of mature OBs, pre-OCs, and HUVECs, maintained with a mixture of each specific culture medium proportional to the respective cell density, in order to reproduce *in vitro* a part of the complex *in vivo* bone environment.

In addition to the usual OB and OC markers, several differentiation markers, including ALP, COL1A1, OPG, RANKL, and TGF-β for OB, CATK for OC, and vascular endothelial growth factor A (VEGF-A) as endothelial-related marker, were evaluated by ELISA assay. A number of data were collected: OB viability increased significantly in presence of OCs or HUVECs, and the tri-culture showed higher values in comparison with OC single culture and OC/HUVEC co-culture, but similar values to OB/OC co-culture. HUVECs do not seem to influence OC activity, but apparently have a role in supporting OB activity by releasing TGF-β, ALP, and Coll I. In addi-

Table 5
Bone cell co–cultures with endothelial cells (tri- or quadri-culture).

OB source	OC source	Cell number OB:OC ratio	EC	SCAFFOLD	Direct/indirect contact	Static/dynamic	Conclusions	Author/year/ref no.
hMSC from hip surgery osteo-differentiated MSC (OD-MSC)	/	HUVEC:MSC:OD-MSC 1:1:0, 10:1:0, 10:1:1 ratio	Primary GFP-transfected HUVEC	Fibrin gel & fibrin-collagen type I gel	Direct	Static	HUVEC:MSC:OD-MSC 10:1:0 ratio selected	S. Bersini, 2016 [176]
Human osteoblast-like MG-63 cells	OC-precursors 2T-110 (Lonza)	$1 \times 10^4/cm^2$ OB & $1 \times 10^4/cm^2$ OC & $1 \times 10^4/cm^2$ HUVECs after 24 hrs	HUVEC (IZSBS, Brescia)	/	Indirect	Static	Advanced model for mimicking bone microenvironment. Synthetic activity of OB and OC stimulated by their coexistence; HUVEC presence promotes OB but inhibitory effect for OC	S. Pagani, 2018 [177]
Osteoprogenitor cells from stromal vascular fraction of human adipose tissue	CD14+ OC-progenitors from human peripheral blood buffy coat	2×10^6 osteoprogenitor cells & 4×10^6 CD14+ monocytes	Endothelial lineage cells from stromal vascular fraction of human adipose tissue	3D porous HA/b-TCP	Direct	Dynamic: perfusion-based bioreactor device	3D human osteoblast-osteoclast-endothelial cell co-culture as advanced *in vitro* model. Fully functional construct following ectopic implantation in nude mice	A. Papadimitropoulos, 2011 [178]
Human osteoblast-like MG-63 cells	OC-precursors 2T-110 (Lonza)	$2 \times 10^4/cm^2$ MG-63 & $4 \times 10^4/cm^2$ OCP & $2 \times 10^4/cm^2$ HUVEC	HUVEC (IZSBS, Brescia)	Quercetin-functionalized hydroxyapatite	Indirect (0.4 um transwell)	Static	Quercetin enhances MG-63 proliferation and differentiation, downregulates osteoclastogenesis of OCP, supports proliferation and differentiation of HUVEC	L. Forte, 2016 [179]
Human bone marrow –derived MSC (hBMSC)	Human peripheral blood mononuclear cells (hPBMC)	hBMSC, hPBMC, EPC-derived MNC at 1:1/3:2 ratio	Endothelial progenitor cell-derived mono-nuclear cells (EPC-derived MNC) from human umbilical cord blood	/	Direct	Static	One step-seeding procedure. Retention of OB (ALP expression), OC (TRAP-positive), and EPC phenotypes, as well as OC resorptive activity (with RANKL) using endothelial culture medium EGM2*/aMEM	A. Grémare, 2019 [180]

tion, the presence of OCs, by increasing the release of RANKL and cathepsin K in the system, could balance osteosynthesis. It is concluded that the behavior of each cell type changes depending on the presence of other cells and culture system, which also modulate the release of the involved mediators over time; therefore, the advanced cellular model described may be used as a starting point for mimicking bone microenvironment *in vivo* [178].

A 3D human-derived OB/OC/endothelial cells co-culture system was developed "to mimic the process of bone turnover" using progenitor cells and a perfusion-based bioreactor device. Following the isolation of osteoprogenitor and endothelial lineage cells from the stromal vascular fraction (SVF) of human adipose tissue, and CD14+ OC-progenitors from human peripheral blood, these cells were co-cultured with osteoclastogenic factors within 3D porous ceramic scaffolds placed in a perfusion-based bioreactor device. The model was developed in two-phases, in order to maintain the osteoclastic cells which in culture show a typically short life span, until a bone-like matrix was deposited in the scaffold pores. By applying non-invasive monitoring techniques, functional interactions among the co-cultured cell types and phenotypical changes were demonstrated, and this "bone organotypic culture" was shown to be "fully functional" following ectopic implantation in nude mice. The authors concluded that the cell-mediated processes of bone-like matrix deposition and resorption can be captured using this co-culture model, which can also be exploited toward the engineering of multi-functional bone substitute implants [179].

Forte et al. provided a tri-culture method, including OB-like cells, OC-precursors and HUVECs to test the effect of adding quercetin, an antioxidant and anti-inflammatory flavonoid, on hydroxyapatite (HA), in order to potentiate the bone forming activity of the bone-like scaffold. The triculture system was assembled using disk-shaped samples, with OBs seeded on the surface and OC-precursors placed within inserts put in the HUVEC seeded-wells: the co-culture was maintained up to 14 days using a mixture of each culture medium according to the cell density proportion. In the presented system, an enhanced proliferation and improved activity of OBs on the quercetin-added scaffolds was found, while osteoclastogenesis was downregulated due to a high OPG/RANKL ratio, and no change for co-cultured HUVECs was recorded [180].

The recent study of Grémare et al. aimed at establishing a "simple" procedure for a direct tri-culture model by one-step seeding of human primary cells on artificial 2D cell culture in plastic or bone-like environment (micro- macro-porous biphasic CaP, MBCP™, Biomatlante). Primary human bone marrow stromal cells (hBMSCs), cord blood-derived mononuclear cells (MNCs) and endothelial progenitor cells (EPCs) were co-cultured and fed with a mix of a-MEM and endothelial basal medium (EBM) added with a series of growth factors, to be assayed at 3, 7 and 11 days. Thanks to an accurate mix of culture media and additives, the different cell phenotypes were maintained in the co-culture. In addition, after 11 days, hBMSCs expressed ALP, while multinucleated TRAP-positive cells apparently increased, with a resorptive activity of the bone-like substrate observed after addition of RANKL [181].

Some authors tried to better understand the OB/OC cooperation in endochondral ossification using co-culture models. It is known that long bones develop through endochondral ossification, where MSCs differentiate into chondrocytes and a cartilaginous anlage is formed to guide blood vessels. In this formation process, blood vessels enter the anlage, recruiting osteoprogenitors and OCs in order to degrade the cartilaginous template that will be replaced by new bone tissue. The progressive substitution of an avascular cartilage template by a highly vascularized bone tissue is the characterizing feature of endochondral ossification. However, few studies investigated the potential of co-cultures to retrace the endochondral ossification. An *in vitro* bone regeneration strategy, involving the use of co-cultures of MSCs, endothelial cells, and chondrocytes, has been explored by Freeman et al. The study evidenced that mimicking the cellular niche existing during endochondral ossification could obviate the need for osteogenic supplements to induce osteogenesis in a 3D cellular aggregate *in vitro*. Considering a non containing osteogenic supplements MSC/HUVEC co-culture, the production of early (ALP) and late (calcium) osteogenic markers of MSCs and the formation of rudimentary vessels *in vitro* were significantly enhanced by applying both chondrogenic and vascular priming [182].

To develop an *in vitro* vascularized 3D MiniTissue bone remodeling model, Bongio et al. proposed the combination of four cell types, *i.e.* HUVECs, hBMSCs, and precursors of human OBs and OCs (10:1:1:2 final ratio), in a tetra-culture which was embedded in collagen type I-fibrin (Col/Fib) hydrogels enriched with different concentrations of calcium phosphate nanoparticles (CaPn). The mixture of collagen and fibrin was prepared as a 3D substrate promoting vasculogenesis, while the presence of CaPn influenced OB and OC differentiation. As reported by the authors, "using minimal amounts of cells and reagents compared with standard macroscale 3D cultures" this system confirmed the mechanism of cooperation of bone cells and their interaction with the endothelial cells, up to a microvascular network formation, and could be used to model a specific disease using patient's cells [183].

7. Discussion

The key-role of cell contact and interactions in determining cell fate and activity was already suggested in a pioneering study, where Ball et al. described the phenotypic changes of MSCs co-cultured with either smooth muscle cells, endothelial cells or dermal fibroblasts using direct and indirect co-culture systems, leading to the conclusion that MSCs both influence, and are profoundly influenced by other cells in direct contact. These findings have fundamental implications for the modulation of MSC phenotype in the vasculature in development and repair [184].

As outlined by Goers, the main reasons for conducting co-culture experiments include the study of natural interactions, the improvement of the culture outcome or the engineering of "synthetic" interactions, such as the reproduction of cell cooperation to achieve tissue regeneration [185].

The importance of considering OCs in BTE, instead of only focusing on bone-forming cells, was further underlined by Detsch and Boccaccini. Since OCs control OBs, co-cultures of OBs and OCs may be beneficial to the bone scaffold micro-environment [186].

More recently, OB-OC co-culture models and the selection of the various parameters involved in the system have been reviewed by Zhu et al. in order to better understand pathological changes in metabolic bone diseases and identify drug targets [187]. In order to hypothesize optimal *in vitro* bone models for basic research, drug development and toxicology, Scheinpflug et al. have recently reviewed the current *in vitro* systems to recreate bone biology [161]. The identification of a standard model of bone cell co-culture is a challenging issue, due to the different cell types and culture parameters that can be selected by different research groups, depending on the main target of the study and the experimental protocol adopted. Starting from the need to culture cells in 3D environments to closely resemble the *in vivo* situation, one of the main challenges is represented by the inherent difficulty of controlling the relevant cell processes, as noted by Papadimitropoulos [179], even if the use of indirect systems presents less issues when compared with direct contact models. An interesting method developed recently, which has been reported to be successful both in direct and indirect modeling, concerns the use of a removable permeable divider for temporally and spatially controlling cellular interactions, both in the case of cell-cell contact and paracrine signaling. These interactions can be monitored in combined or sin-

gle populations [188]. Indeed, both direct and indirect co-culture systems provide information on the OB:OC cooperation *in vivo*, since these cells communicate through ligand-receptor signals and paracrine factors.

With regard to the type of cells that can be selected, both cell lines and primary cells have been widely used, despite the heterogeneity, sensitivity and the inherent complexity of cell isolation and manipulation shown by primary cells. As a matter of fact, the use of human tissue derived cells is a fundamental resource in the design of experimental models for *in vitro* testing, and human induced pluripotent stem cells (hiPSC) have been suggested as a patient-specific alternative [170]. As reported by Caddeo et al., when designing 3D engineered testing systems to reproduce the native tissue and the micro-environment, many issues are still open. Among these are the most appropriate selection of optimal material(s) for the scaffold design, cell source and biofabrication technologies, and the biochemical and physical signals given by the cell culture conditions [189].

The temporal and spatial parameters involved in cell seeding, *i.e.* simultaneous or sequential loading of different cells on the scaffold, in a layer or multi-layer arrangement, can also influence the phenotype of cells and their interactions [190].

Kholi et al. discussed the complex challenge of mimicking the bone remodeling process *in vitro*, highlighting the incorrect sequence of events in several co-culture models often used to test biomaterials. According to these authors, the biomaterials being tested should be put in direct contact with macrophages to mimic the inflammatory phase of bone healing, and be pre-mineralised before bone-forming cells seeding, since *in vivo* bone resorption always precedes tissue formation [191].

The important role of co-cultures in the bone remodeling process and the "constant interaction" between OBs, OCs and macrophages has been recently reviewed, and the use of *in vitro* cellular models has been highly recommended for the preliminary determination of cell-material interactions [192]. Moreover, the culture conditions and some medium additives could positively affect one type of cells, while hampering the other: while the potential presence of substances inhibiting OC differentiation was shown in serum, conversely, the addition of osteogenic promoting factors β-glycerophosphate and ascorbic acid during the latter stage of a primary mouse bone marrow co-culture increased the RANKL-induced osteoclast formation [133,193].

Hypoxia, *i.e.* 2.5% O_2, applied to a primary human PBMNC/OB co-culture apparently triggered the differentiation of PBMNCs to OCs in the presence of OBs in a HIF-dependent manner [194]. In contrast, the conditioned medium derived from MC3T3-E1 osteoblastic cells treated with strontium dose-dependently inhibited OC differentiation and resorption activity, due to an increased expression and secretion of osteoprotegerin. Therefore, strontium, known to promote OB activity, could exert an uncoupling effect on bone [195].

Each co-culture system is normally designed to address one or more specific research questions.

A statistical software was exploited by Bersini et al. in order to identify the best combination of parameters (cell ratio, hydrogel type, culture medium, oxygen gradient, etc.) and the minimum set of experiments required for the generation of a physiological-like vascular network [177]. The study identified a final combination where endothelial cells, MSCs and osteo-differentiated MSCs were co-cultured to generate bone-mimicking pre-vascularized matrices with pervious microvessels, therefore demonstrating that the model employed was able to screen different experimental conditions for use in TE applications.

The co-culture model can also potentiate innovative techniques, such as additive manufacturing technologies and specifically the bioplotting of materials with cells enabling biofabrication of bone tissue, as shown by Zehnder et al., who observed increased levels of OPN, TRAP and vascular endothelial growth factor (VEGF) after plotting ST2 OBs and RAW pre-OCs in oxidized alginate-gelatin crosslinked hydrogels [196].

In conclusion, co-culture patterns allow the exploration of cell-cell interactions, and the replication of those naturally occurring in multi-cellular tissues can increase the possibility of designing a reliable bone engineering model. A shared optimal 3D co-culture protocol for human bone cells to accurately replicate the bone micro-environment is still lacking, but a great deal of helpful data and interesting strategies are currently available.

As fostered by the principles of 3 Rs for human research, *in vitro* co-culture systems using human cells may support or even represent a valid alternative to animal testing in the future. Indeed, the mechano-biological environment can be successfully recreated using 3D co-culture dynamic models, and basic regenerative mechanisms of bone can be identified also thanks to new imaging modalities [197]. Moreover, *in silico* models provide data for tuning the dynamic parameters applied to culture systems, in order to better resemble the native tissue responses to scaffolds [198].

Finally, current *in vivo* models are being designed taking into account all the different factors related to the healing site, such as the presence of macrophages, the angiogenic process and the interaction between the regenerative cells and the immunological counterparts: all these additional components have been identified or characterized in recent studies *in vitro* and should be adopted whenever possible [199–201].

8. Future outlook

Human primary cells can be definitely endorsed as a gold standard in the design of co-cultures, in order to achieve reliable conclusions and potential translation to the clinical settings, despite the patient-to-patient biological variability that hinders a good reproducibility of results.

To decrease the donor heterogeneity of human primary cells, Pennings et al. explored the use of human induced pluripotent stem cell (hiPSC)-derived endothelial cells in co-culture with MSCs and endothelial colony forming cells (ECFCs). They demonstrated the generation of pre-vascularized constructs, suggesting the use of iPSC-derived cell lineages as a uniform cell source for pre-clinical and clinical settings [202]. Similarly, Jeon et al. too, suggested hiPSCs as an exciting prospective cell source for BTE, thanks to their ability to differentiate into OBs and OCs [170].

Quite recently the bone co-culture model has lended itself to cancer research, and preclinical models of breast cancer cells and bone stromal cells, as well as OCs, have been developed to mimic the bone micro-environment of primary tumors and metastases [203].

Most of the studies thus support the use of 3D culture systems for future investigations regarding better understanding of OB/OC cooperation. In this context, the micromass can be considered as an easy method for 3D culture, *i.e.* the pelleting of cells which aggregate thanks to the self-produced ECM [204]. An additional promising strategy is also represented by heterotypic cell spheroids combining ECs, MSCs and their differentiated progeny [109].

Up to now, a growing trend toward the use of hydrogels, such as PEG, gelatin, chitosan, *etc*., has been observed as they can provide a tunable micro-environment for both short- and long-term 3D co-culture settings, with the opportunity to analyze the role of paracrine interactions on the cell phenotype [205,206].

The use of perfusion systems, bioreactors, rotating vessels or microfluidic systems to form dynamic co-cultures able to provide cells with enhanced cell adhesion ability, continuous nutrients and oxygen renewal, as well as mechanical stimuli, is thus highly recommended [207]. According to Vetsch et al. mathematical simula-

tions of TE systems, such as calculation of stress and strain distributions, fluid shear stress and bone ingrowth, as well as scaffolds properties, may allow more realistic design of *in vitro* studies [208]. As recently reviewed by Ahmed et al., the ability of dynamic systems to non-invasively monitor the cellular microenvironment *in vitro* may positively affect the TE field.

Microfluidic technologies have been specifically developed to create micro-engineered platforms that mimic the complex network of cells and the nature of vascularized bone formation: the interplay between co-cultured bone cells or MSCs with ECs and scaffolds, which is ultimately necessary for adequate regeneration of bone defects, is well reproduced by these dynamic systems [209–211].

Three-dimensional (3D) bioprinting is now a versatile technology to fabricate biomimetic substrates reproducing reliable replicas of natural tissues. As an example, a 3D-printed poly(ε-caprolactone) multi-compartment structure realised by means of Fused Filament Fabrication technique, hosting human-TERT MSCs and HUVECs, encapsulated within Sr^{2+} ion-containing gellan gum-based hydrogels, has been exploited as an advanced *in vitro* co-culture model [212]. Instruments, methods, bioinks and cells to achieve 3D or 4D bioprinted tissues, and the translational potential of these resources have been recently reviewed [213].

In particular, the application of co-cultures within the field of BTE requires a special focus on MSC/EC cooperation, considered a necessary step for the construct survival after implantation. Indeed, the vascularization of new bone by new endothelial tubules is essential for regeneration. Several 3D co-cultures are currently designed to simulate the angiogenic support to bone formation in pre-clinical models of tissue regeneration [214,215], and the current vascularization strategies have been reviewed for TE [216], drug screening and predictive toxicology [217].

Another aspect that needs to be considered in future BTE studies is the presence of an inflammatory milieu at the site of implantation. As underlined by Smits et al., any TE construct, even if not immunogenic, will trigger an inflammatory reaction of the host, involving cells of the innate immune system, *i.e.* monocytes-macrophages [218]. Therefore, the introduction of monocytes or macrophages in the co-culture makes the model more similar to the *in vivo* setting, and may also be useful to develop ”immunomodulatory” biomaterials which harness monocyte-macrophage activities toward bone regeneration [219,220]. The immunomodulation ability of MSCs in co-cultures still needs to be explored extensively. As an example, Saldana et al. have recently tested MSCs stimulated with factors secreted from macrophages polarized toward pro-inflammatory or anti-inflammatory phenotype, showing that the immunomodulatory potential of MSCs is activated by macrophage-secreted factors, and further enhanced after MSC encapsulation in hydrogels [221].

Considering that extracellular vesicles (EVs), including exosomes and microvesicles, are well known mediators of cell-cell communication, the bidirectional interplay between OBs and OCs after reciprocal delivery of secreted EVs represents an important aspect that definetely requires further investigation [222,223].

As addressed in the present review, the *in vitro* simulation of the OB/OC coupling in bone tissue may be approached using a number of resources and innovative tools currently available, in order to obtain relevant information on the bone remodeling/regeneration process. It can be concluded that refined and optimised co-culture methods can potentially be associated to the advance of future tissue engineering applications.

Declaration of Competing Interest

None.

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