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Bone marrow/bone pre-metastatic niche for breast cancer cells colonization: The role of mesenchymal stromal cells

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ABSTRACT

Breast cancer is one of the most common oncological pathologies in women worldwide. While its early diagnosis has considerably improved, about 70 % of advanced patients develop bone metastases with a high mortality rate. Several authors demonstrated that primary breast cancer cells prepare their future metastatic niche –known as the pre-metastatic niche- to turn it into an "optimal soil" for colonization. The role of the different cellular components of the bone marrow/bone niche in bone metastasis has been well described. However, studying the changes that occur in this microenvironment before tumor cells arrival has become a novel research field. Therefore, the purpose of this review is to describe the current knowledge about the modulation of the normal bone marrow/bone niche by the primary breast tumor, in particular, highlighting the role of mesenchymal stem/stromal cells in transforming this soil into a pre-metastatic niche for breast cancer cells colonization.

1. Introduction

Breast cancer is the most common cancer type among women, accounting for 24.2 % of all female cancers and more than 2 million new cases worldwide in 2018 (van der Meer et al., 2020). According to the GLOBOCAN Cancer Tomorrow prediction tool, breast cancer global incidence will increase by more than 46 % by 2040 (Heer et al., 2020). Despite advances in early diagnosis, 20%–30% of breast cancer patients (BCPs) in an early clinical stage will relapse and die as a result of the complications generated by the spread of breast cancer cells (BCCs) from the primary tumor to distant tissues, in the process known as metastasis (Chin and Wang, 2016; Kennecke et al., 2010). The most common sites to which BCCs preferentially metastasize include bone, liver, lung, and brain (Wu et al., 2017). In particular, about 70 % of advanced BCPs

Abbreviations: Ab, antibody; ABCPs, advanced breast cancer patients; BCCs, breast cancer cells; BCPs, breast cancer patients; BM, bone marrow; BMDCs, BM-derived cells; CAFs, cancer-associated fibroblasts; CCL-2, chemokine (C—C motif) ligand 2; CFU-F, colony-forming units; DKK-1, factors Dickkopf-1; DTCs, disseminated tumor cells; ECM, extracellular matrix; EMT, epithelial to mesenchymal transition; EVs, extracellular vesicles; FGF, fibroblastic growth factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; HGF, hepatocyte growth factor; HIF1α, hypoxia-inducible factor 1α; HSCs, hematopoietic stem cells; HSPCs, hematopoietic stem and progenitor cells; HV, healthy volunteers; ICAM-1, Intercellular Adhesion Molecule 1; IDO, indoleamine-2,3-dioxygenase; IL-6, interleukin-6; ISCT, International Society for Cellular Therapy; ISEV, International Society for Extracellular Vesicles; LIF, leukemia inhibitory factor; LIFR, LIF receptor; LOX, lysyl oxidase; mAb, monoclonal antibody; M-CSF, macrophage colony-stimulating factor; MDSCs, myeloid-derived suppressor cells; mRNAs, messenger RNAs; miRNAs, microRNAs; MSCs, mesenchymal stem/stromal cells; NB, neuroblastoma; NR2F1, nuclear receptor subfamily 2 group F number 1; OPG, osteoprotegerin; OPN, osteopontin; PDGF, platelets-derived growth factor; PG, propagermanium; PGE2, prostaglandin E2; PMN, pre-metastatic niche; PTHrP, parathyroid hormone-related protein; RANKL, receptor activator of nuclear factor-κB ligand; ROS, reactive oxygen species; RUNX2, RUNX Family Transcription Factor 2; SCF, stem cell factor; sEVs, small extracellular vesicles; TA-MSCs, tumor associated-MSCs; TGF-β, transforming growth factor-beta; TIMP-2, TIMP metallopeptidase inhibitor 2; TLRs, toll-like receptors; TNTs, tunneling nanotubes; TPO, thrombopoietin; TSP-1, thrombospondin-1; VCAM-1, vascular cell adhesion molecule 1; VEGF, vascular endothelial growth factor; VEGFR-1, vascular endothelial growth factor receptor 1; 3D, tridimensional; 5-FU, fluorouracil.

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(ABCPs) develop osteolytic bone metastasis, with a higher mortality rate, median overall survival of 40 months, reduced quality of life, and several clinical complications, including pain, fracture, spinal cord compression, and hypercalcemia (Zhang et al., 2019; Sun et al., 2019; Brook et al., 2018).

The development of bone metastasis, as it happens with all types of metastases, is a complex and inefficient process that involves several steps. First, changes occur in the primary tumor, including angiogenesis, and the epithelial to mesenchymal transition (EMT) that facilitates the migration and invasion of cancer cells into the surrounding stroma. Through EMT, tumor epithelial cells transdifferentiate into a mesenchymal-like phenotype (E-cadherin^{-}, occluding^{-}, α-catenin^{-}, claudins 3/4/7^{-}, N-cadherin^{+}, vimentin^{+}, fibronectin^{+}), with a higher metastatic potential (Guarino et al., 2007). Next, cancer cells intravasate into circulation, either through peripheral blood or lymphatics vessels. Within the circulatory system, disseminated tumor cells (DTCs) can activate platelets to protect themselves from the forces induced by blood flow velocity, avoiding elimination by immune cells -mainly natural killer cells- and promoting their arrest by endothelial cells (Gay and Felding-Habermann, 2011; Huang et al., 2018). Also, macrophages play an essential role in promoting tumor cells survival in the circulation by direct cell-cell contact for the transmission of survival signals (Chambers et al., 2001). The final step includes the bone marrow (BM)/bone extravasation of DTCs, the survival of a small set of them and quiescence -or dormancy- for several years, and their eventual reactivation and progression into micro and macrometastasis (Zarrer et al., 2020).

2. The pre-metastatic niche

Apart from the mentioned processes inherent to the metastatic cascade, Kaplan RN. et al. demonstrated that the formation of a pre-metastatic niche (PMN) is a crucial preceding step for the development of metastases, through an experiment in which vascular endothelial growth factor receptor 1 (VEGFR-1) positive BM-derived cells (BMDCs) were attracted to the second site before tumor cells arrival (Kaplan et al., 2005). The blocking of VEGFR-1 through an antibody (Ab) or the elimination of VEGFR-1-positive cells in wild-type mice blocked the PMN formation and metastasis development (Kaplan et al., 2005). Later, it was proved that cancer cells need a favorable environment in the second site with nutrients, a remodeled extracellular matrix (ECM), and supportive signals from the stromal cells, for their successful colonization (Hill et al., 2020). Interestingly, in 1889 Sir Stephen Paget made the first known reference to the concept of the PMN, in his known "Seed and Soil" theory. He proposed that tumor cells (seeds) can only grow in certain specific and permissive microenvironments (soil) with factors that would be advantageous for the metastatic process (Paget, 1989). Nowadays, it is known that this fertile microenvironment is mainly composed of a remodeled ECM and stromal cells from that particular microenvironment, BMDCs, and paracrine factors secreted by the primary tumor to prepare their second site (Zhou et al., 2020). Those factors secreted by tumor cells, either as soluble or contained in extracellular vesicles (EVs), play an important role in the preparation and conditioning of the PMN, and even in the determination of the organotropism of the metastases through the presence of specific integrins in EV membranes (Peinado et al., 2011).

In general, the main characteristics of any PMN include angiogenesis and vascular permeability, ECM remodeling, an abundance of both inflammatory and immunosuppressive cytokines and chemokines such as interleukin-6 (IL-6), IL-10, transforming growth factor-beta (TGF-β), vascular endothelial growth factor (VEGF), granulocyte-macrophage colony-stimulating factor (GM-CSF), chemokine (C-C motif) ligand 2 (CCL-2), *etc.*; as well as metabolic reprogramming of stromal cells (Zhou et al., 2020; Peinado et al., 2011; Shu et al., 2018). In this way, Liu Y. et al. proposed the existence of different sequential stages in preparing the PMN (Liu and Cao, 2016). First, a "priming phase", with ECM remodeling and stromal cells reprogramming by soluble factors and EVs secreted by the primary tumor. Second, the "licensing phase" involves paracrine recruiting of BMDC and immune regulatory cells to the second site by factors secreted by primary tumor cells to develop a mature niche for DTCs to promote their aggressiveness. Third, an "initiation phase", during which previously "educated" stromal cells facilitate the extravasation and attachment of DTCs, and they collaborate with either the regulation of DTCs proliferation or their entry into a dormancy state. Finally, the PMN can evolve into a "metastatic niche" following tumor cell engraftment (Liu and Cao, 2016). Similarly, Zhou Y. et al. stated the four sequential phases that describe the establishment of the PMN: "I. remote control from primary tumor; II. recruitment of immunosuppressive cells; III. microenvironment preparation; IV. circulating tumor cells colonization" (Zhou et al., 2020).

In particular, the BM/bone niche results suitable for the anchorage of tumor cells and the development of metastasis due to several intrinsic characteristics. First of all, the presence of different soluble factors promotes the survival and proliferation of tumor cells, such as IL-6, IL-8, TGF-β, and Ca^{+2} (Zarrer et al., 2020). In addition, it is a very vascularized tissue with abundant blood flow and the presence of a particular profile of adhesion molecules in endothelial cells -*i.e.*, vascular cell adhesion molecule 1 (VCAM-1) that interacts with α4β1 integrin expressed by DTCs-, facilitating DTCs extravasation (Schneider et al., 2011). BM is also a highly hypoxic tissue, with oxygen levels ranging from <1% to 6% that promote reactivation of dormant DTCs and drug resistance as well (Johnson et al., 2017). Finally, but not less important, the BM is characterized for the presence of specific subniches with various cell types that collaborate in the maintenance of hematopoietic stem and progenitor cells (HSPCs), including endothelial cells, mesenchymal stem/stromal cells (MSCs), osteoblasts, osteoclasts and adipocytes, among others (Crane et al., 2017; Peinado et al., 2017). Hematopoietic stem cells (HSCs) share some molecular features with DTCs, since the latter has similarities with the biology of cancer stem cells (CSCs). So, HSCs may play a relevant role in BM/bone metastatic progression (Hen and Barkan, 2020). Although BM metastasis is considered a subtype of bone metastasis, evidence suggests that the BM involvement in metastatic spread may represent a precondition for bone metastasis, with components of the BM "preparing" the development of PMN in the bone (Pedersen et al., 2012).

3. Normal bone marrow niche

In order to understand the role of the BM niche in its colonization by DTCs, it is necessary to explain the normal physiological dynamics of this microenvironment. The BM is composed of different compartments, which mutual communication is critical for BM/bone integrity. On the one hand, the endosteum contains osteoblasts responsible for bone formation and osteoclasts that resorb bone, as well as MSCs that collaborate regulating hematopoietic homeostasis and osteogenesis (Chen et al., 2018; Bianco et al., 2013). This niche allows the maintenance of the HSC in a quiescent state and its retention through different soluble factors, hypoxia and Ca^{+2} (Li, 2011). On the other hand, the vascular niche contains the sinusoidal endothelium, pericytes, and smooth muscle cells; it recruits MSCs as well as endothelial cells and their progenitors, and promotes HSCs proliferation, mobilization, and differentiation (Haider et al., 2020).

Regarding the vascular niche, the endothelial cells are critical components of this niche. They support the proliferation of HSCs through interaction *via* endothelial selectin, and secrete factors implicated in HSCs maintenance, such as the chemokine CXCL12 - also known as stromal-derived factor-1- an essential factor for the retention and homing of HSCs in the BM (Chute et al., 2006; Winkler et al., 2012; Teicher and Fricker, 2010). Nestin^{+} MSCs also reside in this niche, collaborating with HSCs maintenance (Bianco et al., 2013). Non-myelinating Schwann cells, a type of glial cells, have also been implicated in HSCs regulation as the primary source of TGF-β that induces HSC quiescence *in vitro* (Yamazaki et al., 2011, 2009). Furthermore, the

sympathetic nervous system contributes to HSPCs trafficking from the BM by regulating CXCL12 production (Katayama et al., 2006).

Osteoblasts are one of the key components of the endosteum. They originate from MSCs, and several factors regulate their differentiation process, including parathyroid hormone, the RUNX Family Transcription Factor 2 (RUNX2), osterix transcription factor, and the Wnt pathway (Komori, 2006; Garg et al., 2017). During bone formation, mature osteoblasts are responsible for the secretion of type I collagen, alkaline phosphatase, and osteocalcin, and these cells can be localized in a quiescent state on the bone surface -in the so-called lining cell- or embedded in the bone matrix as differentiated cells: the osteocytes (Maes et al., 2010). Osteocytes are connected through gap junctions, forming a tridimensional (3D) web in the bone matrix, where they can translate mechanical stimuli into biochemical signals to promote bone formation, as well as regulate osteoblasts and osteoclasts differentiation and activity (Capulli et al., 2014). Osteocytes, osteoblasts, and MSCs are the main sources of the receptor activator of nuclear factor-κB ligand (RANKL) that binds to its receptor RANK on the surface of pre-osteoclasts, stimulating their differentiation into osteoclasts (Xiong et al., 2018). In addition, osteocytes can negatively regulate MSCs differentiation into osteoblasts by secreting the factors Dickkopf-1 (DKK-1) and sclerostin, both antagonists of the Wnt pathway (Capulli et al., 2014; Atkinson and Delgado-Calle, 2019). Furthermore, osteoblasts negatively regulate osteoclasts differentiation and activity through the secretion of osteoprotegerin (OPG) -the soluble receptor of RANKL- that attenuates bone resorption by blocking the effects of RANKL (Rahim et al., 2014; Khosla, 2001). Osteoblasts also maintain HSCs dormancy by cell-cell interactions and the secretion of different cytokines, including the stem cell factor (SCF), thrombopoietin (TPO), angiopoietin-1, and CXCL12, among others (Li, 2011; Jung et al., 2006; Qian et al., 2007).

In contrast with osteoblasts, osteoclasts are large multinucleated cells that resorb bone by releasing hydrogen ions that acidify the bone interface, as well as by secreting lysosomal enzymes -such as tartrate-resistant acid phosphatase and cathepsin K- to degrade the organic components of the bone matrix (Park-Min, 2018; Kolb and Bussard, 2019). These cells originate from myelomonocytic progenitors in response to macrophage colony-stimulating factor (M-CSF) and RANKL, both osteoclastogenic factors secreted by osteoblasts, among other factors (Park-Min, 2018; Jacome-Galarza et al., 2019; Boyle et al., 2003). Additionally, osteoclasts secrete different factors that regulate the activity of osteoblasts, such as bone morphogenetic protein-6 and sphingosine-1-phosphate (Sims and Martin, 2015).

Therefore, as can be understood from the intricate molecular communication between osteoblasts and osteoclasts, this process is crucial for maintaining the BM/bone niche under physiological conditions. An imbalance in this communication is responsible for several bone pathologies, including osteoporosis and fractures. Moreover, both osteolytic and osteoblastic bone metastases are related to increased osteoclastic activity and bone architecture changes in patients with multiple myeloma, breast, prostate, and lung cancers (Schneider et al., 2011; Mundy, 2002; Macedo et al., 2017). It is known that BCCs commonly disrupt BM/bone homeostasis by promoting bone resorption and osteoclastogenesis (Kolb and Bussard, 2019). They also educate stromal cells in the BM niche for their own benefit by making them pro-tumoral (Kolb and Bussard, 2019).

Interestingly, in recent years, some similarities were found between osteoimmunological disorders - *i.e.*, osteoporosis and rheumatoid arthritis- and breast cancer. Breast malignant microcalcifications are characterized by the deposition of calcium crystals -mainly containing calcium oxalate or hydroxyapatite- by BCCs that undergo the EMT and differentiate into osteoblast-like cells (Bonfiglio et al., 2020). A positive correlation between the presence of osteoblast-like BCCs and the occurrence of bone metastasis within five years from the diagnosis was recently demonstrated (Bonfiglio et al., 2020). Additionally, evidence suggests that the inflammatory profile of the breast tissue throughout women's life could influence the development of malignant microcalcifications and consequently, breast tumors (Clemenceau et al., 2020). Furthermore, the presence of osteoblast and osteoclast-like cells in breast tumors can give rise to a bone resorptive microenvironment-like in the breast, which was found to promote aggressiveness in BCCs (Clemenceau et al., 2020).

4. Bone marrow as a pre-metastatic niche

The way to establish the permissive BM/bone PMN and its colonization by BCCs is a complex process that is not yet fully understood. The paracrine communication between BCCs in the primary breast tumor and the BM/bone niche promotes the PMN establishment and balances bone metabolism towards bone destruction (Mishra et al., 2011) (Fig. 1). BCCs in the primary tumor secrete different factors that enhance bone resorption, including lysyl oxidase (LOX), parathyroid hormone-related protein (PTHrP), osteopontin (OPN), and CCL-2 (Shevde et al., 2010; Martin and Johnson, 2019; Malanchi et al., 2011; Lu and Kang, 2009). Particularly, LOX is a collagen-cross linking enzyme secreted mainly by estrogen receptor-negative hypoxic BCCs, which directly promotes bone resorption by acting on osteoblasts and osteoclasts in the BM (Cox et al., 2015). Additionally, LOX is involved in ECM remodeling in bone, making this niche more permissive for DTCs (Cox et al., 2015).

Prior to seeding, DTCs must home to the BM/bone PMN. This homing process implies interactions between BCCs and BM endothelial cells in the vascular niche, such as the reported αvβ3/OPN, CD44/OPN, α4β1/VCAM-1 and CXCR4-CXCL12 interactions, respectively (Ponzetti and Rucci, 2019). BCCs overexpress the CXCR4 receptor that interacts with the chemokine CXCL12 secreted by BM cells for HSC homing (Chatterjee et al., 2014). In this way, Zhang X. et al. demonstrated that the expression of CXCL12 was higher in bone metastases than in other metastatic sites (Ridge et al., 2017). Upon their arrival to the BM/bone niche, it was found that BCCs localize preferentially in the vascular niche, where they remain dormant due to their interaction with the endothelium through thrombospondin-1 (TSP-1) (Ghajar et al., 2013). Furthermore, several murine models proved a competition between tumor cells and HSCs in the endosteal and vascular niches by direct interaction with osteoblasts and osteoclasts. These heterotrophic adherent junctions can occur *via* E-cadherin present in BCCs and N-cadherin in osteoblasts -that enhances BCCs proliferation through activation of mTOR and AKT pathways-, as well as *via* αVβ3 integrin expressed by BCCs that interact with BM resident cells and with OPN ligand, promoting osteolysis (Wang et al., 2015; Sowder and Johnson, 2018).

Once tumor cells infiltrate the BM/bone PMN, they can enter a dormancy state by activating the p38 MAPK stress-response pathway, resulting in a high p38 MAPK to ERK1/2 signaling ratio (Clements and Johnson, 2019). In BCCs, this phenotype can be promoted by factors secreted by BM stromal cells in the perivascular niche -such as TSP-1 and MSCs-derived microRNAs (miRNAs) 222/223- and by BCCs intrinsic signaling -including leukemia inhibitory factor (LIF)/LIF receptor, p38, and pro-dormancy gene programs- (Clements and Johnson, 2019). LIF –involved in bone remodeling and hematopoiesis regulation in the BM- is also a pro-dormancy signal in BCCs, as it interacts with the LIF receptor (LIFR) expressed by these cells (Johnson et al., 2016; Santos et al., 2020).

It remains unclear which factors mediate the transition from dormancy state into BCCs proliferation. However, it has been proposed that BM stromal cells can release factors that could reactivate dormant BCCs, such as IL-6 and IL-8, which are also known to be pro-osteoclastogenic (Tivari et al., 2018; Amarasekara et al., 2018). Furthermore, a higher activity of osteoclasts induces VCAM-1 expression in endosteal micrometastatic BCCs (Tivari et al., 2018). It was proved that the overexpression of PTHrP in BCCs causes downregulation of specific dormancy-associated genes as well as bone resorption *in vivo* (Clements and Johnson, 2019). Regarding hypoxia, there are conflicting reports. It was pointed as a promoter of DTCs dormancy escape in the

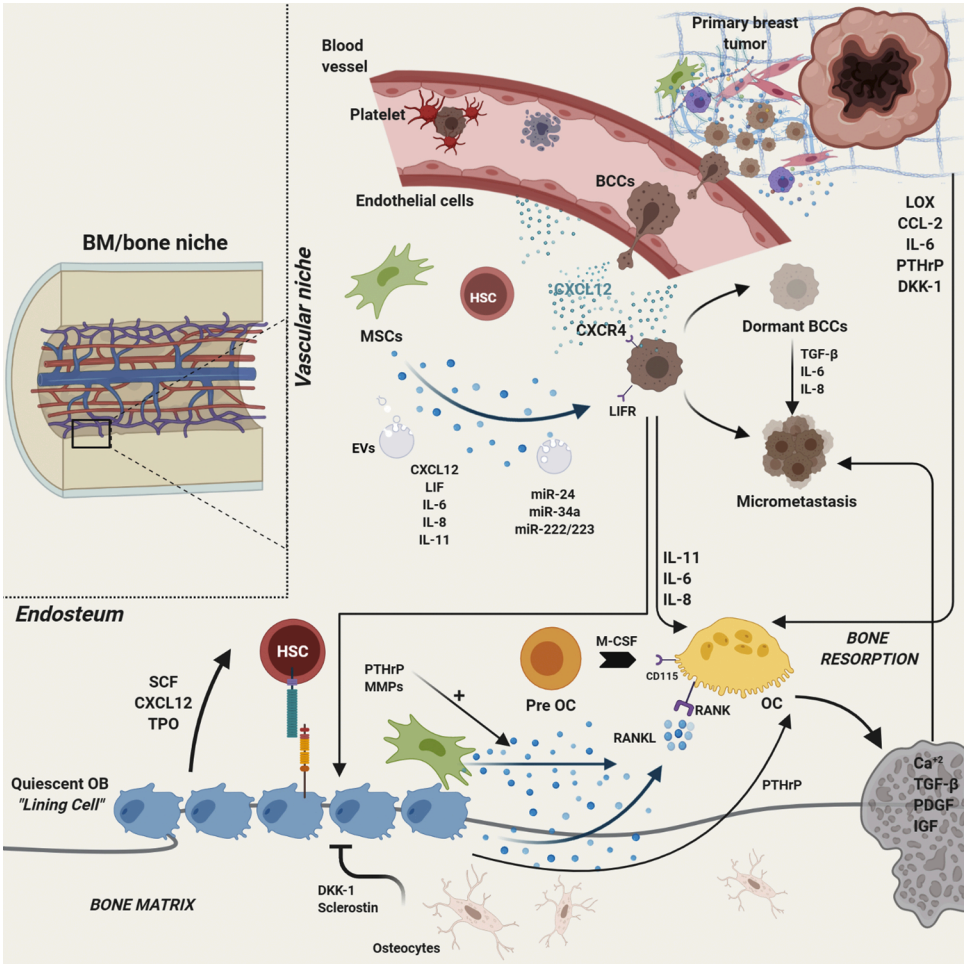

Fig. 1. Summary of the main interactions between breast cancer cells in the primary tumor, disseminated tumor cells, and stromal cells in the bone marrow/bone premetastatic niche. BCCs in the primary tumor secrete different paracrine factors, including LOX, CCL-2, IL-6 and DKK-1 that promote osteoclastogenesis and bone resorption. OB reside in the "lining cell" in the endosteum, where they establish cell-cell contacts with HSCs and secrete CXCL12, TPO and SCF to maintain HSCs in a quiescent state. OB also secrete RANKL that binds its receptor RANK in pre−OC to promote their differentiation into OC. Osteocytes secrete DKK-1 and sclerostin to downregulate MSCs differentiation into OB, and OPG that inhibits osteoclastogenesis by binding RANKL. Endothelial cells in the vascular niche produce CXCL12 that not only participates in HSCs homing and maintenance, but also is involved in disseminated BCCs homing to the BM. Once they arrive to this niche, BCCs can enter a dormancy state and later reactivate to develop micro and macrometastasis. MSCs can secrete soluble factors, such as CXCL12, IL-6, IL-8 and LIF that are known to be pro-tumoral, as well as EVs containing pro-dormancy and pro-tumoral miRNAs. BCCs in the BM secrete IL-11, IL-6 and IL-8 that promote osteoclastogenesis, and in turn, the factors released during bone resorption stimulate BCCs proliferation and metastatic outgrowth, giving rise to the vicious cycle of bone metastasis. Created with BioRender.com.

BCCs: breast cancer cells; BM: bone marrow; CCL-2: chemokine (C-C motif) ligand 2; DKK-1: factors Dickkopf-1; EVs: extracellular vesicles; HSCs: hematopoietic stem cells; IGF: insulin growth factor; IL-6: interleukin-6; LIF: leukemia inhibitory factor; LIFR: LIF receptor; LOX: lysyl oxidase; M-CSF: macrophage colony-stimulating factor; MMPs: metalloproteinases; miRNAs: microRNAs; miR: microRNAs; MSCs: mesenchymal stem/stromal cells; OB: osteoblasts; OC: osteoclasts; OPG: osteoprotegerin; PDGF: platelets-derived growth factor; PTHrP: parathyroid hormone-related protein; RANKL: receptor activator of nuclear factor-κB ligand; SCF: stem cell factor; TGF-β: transforming growth factor-beta; TPO: thrombopoietin;

BM, as it can upregulate TSP-1, but it was also reported that it can induce glucose transporter-1, hypoxia-inducible factor 1α (HIF1α), and key dormancy genes such as nuclear receptor subfamily 2 group F number 1 (NR2F1) (Clements and Johnson, 2019; Tivari et al., 2018). Even neovascularization has been highlighted as a promoter of metastatic reactivation, as endothelial cells secrete factors like periostin and TGF-β that can stimulate micrometastatic outgrowth (Yadav et al., 2018). Nevertheless, it is acknowledged that disrupted bone homeostasis can promote a sustained micro and macrometastatic outgrowth through the so-called vicious cycle of bone metastases (Yang et al., 2020). BCCs positively stimulate endothelial cells through VEGF production and also upregulate osteoclastogenesis through the secretion of IL-11, IL-6, and IL-8 (Amarasekara et al., 2018; McCoy et al., 2013). Furthermore, BCCs secrete PTHrP that promotes RANKL expression by osteoblasts, which indirectly promotes osteoclastogenesis and, in consequence, bone matrix resorption (Guise et al., 2006). Consecutively, the factors released from the bone matrix during bone resorption, including Ca^{+2}, TGF-β, platelets-derived growth factor (PDGF), and insulin growth factor, promote metastatic outgrowth and reinforce the cycle (Yang et al., 2020; Mundy, 1997; Bussard et al., 2010).

Finally, it is important to mention the effect of chemotherapeutic agents on the BM stromal compartment. Some reports demonstrated a toxic effect of various chemotherapeutic agents -commonly used for breast cancer treatment among others tumors- on BM progenitor cells, which are necessary for BM microenvironment maintenance (Somaiah et al., 2018). The damage level depends on the dose of the chemotherapeutic agent, the type of cancer, the illness severity, and the individual BM recovery capacity (Georgiou et al., 2010). It was demonstrated that the expression of CXCL12 increases in the BM after 24−48 hours of exposure to fluorouracil (5-FU) or cyclophosphamide in a murine model of BM transplantation (Georgiou et al., 2010). As we previously mentioned in the present review, the CXCR4-CXCL12 axis plays an essential role in facilitating BCCs homing to the BM-PMN. With regard to the effect of cytotoxic agents on BM-MSCs, there are some contradictory results. On the one hand, Qi Z. et al. reported that both methotrexate and doxorubicin reduce the viability and increase the proportion of senescent BM-MSCs *in vitro* compared to 5-FU (Qi et al., 2012). On the other hand, Li J. et al. showed that human BM-MSCs are resistant to methotrexate and cyclophosphamide, whereas they are sensible to paclitaxel and vincristine with no alteration in their differentiation potential (Li et al., 2004). In this way, Münz F. et al. demonstrated that the exposure of BM-MSCs to low concentrations of paclitaxel *in vitro* decreased their proliferation rate –but not in their viability-, and induced premature senescence and altered functional capabilities (Münz et al., 2018).

Similarly, Somaiah C. et al. found phenotypic and functional alterations in BM-MSCs treated with cytarabine, daunorubicin, and vincristine, including decreased proliferation and osteogenic and adipogenic differentiation capacity, as well as increased expression of pro-tumoral cytokines (Somaiah et al., 2018). It was also reported that chemotherapy with multiple agents at high doses is able to reduce the number of BM osteoprogenitors cells in BCPs and non-Hodgkin lymphoma patients (Banfi et al., 2001), which may impact bone formation and resorption homeostasis.

5. Mesenchymal stem cells and their role in the bone marrow pre-metastatic niche establishment and in the bone metastatic cascade

5.1. General characteristics of mesenchymal stem/stromal cells

MSCs are a heterogeneous group of cells of the non-hematopoietic lineage with different proliferative capacity and plasticity that are located mainly in the BM, and contribute to the maintenance and regeneration of different connective tissues (such as bone, cartilage, adipose, and muscle tissues) (Gregory et al., 2005). They can generate different BM/bone stromal cells such as fibroblasts, endothelial cells, pericytes, osteoblasts/osteocytes, adipocytes, and chondrocytes (Kode et al., 2009). MSCs are essential for maintaining hematopoietic homeostasis as well as for the regulation of immunological processes, osteogenesis, and osteoclastogenesis/bone resorption processes (Bianco et al., 2013; Uccelli et al., 2006). A key feature of MSCs is their rapid expansion *in vitro* and development of colony-forming units (CFU-F) with spindle shape, demonstrating their highly clonogenic nature (Prockop and Oh, 2012). Regarding the identification and characterization of MSCs, in 2006, the International Society for Cellular Therapy (ISCT) proposed the minimum criteria to define human MSCs (Dominici et al., 2006). These guidelines are based on MSCs ability to adhere to culture flasks and develop CFU-F under standard conditions. Additionally, these cells must express (>95 %) CD105, CD73, and CD90 surface antigens, and they must lack (<2%) the expression of CD34, CD45, CD14 or CD11b, CD79a or CD19, and HLA-DR surface molecules. Thirdly, MSCs should differentiate into osteoblasts, chondroblasts, and adipocytes *in vitro* (Dominici et al., 2006). In addition, the expression of CD146 surface molecule is a known marker of osteoprogenitor MSCs or MSCs from the BM vascular niche, associated with multipotentiality and higher self-renewal capacity (Fernandez Vallone et al., 2013a). Other reported stemness markers are the expression of OCT4, conserved telomere length, and telomerase activity, which are essential for MSCs preservation of their cloning capacity and plasticity as well (Samsonraj et al., 2013; Yannarelli et al., 2013; Malvicini et al., 2019).

The expression of the cell surface marker CD271 (low-affinity nerve growth factor receptor), identifies a subpopulation of bone-lining MSCs in healthy adult BM (CD271+ CD146-) (Hochheuser et al., 2020). However, Álvarez-Viejo M. et al. concluded that CD271 cannot be employed as a universal marker for MSCs before culture, since several studies confirmed the presence of this marker in adipose tissue and BM sources, but not in umbilical cord tissue or umbilical cord blood (Álvarez-Viejo, 2015). Additionally, it was demonstrated that $CD45^{low}$ $CD271^{high}$ cells could be used to judge the quality of BM samples applied in clinical settings since this subtype completely confines BM colony forming activity and shows an age-related decline among women (El-Jawhari et al., 2017). Despite this information, it has to be considered that culturing MSCs alters the expression of cell surface markers, including CD146, CD271, among others, and thus cultured cells may not accurately reflect the properties of MSCs *in vivo* (Hochheuser et al., 2021).

In the normal BM niche, quiescent $Nestin^{+}$ MSCs reside over sinusoids -a specific type of blood vessels in BM-, sharing the HSCs niche and expressing HSCs maintenance genes, including *cxcl12*(Bianco et al., 2013; Bara et al., 2014). Additionally, MSCs give rise to osteoprogenitors in the endosteal niche, where both MSCs and osteoblasts regulate HSCs quiescence, and in turn, HSCs can induce osteogenic differentiation of MSCs (Bara et al., 2014; Yin and Li, 2006). BM-MSCs produce several secreted factors, including SCF, FH3 ligand, VCAM-1, TPO, GM-CSF, and the CXCL12, CXCL2, CXCL8, and CCL-3 chemokines (Ahmadzadeh et al., 2015). These factors help create a favorable sheltering environment for HSC maintenance and protect them from differentiation and pro-apoptotic stimuli (Ahmadzadeh et al., 2015). Furthermore, MSCs -as well as osteoblasts- secrete both pro osteoclastogenic factors –including RANKL, M-CSF, VEGF, CCL-2, migration inhibitory factor, PDGF, IL-6, and reactive oxygen species (ROS), among others-, and anti-osteoclastogenic factors –such as OPG, IL-4, IL-10, galectin-3, *etc.*- (Martinez et al., 2014; Manolagas and Jilka, 1995).

In addition to maintain bone homeostasis and provide support to HSPCs, BM-MSCs secrete soluble factors –such as indoleamine-2,3-dioxygenase (IDO), prostaglandin E2 (PGE2), TGF-β, IL-10, *etc.*- and EVs that suppress immune responses by inhibiting B- and T- cell proliferation and monocyte maturation (Nauta and Fibbe, 2007; de Witte et al., 2015; Kim et al., 2013; Jiang and Xu, 2020). Those factors can also promote the generation of regulatory T cells and M2 macrophages (Nauta and Fibbe, 2007; de Witte et al., 2015; Kim et al., 2013). Moreover, BM-MSCs express high levels of toll-like receptors (TLRs) -*i.e.*, TLR3 and TLR4- which activation can change the phenotype and immunomodulatory properties of MSCs depending on the signals from the particular microenvironment (He et al., 2009). Some experiments demonstrated that BM-MSCs polarize into two different subtypes depending on the stimulation of TLR4 (MSC1) or TLR3 (MSC2), respectively (Tomchuck et al., 2008). MSC1 secrete pro-inflammatory factors, such as CXCL1, IL-6, IL-8, and CCL-2, as well as the adhesion molecules VCAM-1 and Intercellular Adhesion Molecule 1 (ICAM-1). In counterpart, MSC2 release immunosuppressive mediators like PGE2, IDO and IL-10 (de Witte et al., 2015; Meisel et al., 2004). Apart from TLR activation, some pro-inflammatory cytokines such as interferon-γ, tumor necrosis factor-α, and IL-17A can also boost the immunomodulatory properties of MSCs (de Witte et al., 2015).

5.2. Mesenchymal stem/stromal cells in the primary breast tumor

In addition to their regenerative capacity through the secretion of soluble factors and EVs (Rani et al., 2015), MSCs have an intrinsic capacity to migrate towards microenvironments enriched in growth factors, cytokines, and chemokines, like inflammatory tissues (Rustad and Gurtner, 2012). Notably, tumor microenvironments are especially rich in these factors. BM-MSCs are recruited by the primary breast tumor, where they influence BCCs metastatic potential, as proved in different breast cancer murine models (Karnoub et al., 2007; Raz et al., 2018) **(**Fig. 2**)**. Different studies showed that several soluble factors and chemokines in the breast tumor microenvironment favor the attraction of BM-MSCs, such as IL-6, CCL-2, CCL-5, and CXCL12, as well as the hypoxia state in the tumor -that promotes the secretion of IL-6 by BCCs- (Rattigan et al., 2010; Chaturvedi et al., 2013; Shi et al., 2017; Spaeth et al., 2008). In a previous study, our research group found an association between the expression of IL-6, CCL-2 and CXCL12, and their corresponding receptors -IL-6 receptor, CXCR-4, and CCL-2 receptor- present in intratumoral spindle-shaped stromal cells like MSCs and cancer-associated fibroblasts (CAFs) (Labovsky et al., 2015). It has also been observed that miRNAs can modulate the migration of BM-MSCs towards the primary breast tumor. In this way, Zhang Y. et al. found that miR-126/miR-126* suppress the sequential recruitment of MSCs and inflammatory monocytes into the breast tumor stroma in a mouse xenograft model (Zhang et al., 2013).

Tumor-recruited BM-MSCs progressively acquire an activated phenotype with the ability to promote breast tumor growth and become tumor-associated-MSCs (TA-MSCs), some of which eventually lose their self-renewal capacity (Shi et al., 2017). However, the mechanisms that

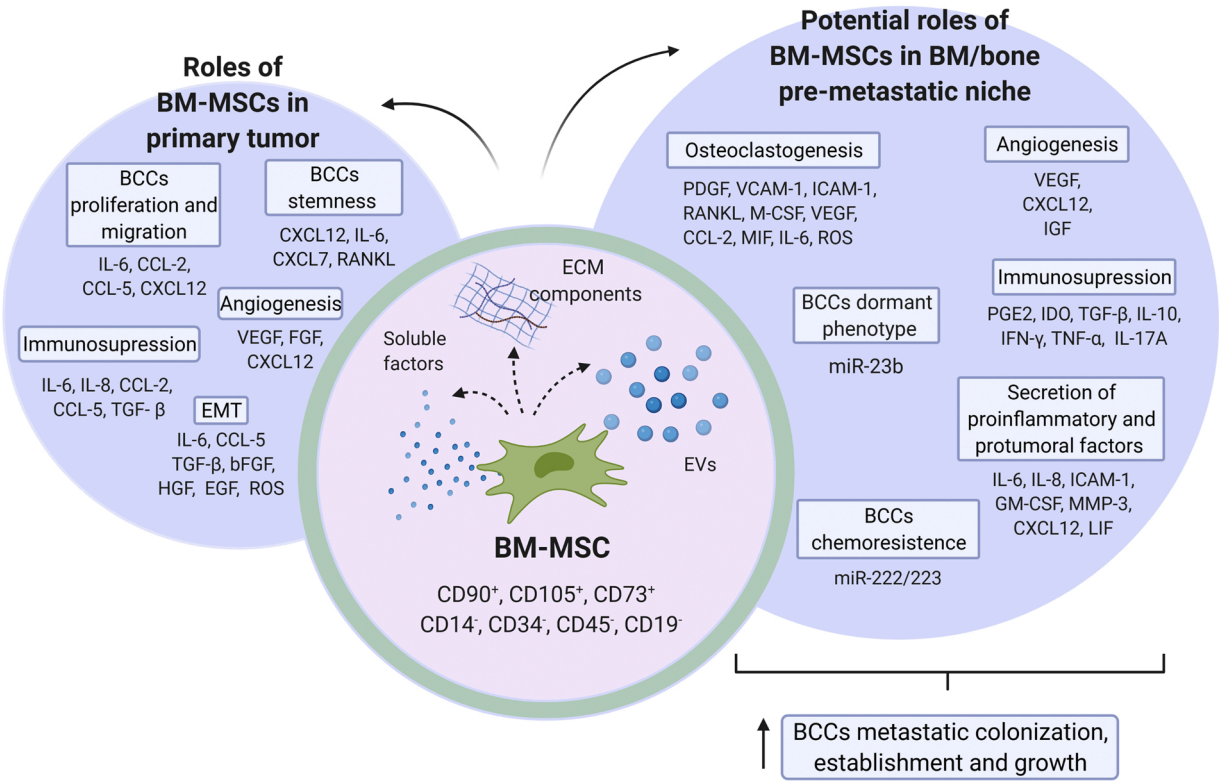

Fig. 2. Principal functions of bone marrow mesenchymal stromal cells (BM-MSC) that modulate breast cancer development, metastatic colonization, establishment and growth. The main known roles of BM-MSCs in the primary breast tumor and their potential roles in BM/bone pre-metastatic niche are shown. Created with BioRender.com. CCL-2: chemokine (C-C motif) ligand 2; CCL-5: chemokine (C-C motif) ligand 5; EGF: epithelial growth factor; EVs: extracellular vesicles; bFGF: basic fibroblastic growth factor; FGF: fibroblastic growth factor; GM-CSF: granulocyte-macrophage colony-stimulating factor; HGF: hepatocyte growth factor; ICAM-1: Intercellular Adhesion Molecule 1; IDO: indoleamine-2,3-dioxygenase; IFN-δ: interferon gamma; ; IGF: insulin growth factor; IL-6: interleukin-6; LIF: leukemia inhibitory factor; M-CSF: macrophage colony-stimulating factor; MIF: migratory inhibitory factor; miR: microRNA; MMP-3: metalloproteinase 3; PDGF: platelets-derived growth factor; PGE2: prostaglandin E2; RANKL: receptor activator of nuclear factor-κB ligand; ROS: reactive oxygen species; TGF-β: transforming growth factor-beta; TNF-α: tumor necrosis factor alpha; VCAM-1: vascular cell adhesion molecule 1; VEGF: vascular endothelial growth factor.

promote the pro-tumoral phenotype of TA-MSCs have recently begun to be elucidated. Recently, Blache U. et al. used 3D hydrogels to investigate the role of MDA-MB 231 cells secretome in the activation of MSCs (Blache et al., 2019). They found that MDA cells secretome upregulated several pro-tumoral chemokine and metalloproteases genes, which are also MSCs secretion molecules (Blache et al., 2019). Since tumors are analogous to chronic inflammation, the factors released by the primary breast tumor could modify the responses of MSCs and polarize them, promoting tumor progression and metastasis (Barcellos-de-Souza et al., 2013). MSC1 showed a pro-inflammatory secretome and inhibition of tumor cell growth either *in vitro* and *in vivo*, whereas MSC2 secreted immunosuppressive factors and showed a pro-tumoral effect both *in vitro* and *in vivo* (Waterman et al., 2010, 2012). There are opposing results regarding the TLRs stimulation effects on MSCs, due to differences in stimulation and *in vitro* culture conditions, as well as in the tissue of origin of the MSCs (Shojaei et al., 2019).

The activated pro-tumoral BM-MSCs also promote immunomodulation in the primary tumor by upregulating diverse soluble factors, including IL-6, IL-8, CCL-2, CCL-5, and TGF- β (Liu et al., 2011; Khalid et al., 2015). While CCL-2 is a crucial factor for the recruitment of monocytes (Qian et al., 2011), IL-6, TGF-β, and CCL-5 are relevant factors for the recruitment of myeloid-derived suppressor cells (MDSCs) involved in the coordination of the immunosuppressive tumor microenvironment (Chaturvedi et al., 2013; Zhang et al., 2018). In the context of a hypoxic microenvironment, CCL-5 favors the secretion of colony-stimulating factor 1 by BCCs, which in turn allows the recruitment of tumor-associated macrophages and MDSCs to the microenvironment (Chaturvedi et al., 2013). In particular, TGF-β plays a crucial role in the repression of the immune system. It can affect the differentiation of dendritic cells and T lymphocytes, promote the recruitment of MDSCs and monocytes, and induce monocytes differentiation to M2 macrophages with immunosuppressive capacities (Stuber et al., 2020; Hargadon, 2016).

TA-MSCs even have the ability to differentiate into CAFs (Mishra et al., 2008). A study showed that breast TA-MSCs, as well as CAFs, secrete a plethora of cytokines and growth factors that upregulate tumor growth and angiogenesis, including IL-6, IL-8, TIMP metallopeptidase inhibitor 2 (TIMP-2), CCL-2, CXCL12, fibroblastic growth factor (FGF), VEGF, among others (Park et al., 2009; Giorello et al., 2021).

Notably, several studies show that BM-MSCs also promote CSCs phenotype in BCCs (Chan et al., 2019). This can be achieved directly through the secretion of pro-stemness factors –mainly IL-6, CXCL-7, and RANKL-, or indirectly through MSCs differentiation to pro-stemness CAFs (Chan et al., 2019; Li et al., 2012; Infante et al., 2019). Furthermore, some studies recently showed that MSCs are actively involved in inducing the EMT phenotype of tumor cells, which is also associated with stemness (Hill et al., 2017; Hass et al., 2019; Cannito et al., 2010). MSCs secrete several factors that are known to induce EMT, such as IL-6 (Sasser et al., 2007), CCL-5 (Karnoub et al., 2007), TGF-β, basic FGF, hepatocyte growth factor (HGF), and epidermal growth factor (McAndrews et al., 2015; Berger et al., 2016), as well as ROS (Ridge et al., 2017; Schieber and Chandel, 2013).

5.3. Mesenchymal stem/stromal cells in the context of the bone marrow pre-metastatic niche

As previously mentioned, primary tumors interact paracrinally with stromal cells present at the site of metastases to prepare the "soil" for their colonization. Thus, the BM/bone is considered a microenvironmental unit that regulates and modulates both HSPCs and metastatic cancer cells (Peinado et al., 2017). Particularly, BM-MSCs play a significant role in generating the BM/bone PMN and the development of BCCs bone metastases **(**Fig. 2**)**.

Tumor cells and stromal cells in the BM/bone PMN can establish cell-cell contacts through adhesion molecules. Cell to cell interactions between BCCs and MSCs, similar to those between MSCs-HSCs, are necessary for BCCs retention in the BM/bone PMN (Verfaillie, 1998). BCCs can bind directly to osteoblasts through OB-cadherin (Kimura et al., 2017), and as MSCs also express OB-cadherin, it could mediate DTCs homing at the perivascular niche (Hajra and Fearon, 2002). As some groups reported, cancer cells and MSCs can also establish cell-cell contacts through specific and dynamic structures called tunneling nanotubes (TNTs) (Soundara Rajan et al., 2020). In particular, Pasquier J. et al. demonstrated that TNTs-mediated mitochondrial transfer and exchange of cytoplasmic components –proteins and genetic material- between MSCs and BCCs promote chemoresistance in BCCs (Pasquier et al., 2013). Additionally, an extensive paracrine communication network occurs between BCCs and BM stromal cells through the secretion of soluble factors. These molecules play an essential role in hematopoiesis and bone remodeling, and in the process of colonization of the BM/bone by BCCs. Saki N. et al. reported that BM- MSCs secrete growth factors that are known to be pro-tumoral, such as IL-8, IL-6, LIF, GM-CSF, ICAM-1, CXCL12, among others (Saki et al., 2011). BM-MSCs in the perivascular niche also secrete CCL-2 and CXCL12 that mediate DTCs chemoattraction to the BM and DTCs homing to this niche, respectively (Corcoran et al., 2008; Esposito and Kang, 2014).

Furthermore, as some authors demonstrated, MSCs with a senescent state – that means in cell cycle arrest, usually in response to DNA damage- are closely related to aging and age-related diseases, including cancer (Minieri et al., 2015). MSCs can become senescent under different stresses, including chemotherapeutic agents, ROS, heat shock, and ionizing radiation (Minieri et al., 2015). This state negatively affects MSCs secretome, as well as stemness, immunomodulatory and differentiation capacities (Turinetto et al., 2016; Liu et al., 2020). Interestingly, it was reported that the expression of several pro-inflammatory cytokines increases in senescent MSCs, such as IL-6, IL-8, GM-CSF, MMP-3, and ICAM-1, which are known to have pro-tumoral effects (Minieri et al., 2015). Furthermore, IL-6, IL-8, and CCL-2 factors -relevant in the BM/bone PMN context- were found to be increased in the conditioned medium of *in vitro* cultures of aged MSCs compared to young MSCs (Gnani et al., 2019). Accordingly, Di G. et al. reported that IL-6 cytokine secreted by senescent MSCs promoted BCCs proliferation and migration *in vitro*, as well as an enhancement of tumor growth in a xenograft mouse model when compared with normal MSCs, providing some evidence about the pro-tumoral effects of senescent MSCs (Di GH et al., 2014). Similarly, Saeed H. et al. described a telomere shortening after successive BM-MSCs subcultures, as well as a senescent phenotype and lower osteogenic differentiation *in vitro* of those MSCs (Saeed et al., 2011). To support these observations, they used a telomerase-deficient (Terc $^{-/-}$) murine model that showed impaired bone formation *in vivo*. Furthermore, serum from Terc $^{-/-}$ animals enhanced osteoclastogenesis of *in vitro* BM mononuclear cells cultures, compared with serum from wild-type animals (Saeed et al., 2011).

Considering what was previously described in the literature and the concept of the PMN, we hypothesized that BM-MSCs isolated from ABCPs -without surgery and treatment-, would show an altered phenotype compared with BM-MSCs from healthy volunteers (HV). In this way, our group aimed to demonstrate that the components of the BM microenvironment, in particular MSCs, of ABCPs (menopausal women with infiltrative breast ductal carcinoma, clinical-pathological stage III-B without BM/bone metastases and osteoporosis, before surgery, irradiation, and chemotherapy protocols) represent an optimal PMN that favors BCCs metastatic colonization, establishment, and outgrowth. We found that MSCs from ABCPs have a reduced number of CFU-F (1 CFU-F = 1 MSC) and inefficient self-renewal and proliferation capacity compared with HV (Hofer et al., 2010). Moreover, our results showed that the osteogenic and adipogenic differentiation is impaired in those MSCs. Accordingly, they had a significantly lower expression of CD146 per cell, indicating that these cells may not accomplish self-renewal and bone regeneration correctly (Fernandez Vallone et al., 2013b).

On the other hand, we found a significant increase in the capacity of peripheral blood plasma from ABCPs to induce the transendothelial migration of MCF-7 human luminal BCCs compared with plasma obtained from HV (Martinez et al., 2014). In addition, we observed a significant increase in patients' BM plasma capacity to induce transendothelial migration of MDA-MB 231 and MCF-7 human breast tumor cells, as well as a significantly higher MDA-MB 231 cell proliferation rate when compared with HV BM plasma (Martinez et al., 2014). Furthermore, significantly lower levels of OPG were detected in the conditioned media from 14 day-subcultures of CFU-F from ABCPs, compared to HV (Martinez et al., 2014). Since OPG inhibits osteoclastogenesis by binding soluble RANKL, MSCs might promote bone resorption in ABCPs through impaired OPG production. In this way, the conditioned media from CFU-F cultures of these patients induced a higher transwell migration of the MDA-MB 231 and MCF-7 cell lines (Martinez et al., 2014). Interestingly, PDGF-AB, ICAM-1 and VCAM-1 factors -key molecules for pre-osteoclasts recruitment and osteoclastogenesis, as well as BM extravasation- were significantly higher in patients' BM plasma than HV, suggesting that they could be involved in BCCs extravasation and proliferation, as well as in bone resorption process (Martinez et al., 2014). There is growing evidence suggesting that ICAM-1 plays a relevant role in the adhesion of breast DTCs to endothelial cell monolayers and their subsequent transendothelial migration (Strell et al., 2007; Li and Feng, 2011). Our data reveal new information about the alterations that happen in the BM of untreated ABCPs before bone colonization, changes that create optimal soil for the metastatic cascade progression.

While the establishment of the BM/bone PMN is increasingly acknowledged in some types of cancers like breast cancer as we described before, it has not been well studied yet in other cases such as neuroblastoma (NB) since the majority of NB patients already present BM metastasis at the time of diagnosis (Hochheuser et al., 2021). However, it was reported that EVs derived from NB cell lines turn BM-MSCs into a pro-tumoral phenotype *in vitro* (Hochheuser et al., 2021), supporting the concept of the distant preparation of the PMN by NB cells.

5.4. Mesenchymal stem cells derived extracellular vesicles

As previously mentioned, the paracrine communication between the primary tumor and the PMN is crucial for establishing the 'fertile soil' for cancer cells. In this way, many research groups recently proved that EVs mediate a significant part of that paracrine interaction (Lobb et al., 2017). Particular attention has been focused on exosomes, a group of very small EVs (30−200 nm in diameter) derived from the multivesicular bodies found in the endomembrane system of eukaryotic cells. Due to their biogenesis, exosomes contain CD9, CD63, and CD81 tetraspanins and other endosomal proteins like Alix and Tsg101 (Pegtel and Gould, 2019; Pacienza et al., 2019). However, analytical techniques can not differentiate between exosomes and other types of EVs, such as microvesicles, as their size range overlaps. Thus, following the International Society for Extracellular Vesicles (ISEV) recommendations (Witwer et al., 2019), we will use the term small extracellular vesicles (sEVs) from herein.

Notably, EVs can deliver different molecules such as DNA, RNA,

proteins, and lipids into target cells to modulate their phenotype (Raposo and Stahl, 2019; Xu et al., 2018). In this way, EVs play critical roles in cell-cell paracrine and systemic communication and in several biological processes, either under physiological and pathological conditions, including cancer (Xu et al., 2018). For example, the release of sEVs containing miRNAs mir23-b, mir-940, and miR-5a-20p is involved in breast cancer metastasis to bone (Wong et al., 2020). Moreover, high levels of EVs were found in the serum of cancer patients, and their concentration positively correlates with malignancy (Li et al., 2019). In this way, Yuan X. et al. demonstrated that single breast cancer cell populations (SCP28) with lung and bone tropism and derived of the MDA-MB 231 cell line could transfer sEVs to BM osteoclasts *in vivo* (Yuan et al., 2021). Those sEVs contained factors -in particular miR-21- that showed to promote osteoclastogenesis and bone resorption (Yuan et al., 2021).

Different cell types, including MSCs, release EVs into the extracellular microenvironment, which contain messenger RNAs (mRNAs) and miRNAs that are transferred into cancer cells to trigger the expression or silencing of specific genes, respectively (Li et al., 2018). The results reported by Zhou X. et al. indicate that EVs derived from stressed human umbilical cord MSCs promote an invasive phenotype and EMT in MCF-7 and MDA-MB 231 cells through the activation of the ERK pathway (Zhou et al., 2019). Similarly, Vallabhaneni KC. et al. identified miRNA-24 and 34a, as well as platelet-derived growth factor receptor β, TIMP-1, and TIMP-2 present in MSCs-derived sEVs, as tumor supportive miRNAs and factors -respectively-, and they also proved the tumor supportive function of those sEVs when they were co-injected with MCF-7 cells *in vivo* (Vallabhaneni et al., 2016).

The implications of EVs in the malignancy and metastatic spread of different tumor cell types have been well described (Dostert et al., 2017). However, the establishment of their role in the generation of PMN and in the development of bone metastases is a nascent field in research. The results obtained to date suggest that EVs derived from tumor cells, including BCCs, can modify the behavior of BM stromal cells towards a pro-tumoral phenotype to support tumor cells homing and outgrowth (Chin and Wang, 2016; Cappariello and Rucci, 2019). For example, Bliss S. et al. found that MDA-MB 231 and T47D BCCs are capable of "educating" BM-MSCs to release EVs, which in turn promote chemoresistance and dormancy in $Oct4^{high}$-BCCs *in vivo*, through the delivery of miR-222/223 (Bliss et al., 2016). Accordingly, Ono M. et al. demonstrated that miR-23b is increased in BM-MSC-derived sEVs and can induce a dormant phenotype in MDA-MB 231-bone metastatic cells (Ono et al., 2014). Regarding the role of BM-MSCs in immunomodulation in the context of bone metastases, Walker N. et al. described that sEVs from differentially activated macrophages to an M1 phenotype positively influence dormant $Oct4^{hi}$ MDA-MB-231 and T47D cells reactivation and chemosensitivity to cisplatin within the BM stroma *in vivo* (Walker et al., 2019). Notably, the M1 polarization of macrophages can occur indirectly by TLR-4-activated BM-MSCs (Walker et al., 2019). Overall, these results highlight the relevance of the MSCs-derived EVs in the preparation of the PMN and in the different steps of the metastatic cascade.

6. Therapeutics: targeting the bone marrow/bone pre-metastatic niche in breast cancer patients

As previously mentioned, once osteolytic lesions occur in BCPs, bone metastasis remains incurable and the treatment is limited to palliative care. The standard of care includes the administration of targeted drugs such as the anti-resorptive bisphosphonates (*i.e.*, Zoledronic acid), or the anti-RANKL monoclonal antibody (mAb) (*i.e.*, Denosumab) that negatively regulates osteoclastogenesis and osteoclasts activity –by inhibiting RANKL/RANK interaction-, restores bone integrity and reduces the lesions induced by cancer cells (Haider et al., 2020). The clinical trials ABCSG-12 and AZURE showed that the combination of Zoledronic acid with standard adjuvant treatment improved disease-free survival in pre and postmenopausal women, respectively (D'Oronzo et al., 2019). Bisphosphonates may have a dual function as adjuvant therapy: they can inhibit farnesyl pyrophosphate synthase, which is essential for osteoclasts survival and activity, and they may also accelerate BM-MSCs osteogenesis by inhibiting oxidative stress *via* the SIRT3/SOD2 pathway (Jin et al., 2020). At low doses, Zoledronic acid improved the *in vitro* mineralization process of MSCs- derived osteoblasts (Fliefel et al., 2020). This dual activity makes bisphosphonates great candidates for the normalization of the BM/bone PMN. The mentioned pro-osteogenic effect over osteoblasts activity was also seen with Denosumab, showing a positive effect on osteogenic differentiation at low doses (Mosch et al., 2019). The binding of the mAb to RANKL may also block the interaction between osteoblasts and osteoclasts (Portal-Nunez et al., 2017). Although other antiresorptive drugs are being tested in several clinical trials (Rossi et al., 2020), more and more attention has been focused on improving therapies for prevention of the development or evolution of the PMN, and in consequence, of bone metastases.

Other possible targets may be the soluble factors secreted by the primary tumor that prepare the BM/bone PMN. For example, Erler J. et al. showed that the LOX inhibitor β-aminopropionitrile inhibits BCCs pulmonary metastasis by preventing CD11b + cell recruitment to the PMN (Erler et al., 2009). As LOX enzyme is involved in ECM remodeling in the BM PMN in the context of breast cancer, LOX inhibitors could be an option to help prevent PMN development. Also, Natalizumab Ab -which prevents VCAM-1/VLA-4 interaction- has been approved in the United States to treat inflammatory bowel disease and multiple sclerosis (Zhou et al., 2020). In this way, a neutralizing Ab of VCAM-1 showed to reduce metastasis of melanoma cells as well as diminished *in vitro* osteolysis by decreasing osteoclasts activity in a myeloma model (Schneider et al., 2011). Since VCAM-1/VLA-4 is described as one of the relevant interactions between breast DTCs and BM stromal cells for DTCs homing to the BM/bone PMN, inhibitors such as Natalizumab could be tested in breast to bone metastasis models for the prevention of BM/bone PMN establishment.

Some authors proposed CCL-2 and DKK-1 factors as possible targets for the PMN prevention therapy in BCPs. In the first place, higher expression of the pro-osteoclastogenic factor CCL-2 correlates with decreased survival in BCPs with bone metastases (Ueno et al., 2000). Bonapace L. et al. showed that the treatment with anti−CCL-2 Ab decreased BCCs bone metastasis in mice, but the interruption of this treatment triggered a lethal outcome, with enhanced IL-6 levels and monocytes invasion, which favored higher angiogenesis (Bonapace et al., 2014). In this way, Masuda T. et al. carried out a phase I trial with oral propagermanium (PG), an organic germanium compound that targets glycosylphosphatidylinositol-anchored proteins and selectively inhibits CCL-2/CCR2 signaling (Masuda et al., 2020). This compound might have the potential to prevent metastasis by inhibiting the formation of PMN in BCPs. In contrast with the anti−CCL-2 Ab, IL-6 serum levels did not increase after PG treatment, turning it into a very promising therapy (Masuda et al., 2020). On the other way, anti-DKK-1 Ab -like BHQ880 and DKN-01- are currently being tested in clinical trials for myeloma and esophagic cancer patients, as well as in osteoporotic postmenopausal women (Katoh and Katoh, 2017). Moreover, Heath D. et al. showed that treatment of a mouse model with an anti-DKK-1 Ab could inhibit myeloma bone disease and prevent the development of osteolytic bone lesions, apparently by stimulation of MSCs differentiation into osteoblasts (Heath et al., 2009). Further studies will elucidate the clinical value of BM/bone PMN therapy in BCPs.

Another possible target in the BM/bone PMN is the normalization of the cellular redox state, as oxidative stress in MSCs is associated with the pathogenesis of bone loss. This happens due to an overproduction of ROS that is not balanced by an adequate level of antioxidants, causing an imbalance between osteoclast and osteoblast activity (Domazetovic et al., 2017). Domazetovic V. et al. showed that antioxidants have an important role in maintaining the normal bone remodeling process by inhibiting osteocytes apoptosis, increasing osteoblast activity and

reducing osteoclast activity (Domazetovic et al., 2017). Additionally, Moon H. et al. observed that Coenzyme Q10 can act both as an inhibitor of osteoclast differentiation and as a stimulator of osteoblast differentiation as well (Moon et al., 2013).

Finally, it is important to mention that Takahashi A. et al. identified TNTs as essential cellular structures for intercellular communication among osteoclast precursors, and as an essential process for the regulation of osteoclastogenesis (Takahashi et al., 2013). Additionally, as previously described in this review, TNTs represent a type of cell-cell contact between MSCs and BCCs. The blocking of the TNTs could be a promising novel target for inhibiting the osteoclastogenesis imbalance in the BM/bone PMN. Although Wang J. et al. published some promising *in vitro* results about the anti-tumoral inhibition of TNTs cell-cell contact between MSCs and a leukemia cell line (Wang et al., 2018), further studies are necessary to establish the effect of these therapies in preventing the development of the BM/bone PMN in BCPs.

7. Conclusions and perspectives

The metastatic process is complex and only a few disseminated BCCs manage to colonize their secondary site successfully. In order to facilitate this process, it has been proved that BCCs in the primary tumor prepare their future site of metastasis by secreting pro-tumoral soluble factors and EVs, as well as by educating resident stromal cells to their own benefit. Here we highlighted the specific characteristics that make the BM/bone niche suitable for BCCs metastatic spread. This niche contains different types of cells that not only collaborate to maintain the normal bone remodeling process but also support hematopoiesis homeostasis. Since disseminated BCCs show a stemness phenotype, with characteristics of CSCs -many of them shared with HSCs-, this may explain the particularities of BM/bone niche in supporting BCCs metastatic outgrowth.

Although the *in vitro* and *in vivo* studies carried out so far allowed scientists to begin to understand the mechanisms underlying BCCs bone metastasis, further studies are needed to clarify the development of the BM/bone PMN, as well as the cell types and factors involved. In order to study the dynamics in the establishment of the liver PMN, Kim J. et al. developed a 3D human microfluidic device to emulate the PMN under the effects of BCCs-derived EVs (Kim et al., 2020). However, the BM/bone physiology is more complex, including many different cell types that may be involved in the PMN establishment.

Even though MSCs role as enhancers of primary breast tumor aggressiveness and metastatic spread has been well described, it is less clear their participation in the BM/bone PMN establishment. In this review, we summarized what has been reported to date about the involvement of BM-MSCs in the formation of the BM/bone PMN. MSCs were described as modulators of BCCs dormancy reactivation and chemoresistance, immunomodulation as well as promoters of osteoclastogenesis and bone resorption in the BM. Hence, our results here exposed indicate that BM-MSCs from ABCPs are crucial components of the BM/bone PMN since they showed an altered phenotype, with inefficient self-renewal and proliferation capacity, an impairment in osteogenic differentiation, as well as the secretion of pro-osteoclastogenic factors. Some other authors demonstrated that part of the reciprocal communication between BCCs and MSCs depends on both types of cells secreting EVs that contain proteins and miRNAs -among others- with a relevant role in the BM/bone PMN. Therefore, a better understanding of the biology of those EVs could contribute to developing alternative strategies to prevent BM/bone colonization by BCCs. However, most of the experiments reported to date were made by employing MSCs isolated from HV. We consider that the use of MSCs isolated from advanced patients whose tumors were not removed is critical to investigate the bidirectional communication between BCCs and MSCs that leads to the development of the BM/bone PMN. Finally, it is essential to study further the mechanisms involved in BM/bone PMN formation to develop new therapies to prevent it.

Authors contributions

María Cecilia Sanmartin: investigation, writing - original draft, visualization. Francisco Raúl Borzone: investigation, writing - original draft. María Belén Giorello: investigation, writing - original draft, visualization. Natalia Pacienza: writing - review & editing. Gustavo Yannarelli: conceptualization, writing - review & editing, supervision, funding acquisition. Norma Alejandra Chasseing: conceptualization, writing - review & editing, supervision, funding acquisition.

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Declaration of Competing Interest

All authors declare that they have no conflicts of interest.

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