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RESEARCH PAPER

Behaviour of mesenchymal stem cells from bone marrow of untreated advanced breast and lung cancer patients without bone osteolytic metastasis

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Abstract Tumour cells can find in bone marrow (BM) a niche rich in growth factors and cytokines that promote their self-renewal, proliferation and survival. In turn, tumour cells affect the homeostasis of the BM and bone, as well as the balance among haematopoiesis, osteogenesis, osteoclastogenesis and bone-resorption. As a result, growth and survival factors normally sequestered in the bone matrix are released, favouring tumour development. Mesenchymal stem cells (MSCs) from BM can become tumour-associated fibroblasts, have immunosuppressive function, and facilitate metastasis by epithelial-to-mesenchymal transition. Moreover, MSCs generate osteoblasts and osteocytes and regulate osteoclastogenesis. Therefore, MSCs can play an important pro-tumorigenic role in the formation of a microenvironment that promotes BM and bone metastasis. In this study we showed that BM MSCs from untreated advanced breast and lung cancer patients, without bone metastasis, had low osteogenic and adipogenic differentiation capacity compared to that of healthy volunteers. In contrast, chondrogenic differentiation was increased. Moreover, MSCs from patients had

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lower expression of CD146. Finally, our data showed higher levels of Dkk-1 in peripheral blood plasma from patients compared with healthy volunteers. Because no patient had any bone disorder by the time of the study we propose that the primary tumour altered the plasticity of MSCs. As over 70 % of advanced breast cancer patients and 30–40 % of lung cancer patients will develop osteolytic bone metastasis for which there is no total cure, our findings could possibly be used as predictive tools indicating the first signs of future bone disease. In addition, as the MSCs present in the BM of these patients may not be able to regenerate bone after the tumour cells invasion into BM/bone, it is possible that they promote the cycle between tumour cell growth and bone destruction.

Keywords Mesenchymal stem cell · Bone marrow · Plasticity · Cancer

Introduction

Bone is a dynamic tissue that is constantly remodelled through the resorption of old bone by osteoclasts and the subsequent formation of new bone by osteoblasts [1, 2]. Mammalian bone consists of bone cells at different developmental stages, collagen fibrils and mineral deposits such as calcium and phosphate [3]. Moreover, the bone cavity is filled with soft bone marrow (BM) and blood vessels. Inside the BM, hematopoietic stem cells and their progeny are surrounded by stromal cells, including mesenchymal stem cells (MSCs) [1, 4]. MSCs not only regulate haematopoietic development but also give rise to a majority of BM stromal cell lineages. These lineages include osteoblasts, adipocytes, chondrocytes, fibroblasts, endothelial cells and myocytes, as demonstrated in vitro and in vivo [1, 2].

Bone and BM are closely related, not only as contiguous anatomic organs but also because their functions are closely shared. The interdependence of the haematopoietic system and bone is clearly evident [1, 4]. Inside the BM, haematopoietic stem cells (HSCs) residing near the endosteal bone surface produce progenitors that migrate to blood vessels at the centre of the BM cavity where they mature and differentiate [5]. Therefore, two specific microenvironments are defined inside the BM: the "osteoblastic niche" (or "quiescent microenvironment") which is located near the bone and the "vascular niche" which is situated near the BM sinusoids [1, 5–7].

Bone and BM are connected by the vasculature and, as skeletal structure and function are related to medullar composition, many of the pathologies that affect BM have a high incidence in bone and vice versa [1, 4]. Recent progress in the understanding of the stem cell niche has strengthened the tight interplay between BM and bone remodelling.

Bone metastases are often observed in patients with advanced solid tumours such as breast cancer and lung cancer [8]. Bone metastases due to breast and lung cancer are usually of the osteolytic type and appear within a year following the diagnosis of the primary tumour in most patients. These metastases are a common cause of morbidity and mortality [9–11]. They are often detected as the first sign of illness, such as pathologic fractures as consequence of a slight trauma. In most cancer metastases, however, there is not pure osteolysis or osteogenesis but a combination of both processes [8, 12–14]. Bone metastases are responsible for most of the complications of breast and lung cancer. They trigger fractures, spinal cord compression, pain and hypercalcaemia related to the elevated levels of calcium released from the extracellular matrix [9, 15–17]. Those consequences significantly alter patients' quality of life and are markers for poor prognosis with an average 5- and 2-year survival of 20 % and 3 % of the breast cancer patients (BCPs) and lung cancer (LCPs), respectively [8]. However, the spread and incidence of bone metastases are difficult to determine [18].

As MSCs may have an essential role in invasion and proliferation of cancer cells, there is close interaction among metastatic cancer cells, the BM microenvironment and bone [8]. Consequently, BM and bone may provide a favourable microenvironment for cancer cells to migrate and proliferate becoming, in essence, a disease reservoir.

Osteolytic lesions seem to be result of overactive osteoclastogenesis and patients are treated with biphosphonates to stop the osteoclastic activity. However, bone does not regenerate after treatment, suggesting that cancer cells may have directly or indirectly altered the behaviour of osteoblasts and/or osteocytes. Some studies have shown that when osteoclastic activity was increased, osteocytic activity was reduced in patients with hypercalcaemia [19, 20]. Osteoid surface and volume were also decreased [19]. Other in vitro studies have demonstrated that conditioned medium from a breast cancer cell line (MCF-7) inhibited osteoblastic proliferation and that the presence of FAS-L in conditioned medium favoured apoptosis in preosteoblastic BM stromal cells [21]. Additionally, Mercer and Mastro [19] showed that conditioned medium from another breast cancer cell line (MDA-MB231) inhibited osteogenic differentiation via PDGF, IGF and TGFβ. Because breast and lung cancer metastases are osteolytic, breast and lung cancer cells in the BM might stimulate osteoclasts and directly inhibit osteoblasts maturation. Moreover, immature osteoblasts are rich sources of receptor activator of nuclear factor-kB ligand (RANKL), an osteoclastogenic factor [22]. However, we cannot disregard a possible indirect effect through soluble factors released by cells

from the primary tumour or circulating cancer cells, such as Dickkopf-1 (Dkk-1), which may modify the behaviour not only of cancer cells but also of BM microenvironment components. In particular, Dkk-1 may impact the osteogenic differentiation of MSC [23, 24]. Voorzanger-Rousselot et al. [23] reported that serum Dkk-1 levels from patients with breast cancer and bone metastases were higher than in patients with breast cancer in complete remission, breast cancer at non-bone sites or healthy volunteers (HVs). Furthermore, Sheng et al. [24] have shown that although Dkk-1 function in lung cancer remains unclear, serum concentrations were higher in LCPs than in patients with other malignant tumours or benign lung diseases. Moreover, increasing serum concentrations of Dkk-1 were significantly associated with tumour progression, bone metastasis and decreased survival of advanced BCPs and LCPs, suggesting that Dkk-1 expression in cancer cells could promote dissemination of tumours by stimulating cell invasion [24, 25].

Moreover, MSCs release soluble factors that regulate the development and function of osteocytic and osteoclastic lineages, such as IL-1β, IL-6, IL-11, Dkk-1, Wnt proteins (Wnt 2, 4, 5, 11, 16), TGF-β, FGF-2, PDGF, PGE2, RANKL, LIF, OPG, M-CSF, MIP-1α and HA [26–28]. Thus, bone formation depends on MSC progenitor cells committed to osteocytic lineage that can synthesise an optimum mineralisation matrix [29]. Consequently, osteoclastogenesis is also implied because bone formation and bone resorption are tightly related mechanisms: pre-osteoblasts/osteoblasts release activating and inhibiting factors for osteoclastogenesis and osteoclasts release, from the mineralisation matrix, factors that regulate proliferation, differentiation and activation of osteoblasts [30]. A bone disorder preceding BM or bone metastasis may be related to an imbalance between bone formation and bone resorption resulting from an MSC defect. Previous reports have demonstrated that deficiencies in MSC self-renewal, MSC plasticity and cloning capacity, which generates colony forming units-fibroblastic (CFU-F), may represent signs of osteolysis or another bone disorder [31]. Each CFU-F observed in vitro is representative of one MSC obtained in vivo with self-renewal, proliferation and differentiation capacities [32, 33]. Therefore, the CFU-F assay measures the number and potentiality of in vivo MSCs [34].

The process of differentiation is very complex; many factors are involved, MSCs from the same individual are variable in plasticity [35]. It has been reported that individual colonies derived from a single MSC are heterogeneous in their multilineage differentiation potential [36]. Pittenger et al. [37] observed that only one-third of initially adherent BM-derived MSC clones are pluripotent for osteo/chondro/adipogenic lineages. Moreover, Muraglia et al. [38] showed that 30 % of MSC clones derived in vitro exhibited a tri-lineage differentiation potential, whereas the remainder displayed a bi-lineage (osteo/chondro) or uni-lineage (osteo) potential.

Recently, CD146 has been described as a marker of MSCs from the "vascular niche" [39]. In contrast, CD146 expression near the endosteal surface is absent or very weak as a consequence of the low oxygen level [39]. In addition, Russell et al. [40] have shown that CD146 may be a marker of multipotency, explaining that the differences in mean fluorescence intensity discriminate between tri- and unipotent cells.

Today we know that for bone metastasis to occur in patients with advanced lung and breast cancer, there must be changes in osteogenic and osteoclastogenic processes that result in the development of the osteolytic type of metastasis. However, the role of MSCs in preparing a suitable microenvironment for tumor invasion and the development of the premetastatic niche in bone is not understood.

To address these questions, we studied MSCs from BM of untreated advanced BCPs and LCPs prior to surgery, any adjuvant treatment and bone metastasis. For this purpose, we investigated the pluripotential of MSCs to give rise to the three characteristic lineages, osteogenic, adipogenic and chondrogenic, and the involvement of CD146 in these differentiation pathways. Additionally, the presence of Dkk-1 in peripheral blood plasma (PBP) was examined as an indicator of bone disturbance. Our findings may help to predict and prevent future bone and BM metastasis in BCPs and LCPs.

Materials and methods

Patients

This study included 7 BCPs with infiltrative ductal carcinoma, 7 LCPs with non-small cells (squamous cell) lung cancer, all in advanced clinical stage IIIb, and 7 HVs as controls. The International Union against Cancer TNM classification was used. All advanced cancer patients were untreated (no radio- or chemotherapy) and did not have BM metastases, bone metastases or osteoporosis. Bone metastases were assessed by X-ray procedures and bone scintigraphy. BCPs and LCPs were in menopause with an age range between 50 and 65 years old. Patients were free of metabolic bone disease, such as vitamin D deficiency, thyroid disease, parathyroid disease or kidney damage. HVs were donors for allogeneic BM transplantation with an age range of 40–67 years old and were matched for menopausal status with patients.

All individuals gave consent to participate in this study, which was performed in accordance with the principles of

Helsinki Declaration. Ethical committees from every participating institution approved this investigation.

BM metastases

BM infiltration with neoplastic cells in the aspirate was detected by immunocytochemistry staining (Universal Dako LSAB System, Carpinteria, CA, USA), and the analysis of cell morphology was performed using the Pappenheim technique. BM samples were stained with monoclonal antibodies (MAb) for epithelial membrane antigen (EMA, IS629-Dako), cytokeratin AE1-AE3 (IS053-Dako), cytokeratin 7 (CK7, IS619-Dako) and cytokeratin 20 (CK20, IS777-Dako). Patients' BM was considered positive for metastases if cells expressed EMA (breast cancer), cytokeratin AE1-AE3 (lung and breast cancer), CK7 (lung cancer) and CK20 (lung cancer) and if cells were also morphologically malignant.

Isolation and preparation of BM mononuclear cells (MNC)

BM samples were collected under local anaesthesia from the posterior iliac crest into heparinized saline without preservatives (25 units/ml, Gibco, Grand Island, NY, USA). BM aspirates were half diluted with phosphate–buffered saline (PBS) and layered on Histopaque (density = 1,075 gr/cm^3, Sigma, St. Louis, MO, USA). After centrifugation for 25 min at 340 g, BM light density MNC were harvested from the interface, washed twice in PBS and resuspended in α-minimal essential medium (Gibco) containing 2 mM L-glutamine (Gibco), 100 IU/ml penicillin (Gibco), 100 μg/ml streptomycin (Gibco), 25 μg/ml of amphotericin B (Gibco) and 20 % heat-inactivated FBS (16000-044-Gibco), hereafter referred to as "supplemented α-medium". The MNC suspension was counted in a 3 % acetic acid solution and cell viability was determined by 0.04 % trypan blue dye exclusion.

MSC cloning capacity: colony forming units-fibroblastic (CFU-F) assay

The CFU-F assay was performed according to Castro-Malaspina et al. [41]. Viable MNC (2×10^6) from 7 HVs, 7 BCPs and 7 LCPs were isolated and placed in 25 cm^2 tissue culture flasks containing 10 ml of supplemented α medium suitable for blastogenesis of fibroblast progenitors. Cells were incubated in a 37 °C, 5 % CO_2 humidified environment for 7 days. After this period, non-adherent cells were removed and the medium was renewed. After another 7 days, the adherent stromal cells were washed twice with PBS fixed with 100 % methanol (Merck, Darmstadt, Germany) for 15 min and stained with pure Giemsa (Sigma) for 5 min. Clones of more than 50 fibroblast-like cells containing a compact nucleus were scored as CFU-F.

The fibroblastic nature of the mesenchymal stromal cells was demonstrated by immunocytochemistry using MAb against the human β-subunit of Prolyl-4-hydroxylase (M0877-Dako), CD45 (M0701-Dako), and CD34 (M7165-Dako) and visualised as previously described. Negative controls were stained with isotype control MAb (X0931-Dako). Each sample was performed in duplicate.

We also studied the presence of tumour cells in CFU-F cultures from BCPs and LCPs by immunocytochemistry staining as previously described.

BM-MSC cultures and plasticity study

Viable MNC (10×10^6) obtained from BM of 7 HVs, 7 BCPs and 7 LCPs were plated in 25 cm^2 tissue culture flasks with 10 ml of supplemented α-medium. Cells were incubated in a 37 °C, 5 % CO_2 humidified environment. After 24 h, non-adherent cells were removed and the medium was renewed. Primary cultures were incubated until they reached 70 % sub-confluence, renewing the medium every 7 days. Stromal cells from one flask of this first passage were washed twice with PBS, harvested with a solution of trypsin-EDTA (0.05–0.02 % in PBS, respectively, Gibco) and replated in two 25 cm^2 tissue culture flasks to proliferate until again reaching 70 % confluence. After the second passage, stromal cells were plated at low density (240 cells/cm^2) in 25 cm^2 flasks and incubated in supplemented α-medium for 12 days (medium was renewed at day 6). This low cell density condition favours growth of the MSC population. After this period, mesenchymal stromal cells enriched in MSCs were isolated and counted with trypan blue. Before using MSCs for the plasticity assay, the presence of a number of human MSC phenotypic markers (including β-subunit of Prolyl-4-hydroxylase, CD73, CD105, CD44, CD49b, CD54, CD49e and CD68) and the absence of CD45 and CD34 were confirmed by immunocytochemistry as previously described by Lavobsky et al. [42].

Osteogenesis

Mesenchymal stromal cells enriched in MSCs from third passage (3×10^3 cells/cm^2) were plated in 35 mm Petri dishes and incubated in osteogenic differentiating medium consisting of α-medium with 10 % FBS, 10^{-8} M dexamethasone (Sigma), 0.2 mM ascorbic acid (Sigma), 10 mM beta glycerol phosphate (Sigma), plus 2 mM L-glutamine (Gibco), 100 IU/ml penicillin (Gibco), 100 μg/ml streptomycin (Gibco) and 25 μg/ml of amphotericin B (Gibco). The medium was renewed every 3–4 days until day 21 when cultures were washed with PBS and fixed with 70 % cold ethanol (Merck) for 1 h. Giemsa (Biopure, Cambridge,

MA, USA), Von Kossa (5 % silver nitrate, Sigma-5 % sodium thiosulphate, Sigma) and Alizarin Red-S (40 mM, pH 4.1, Sigma) stains were performed. Immnunocytochemistry for human osteocalcin (SC18319-Santa Cruz Biotechnology, Santa Cruz, CA, USA) was also performed.

For the analysis of the results we have taken into account three parameters according to the recommendations of a pathologist: (1) *Percentage of differentiation*: % of Petri dish surface with differentiated cells (considering "differentiated" cells to be those mesenchymal stromal cells enriched in MSCs with a different morphology from the classical fusiform shape of the stromal fibroblasts); (2) *colonies with osteogenic-like cells*; and 3) *pattern of differentiation (grade 0–3)*: 0 = fusiform morphology (fibroblast-like), 1 = precursor to osteoblast morphology; 2 = osteoblastic morphology and 3 = osteocytic morphology. Each sample was analysed in quadruplicate.

Adipogenesis

Mesenchymal stromal cells enriched in MSCs from the third passage (3×10^3 cells/cm^2) were plated in a 35 mm Petri dish and supplemented with α-medium until confluence (medium was renewed every 3 days). Adipogenic differentiation was performed with three induction/maintenance cycles using PT3004 medium (CAMBREX Bio Science Walkersville, Charles City, IA, USA) over the course of 3 weeks. Each cycle consists of 3 days of culture in induction medium (α-medium with 10 % FBS, 0.5 μM dexamethasone, 50 μg/ml 3-isobuthyl 1-methylxanthine and 50 μg/ml indomethacin plus antibiotic, antimycotic and L-Glutamine as previously described) followed by 3 days of culture in maintenance medium (same composition as induction medium without dexamethasone).

For adipocyte identification, intracellular lipid accumulation was visualised with Oil Red-O staining (Sigma) using the following procedure: cells were fixed for 1 h with 10 % formaldehyde/PBS (Merck) and stained with a fresh solution of Oil Red-O (0.12 %, Sigma) and Giemsa. Finally, the total number of Oil red-O positive adipocytes was counted in each Petri dish. Each sample was stained in quadruplicate.

Chondrogenesis

Mesenchymal stromal cells enriched in MSCs from the third passage (2.5×10^5 cells per sample) were centrifuged at 450 g for 10 min to generate a micro-mass pellet culture in polypropylene 15 ml tubes. Pellets were cultured for 21 days in α-medium containing 10 % FBS, antibiotic, antimycotic and L-Glutamine as previously described plus chondrogenic factors [500 ng/ml BMP-6, 25 mM glucose, 10 ng/ml TGFβ-3 (Genzyme, MA, USA), 10^{-7} M dexamethasone, 50 μg/ml ascorbic acid 2-phosphate, 40 μg/ml proline, 100 μg/ml pyruvate and 50 mg/ml ITS + Premix (PT 3003-CAMBREX)]. The medium was changed every 3 days. The weight of the pellets was recorded to analyse cartilaginous matrix formation. Pellets were embedded in paraffin and 5 μm sections were stained for glycosaminoglycane with 1 % Toluidine blue (Richard Allan Scientific, Kalamazoo, MI, USA) and 1 % Borate sodium (Sigma) for 5 min. Chondrogenic differentiation was confirmed by staining the slides, previously fixed with 50 % methanol (Merck), with anti-human Ab against collagen II (SC7763, Santa Cruz). We visualised the samples using immunocytochemistry as previously described. Negative isotype controls were also performed (X0936, Dako). Each sample was analysed in quadruplicate.

MSC control cultures for the three lineages were performed in parallel. For controls, cells were incubated only in supplemented α-medium without specific stimulation factors.

Flow cytometry for MSC characterisation and CD146 analysis

Mesenchymal stromal cells enriched in MSCs from the third passage were detached with Trypsin/EDTA and counted as previously described. Approximately 2.5×10^5 mesenchymal stromal cells enriched in MSCs from 4 HVs, 4 BCPs and 3 LCPs were centrifuged at 450 g for 5 min, suspended in 50 μl PBS containing 1 % bovine serum albumin (BSA), and labelled with the primary mouse MAb against the following human antigens: CD146 (IgG2a-PE, AO7483-Beckman Coulter, Miami, FL, USA), CD105 (IgG3-PE, AO7414-Beckman Coulter), CD90 (IgG1-PE-Cy5, IM3703-Beckman Coulter), CD73 (IgG1-PE, 550 257-BD Biosciences), CD59 (IgG2a-FITC, IM3457-Beckman Coulter), CD79a (IgG1-PE-Cy5, IM3456U-Beckman Coulter), CD45 (IgG1-PE-Cy7, IM3548- Beckman Coulter), CD11b (IgG1-PE-Cy5, IM3611-Beckman Coulter), CD34 (IgG1-PE, IM1871U-Beckman Coulter), CD19 (IgG1-ECD, IM2708-Beckman Coulter). Isotype controls (mouse MAb: IgG1-PE, 550617-BD Biosciences, San Jose, CA, USA); IgG3-PE, sx2869-Santa Cruz; IgG1-PE-Cy5, IM2663U-Beckman Coulter; IgG2a-FITC, 554647-BD Biosciences; IgG1-PE-Cy7, 6607099-Beckman Coulter; IgG1-ECD, IM2714U-Beckman Coulter) were run in parallel using the same concentration of each antibody tested. The samples were incubated for 30 min at room temperature, and 350 μl BSA 1 % was then added. At least 10,000 events were analysed and compared with isotype controls by flow cytometry (FC; FACScalibur, BD Biosciences) with CellQuest software. WinMDI 2.9 software was used to create the histograms and density plots. Results were expressed as the percentage and relative fluorescence index (RFI = marker mean fluorescence index/corresponding

isotype control mean fluorescence index). Experiments were repeated three times with different MSC preparations for each individual.

Real-time reverse transcription PCR for CD146

For these experiments the third passage of mesenchymal stromal cells enriched in MSCs from 4 HVs, 4 BCPs and 3 LCPs were used. Approximately 20 ng of total RNA was isolated from each sample with the RNeasy mini kit (Qiagen, Valencia, CA, USA), the QuantiTect Probe RT-PCR Kit (Qiagen) was used for sequential reverse transcription, and PCR was used with specific primers and probe for CD146 mRNA (Hs00174838_m1, Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. For relative quantification of gene expression, eukaryotic 18S rRNA endogenous control primers and probe (4352930, Applied Biosystems) were used. Negative controls were run without RNA and reverse transcriptase from the PCR reaction. Reactions were performed in an ABI 7900 Sequence Detector (Applied Biosystems). Data were analysed using the comparative threshold cycle (C_T) method. The experiments were repeated three times with different MSC preparations for each individual.

ELISA for Dkk-1

PBP was obtained from 10 ml of peripheral blood collected over heparin without preservatives (25 units/ml, Gibco). Samples were centrifuged at 1,125 g for 45 min at 4 °C. Plasma obtained was aliquoted and stored at -20 °C until use. Dkk-1 was determined by ELISA according to Labovsky et al. [42].

Statistical test

Results are given as the mean ± standard error (SE) when appropriate. Statistical analysis was performed using Parametric and Nonparametric tests as needed (Graph Pad Prism 4 Software). Differences were considered statistically significant when $p < 0.05$.

Results

Routine tests for BM aspirates of untreated advanced LCPs and BCPs and HVs: metastasis study, CFU-F assay and number of MNC isolated

Non-evidence of infiltration of the BM by neoplastic cells was observed in any of the cancer patients studied. Consistent with previous studies, the number of MNC isolated from BM of these 7 BCPs and 7 LCPs was significantly lower compared with those from the BM of 7 HVs. The number of MNC/ml BM obtained for each group was (mean ± SE) × 10^6; BCP = 2.94 ± 0.56 (a), LCP = 2.14 ± 0.53 (b), and HV = 16.85 ± 3.90 (a, b) (a: $p < 0.05$ *b*: $p < 0.05$; Mann–Whitney test).

Moreover, colony forming efficiency (CFE = # of CFU-F/2 × 10^6 MNC) and CFU-F size of BCPs and LCPs were also lower than those of HVs (Fig. 1), in accordance with previous observations that indicate a deficiency in the cloning capacity of BM-MSCs from cancer patients compared to BM-MSCs from HVs [43].

Stromal cells from CFU-F were positive for β-subunit prolyl-4-hydroxylase and negative for CD45 and CD34, confirming their fibroblastic nature [43]. Furthermore, we have not observed the presence of malignant cells in the CFU-F of any patient (data not shown). Cells from the third passage cultured at low density were positive for β-subunit prolyl-4-hydroxylase, CD73, CD105, CD44, CD54, CD49b, CD49e; they presented low expression of CD68 and were negative for CD45 and CD34, indicating their mesenchymal nature (data not shown).

Plasticity of BM-MSCs from untreated advanced BCPs and LCPs

Osteogenesis

The primary analysis of the samples under our culture conditions for osteogenic differentiation was visualised by

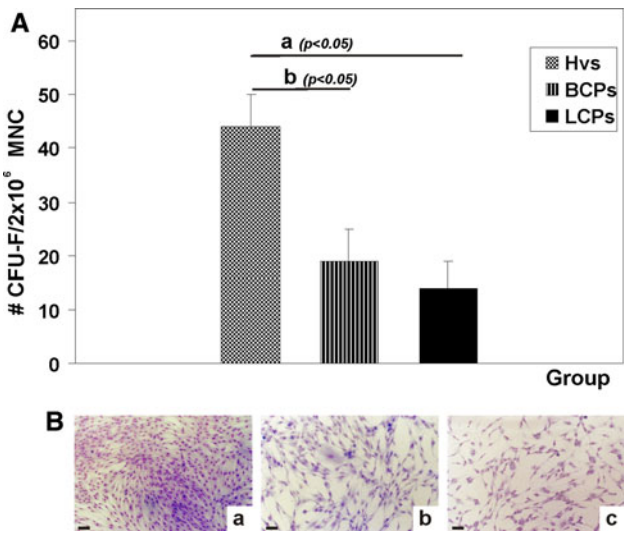


Fig. 1 Colony Forming Units-Fibroblastic (CFU-F) assay: **A** Number of CFU-F/2 × 10^6 MNC observed in 7 healthy volunteers (HVs), 7 breast cancer patients (BCPs) and 7 lung cancer patients (LCPs). Values are expressed as mean ± SE. Statistical analysis: Mann–Whitney test; *a*: $p < 0.05$; *b*: $p < 0.05$. **B** CFU-F size observed for a HV (*a*), a BCP (*b*) and a LCP (*c*); Giemsa staining (100×). *Scale bars* 200 μm

the Giemsa and Von Kossa techniques to evaluate the mineralisation matrix and cellular morphology (osteoblast/osteocyte).

As this was the first time that osteogenic differentiation between MSCs from patients and MSCs from HVs were compared, we developed the classification system to describe the osteogenic differentiation stages based on distinguishing morphological changes (Fig. 2A). Fibroblasts (grade 0 according to our pattern of differentiation) have a morphology consisting of a fusiform shape with variable cytoplasmic processes and an elliptical nucleus with one or two nucleoli. When MSCs commit to the osteogenic lineage (precursors to osteoblasts or grade 1 according to our pattern of differentiation), the cytoplasm condenses, and the nucleus acquires a rounded shape and shifts to become slightly off-centred. In the more differentiated stages, cells become smaller. Osteoblasts (grade 2 according to our pattern of differentiation) have an oval to round shape with a large amount of basophilic cytoplasm and slightly off-centred nuclei. Osteocytes (grade 3 according to our pattern of differentiation) are smaller than osteoblasts, with condensed cytoplasm and an off-centred dark nucleus and one or two nucleoli. Cells committed to the osteoblast lineage are interpreted as a hybrid between the well-defined fibroblast and osteoblast cell type with admixed characteristics of both.

In control cultures from the three groups, the observed cell morphology was the classic spindle shape of mesenchymal stromal cells enriched in MSCs with fibroblastic characteristics (prolyl 4-hydroxylase+).

After 21 days of culture in osteogenic differentiation medium, 100 % of the cultures from HVs in the osteogenic

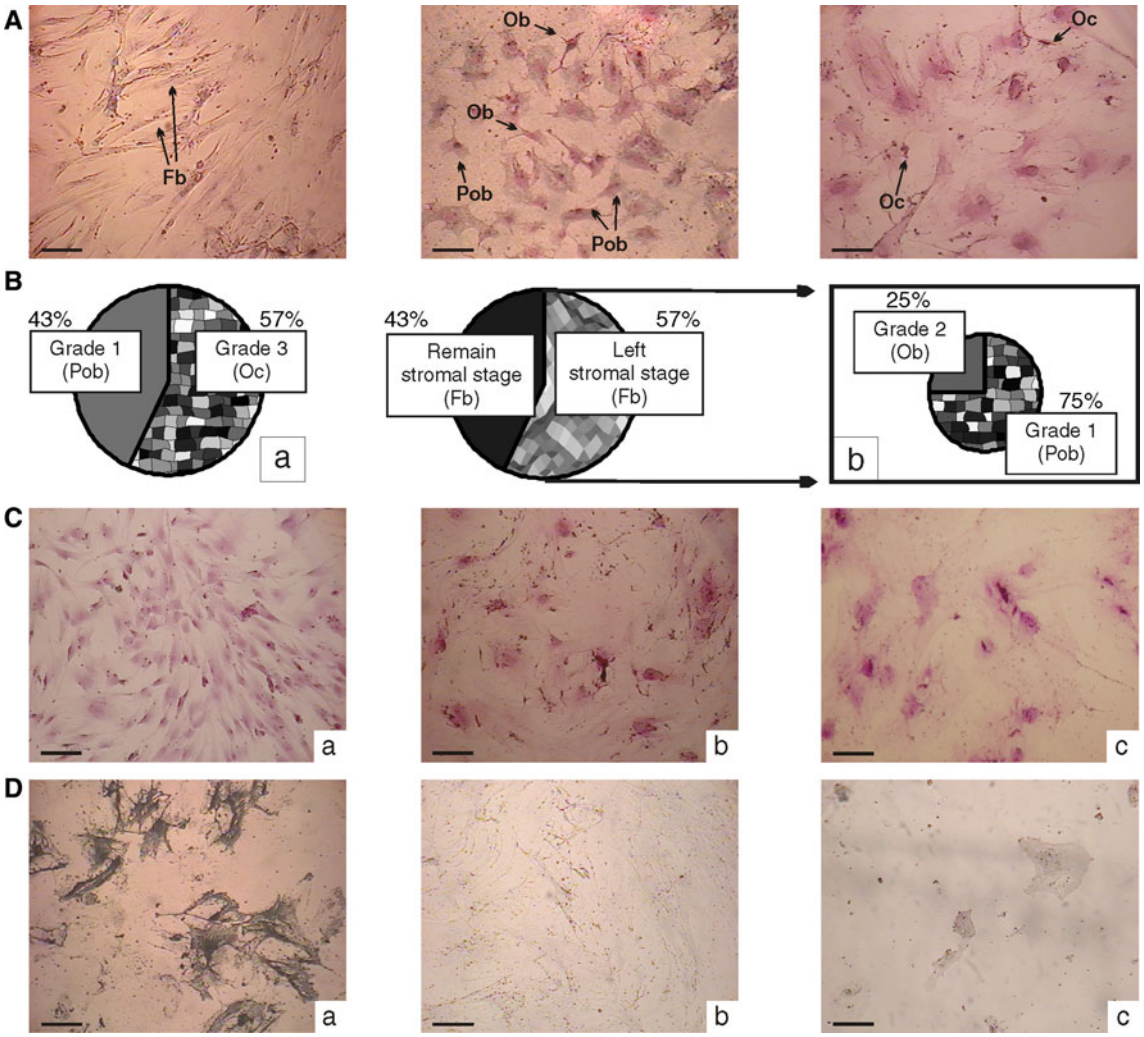


Fig. 2 Osteogenic differentiation: **A** Healthy volunteer (HV). Grade 0 = Fibroblasts (Fb), 1 = osteoblast precursor (Pob), 2 = osteoblast (Ob), 3 = osteocyte (Oc). Von Kossa-Giemsa stains (400×). **B** Differentiation pattern for HV cultures (*a*) and BCP cultures (*b*). **C** HV-MSCs without differentiation medium (*a*), and HV (*b*) and LCP (*c*) cultures with osteogenic medium; Von Kossa-Giemsa stain (400×). **D** Mineralisation matrix after differentiation for a HV (*a*), a BCP (*b*) and a LCP cultures (*c*); Von Kossa stain (400×). *Scale bars* 100 μm

medium developed osteogenic lineage cell colonies, covering 24 ± 4 % of Petri dish surfaces with differentiated cells. In contrast, only 57 % (4/7) of BCP cultures developed colonies with osteogenic cells, covering 15 ± 7 % of the Petri dish surfaces with differentiated cells. These results depended on the sample, and there was much variation at the moment of the comparison; however, when each parameter was analysed for each case, the deficiency in osteogenic differentiation for BCPs became clear. For example, 2 BCP samples had a high percentage of Petri dish surface containing differentiated cells (40 %) but achieved a very low grade of differentiation (grade 1).

From 100 % of osteogenic lineage differentiated HV cultures, 43 % were scored as grade 1 (osteoblasts precursors) based on the pattern of differentiation, and 57 % were scored as grade 3 (osteocytes). In contrast, from the 57 % of the BCP cultures that differentiated beyond the stromal stage, 75 % of the samples scored as grade 1 and the other 25 % as grade 2 (Fig. 2B).

In analysing the osteogenic potential of LCP-BM samples, the percentage of Petri dish surface with differentiated cells was 10 ± 3 %, achieving only grade 1 (osteoblast precursors) except for 1/7 that reached 1 % of Petri dish surface with differentiated cells of grade 3 (osteocytes). None of the LCP cultures developed colonies with osteogenic cells.

All patient cultures were hypocellular compared with the HV cultures that expanded almost to confluence, showing classical fibroblast morphology. These control cultures from the three groups were negative for Von Kossa-Giemsa staining (Fig. 2C, a).

The anti-osteocalcin stain demonstrated weak expression levels in each sample from HVs, BCPs and LCPs under our culture conditions; for this reason, it could not be used to obtain important information regarding the different grades of osteogenic differentiation. Alizarin S Red staining (data not shown) confirmed data obtained using Von Kossa-Giemsa staining.

Finally, it is interesting to note that in the BCP and LCP osteogenic cultures, where the cells were in different stages of differentiation and had altered nucleus/cytoplasm ratios, cells grouped together and developed calcium deposits in an isolated behaviour and a heterogeneous pattern. Furthermore, the mineralisation matrix distribution was scattered and random, whereas HV cultures showed a homogeneous distribution over the complete surface of the cultures (Fig. 2D).

Adipogenesis

After 3 weeks of induction/maintenance cycles and Oil red-O stain, 100 % of HV cultures differentiated into adipocytes, whereas only 57 % of the samples from BCPs and LCPs did so (Fig. 3A, B). Moreover, as shown in Fig. 3C, we found a significant decrease in the number of adipocytes present in BCP (a) and LCP (b) cultures compared with those from HVs (a, b) (a: $p < 0.05$, b: $p < 0.05$; Mann–Whitney test).

Control cultures from the three groups showed cells with the classic spindle shape morphology of mesenchymal stromal cells with fibroblastic characteristics and negative for Oil red-O stain.

Chondrogenesis

In contrast to osteogenesis and adipogenesis, the results for chondrogenesis differentiation showed that all samples from HVs, BCPs and LCPs developed optimal chondrogenic pellets. All showed homogeneous distribution for collagen type II staining and typical metachromasia with Toluidine Blue staining (Fig. 4A, B and C). The presence of chondrogenic tissue morphology was confirmed by a pathologist for all the samples.

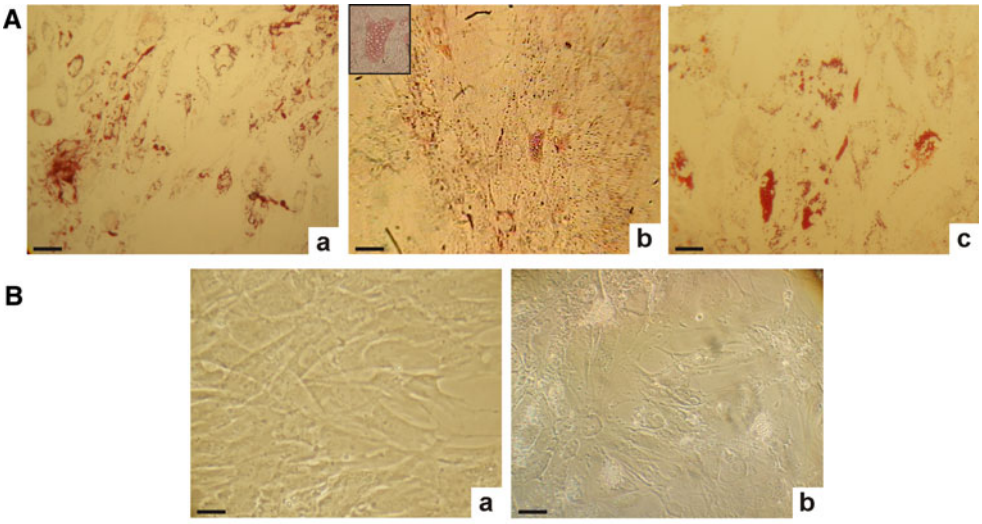
As we have previously described, we evaluated the differentiation process by counting chondrocytes (cells in the final stage of differentiation) over 5 fields from each pellet at random. This procedure was repeated over 3 different slides from the same sample. The media for each group of the number of chondrocytes observed in 5 fields per pellet, and the media for each group of the weight of the pellets are summarised in Fig. 4D. From these data, we observed a significant increase in the number of chondrocytes present in BCP and LCP pellets compared with those from HVs. Moreover, a higher number of chondrocytes was correlated with an increase in the weight of the BCP and LCP pellets, with significant differences only seen between data from BCPs and HVs.

Control cultures from the three groups showed cells with the classic spindle shape morphology of mesenchymal stromal cells with fibroblastic characteristics and negative for Toluidine Blue stain.

CD146 analysis

BM-MSCs from BCPs, LCPs and HVs, were in agreement with the immunophenotyping criteria proposed by the International Society for Cellular Therapy [44]. Results indicated that BM-MSCs from HVs, BCPs and LCPs were positive for CD73, CD90 and CD105 surface molecules and negative for CD11b, CD19, CD34, CD45 and CD79a. We also determined that the MSCs in this study were positive for expression of CD59 and CD146 surface molecules (Fig. 5).

Recent findings support the idea that BM-MSCs that present triple plasticity have elevated expression of CD146 antigen when compared with unipotential MSCs. It has



C

Total number of adipocytes observed over Petri Dish surface

Sample	Hvs	BCPs	LCPs
X±SE	467.85 ± 214.56 (a, b)	3.71 ± 2.16 (a)	98.14 ± 66.27 (b)

Statistical analysis: (a) $p<0.05$; (b) $p<0.05$. Non-parametric Mann Whitney Test. Hvs healthy volunteers, BCPs: breast cancer patients, LCPs: lung cancer patients.

Fig. 3 Adipogenic differentiation: **A** MSCs from a healthy volunteer (HV) (*a*), a breast cancer patient (BCP) (*b*) and a lung cancer patient (LCP) (*c*); Oil red-O stain (400×, detailed adipocyte in *b* at 600×). **B** HV: fresh culture without (*a*) and with (*b*) adipogenic medium (400×). **C** Number of adipocytes/Petri (mean ± SE) for HVs, BCPs and LCPs samples analysed. *Scale bars* 100 μm

been shown that there is an approximately 2-fold difference in mean fluorescence intensity between tri-and unipotent cells [40, 45]. CD146 has also been identified as a marker for clonogenic osteoprogenitor cells responsible for new bone formation [40, 45]. As shown in the present study, BM-MSCs from untreated advanced BCPs and LCPs have a reduced capacity to generate osteogenic and adipogenic lineages compared to BM-MSCs from HVs; therefore, we hypothesised that the expression of CD146 may be altered in MSCs.

Results showed a significantly lower percentage of MSCs expressing CD146 in BCPs compared with HVs; moreover, the expression per cell scored as relative fluorescence index (RFI = marker mean fluorescence index/corresponding isotype control mean fluorescence index) was significantly lower in MSCs from BCPs than from HVs (Table 1). While results showed a similar trend with respect to RFI for BM-MSCs from LCPs, the difference between HVs and LCPs was not significant (Table 1). Density plots and histograms showing the percentage of MSCs expressing CD146 and RFI are shown in Fig. 6A and B, respectively.

We also quantified expression levels of CD146 mRNA in BM-MSCs for each sample using real-time quantitative PCR (qPCR). Although mRNA expression does not always correlate with final protein expression levels, qPCR results showed a decrease in the expression of CD146 mRNA in MSCs from advanced BCPs and LCPs compared with MSCs from HVs (Fig. 6C), consistent with our immunostaining results.

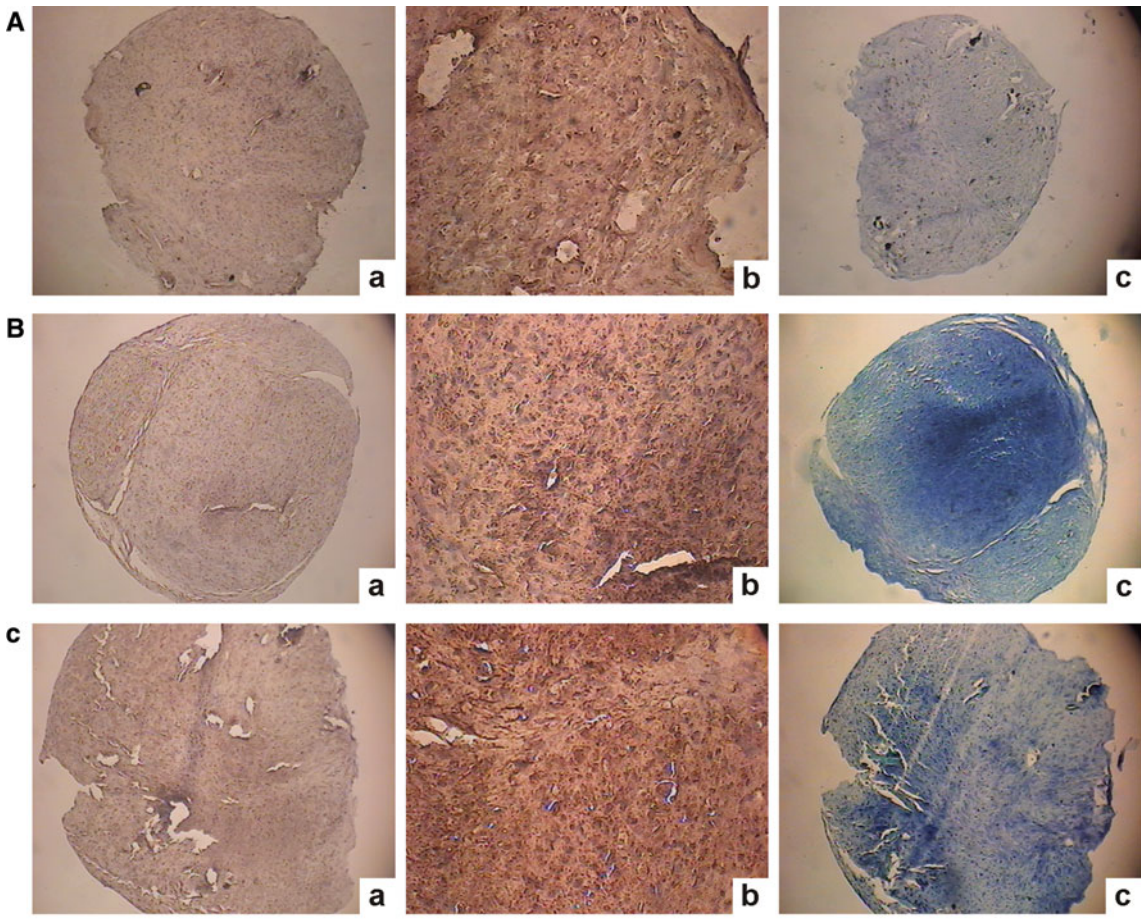
Dkk-1 analysis in peripheral blood plasma (PBP): an inhibitor of osteogenesis

Finally, results showed that PBP levels of Dkk-1 were significantly higher in BCPs and LCPs than in HVs even before observable bone metastasis (Fig. 7). Values are expressed as mean ± SE (pg/ml): BCP = 11,361 ± 1,219 (a); LCP = 12,320 ± 1,394 (b) and HV = 7,268 ± 1,221 (a, b) (a: $p < 0.05$ and b: $p < 0.05$; Unpaired *t* test).

Discussion

Crosstalk between metastatic cancer cells such as breast and lung cancer cells and BM-MSCs is critical to the development and progression of BM/bone metastasis [46]. The process of breast and lung cancer BM/bone metastasis, including tumour cell seeding, tumour dormancy and metastatic growth, is only partly understood.

Recent studies have demonstrated that the interaction with BM-MSCs results in changes in breast and lung



D

 # Chondrocytes and pellet weights means

	Hvs	BCPs	LCPs
#Chondrocytes	8.41 ± 0.78 (a, b)	20.36 ± 2.16 (a)	15.78 ± 2.50 (b)
Pellet weights	0.02 ± 0.08 (c)	0.10 ± 0.01 (c)	0.02 ± 0.01

Statistical analysis: Results are expressed as X±SE (a) $p<0.05$; (b) $p<0.05$ and (c) $p<0.05$. Non - parametric Mann Whitney Test. Hvs: healthy volunteers, BCPs : breast cancer patients, LCPs : lung cancer patients.

Fig. 4 Chondrogenic differentiation: Pellets obtained from a healthy volunteer (HV), a breast cancer patient (BCP) and a lung cancer patient (LCP) culture (**A**, **B** and **C**, respectively) positive for collagen type II (*a*, *b*; 100× and 400×, respectively) and stained for glycosaminoglycane with Toluidine Blue (*c*; 100×). **D** Chondrocytes and pellet weight means for HVs, BCPs and LCPs. Non-chondrogenic pellet positive for Toluidine Blue or Collagen II was observed in control cultures without differentiation medium

cancer cell migration, proliferation, morphology, aggregation and adherence, conferring more invasive and metastatic properties on the tumour cells [13, 14, 47–52]. These observations and the ability of MSCs to generate osteoblasts and osteocytes and regulate the osteoclastogenesis process indicate that MSCs in the BM can play an important pro-tumorigenic role contributing to the formation of a microenvironment that promotes BM and bone metastasis [1, 2]. However, it is unclear how MSCs behave before the appearance of BM and bone metastases in BCPs and LCPs in advanced clinical-pathological stages. Therefore, in the present work we studied the osteo-chondro-adipogenic differentiation of BM-MSCs from untreated advanced BCPs and LCPs without BM and bone metastasis or any metabolic bone disease, as well as from HVs. Moreover, because previous reports indicated that BM-

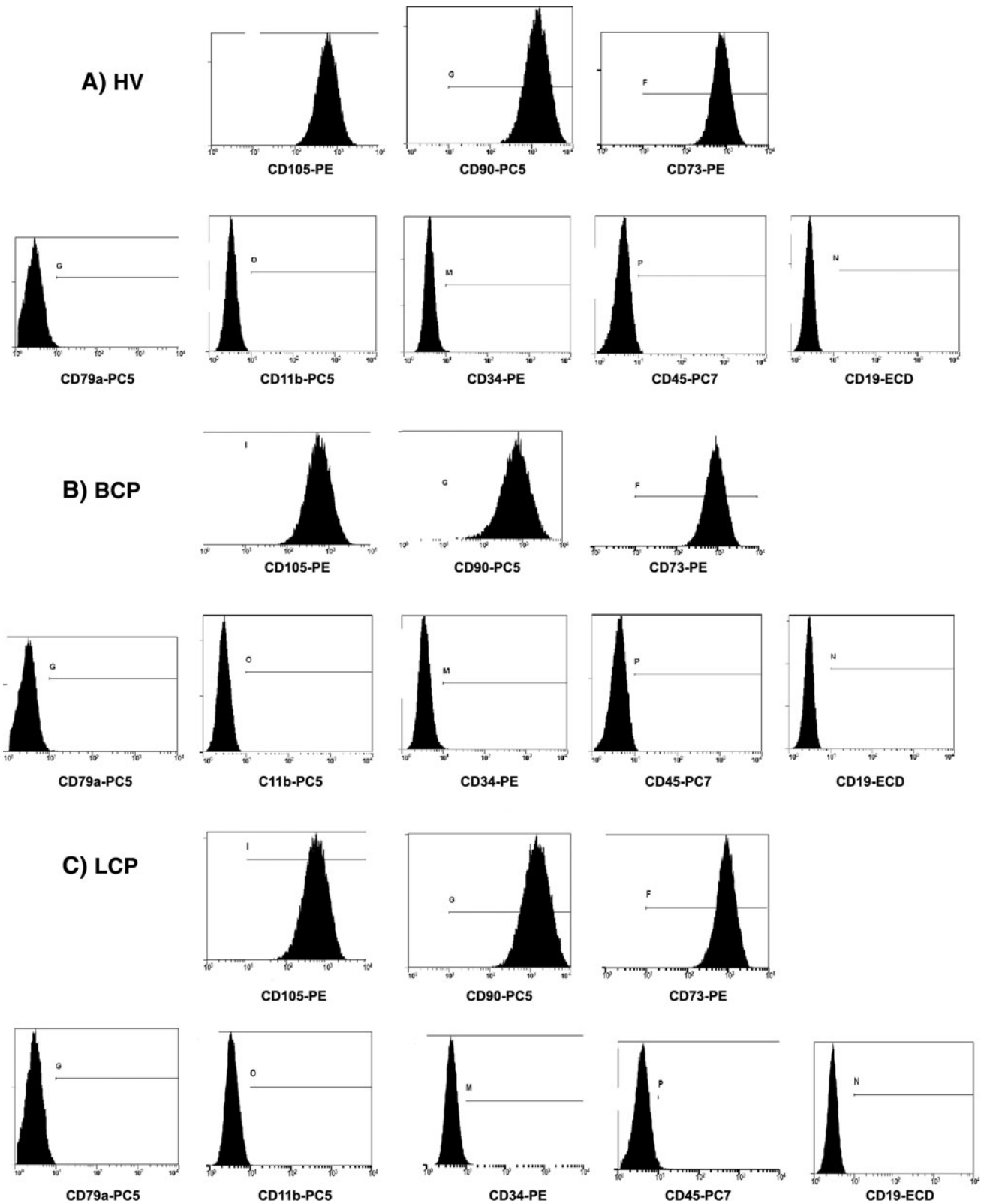


Fig. 5 BM-MSC phenotypic characterisation: Results from healthy volunteer's MSCs (HV), breast cancer patient's MSCs (BCP) and lung cancer patient's MSCs (LCP) are shown as examples in **A**, **B** and **C**, respectively

Table 1 CD146 surface molecular analysis: Percentage and relative fluorescence index (RFI) of MSC expressing CD146 for healthy volunteers (HVs), breast cancer patients (BCPs) and lung cancer patients (LCPs)

	HVs	BCPs	LCPs
% MSC	99.80 ± 0.14(a)	86.60 ± 5.21(a)	99.23 ± 0.38
RFI	82.68 ± 12.24(b)	18.48 ± 3.19(b)	66.08 ± 10.81

Statistical analysis: Results are expressed as X ± SE (a) $p < 0.05$ and (b) $p < 0.05$. Parametric unpaired t test. *HVs* healthy volunteers, *BCPs* breast cancer patients, *LCPs* lung cancer patients, *RFI* relative fluorescence index

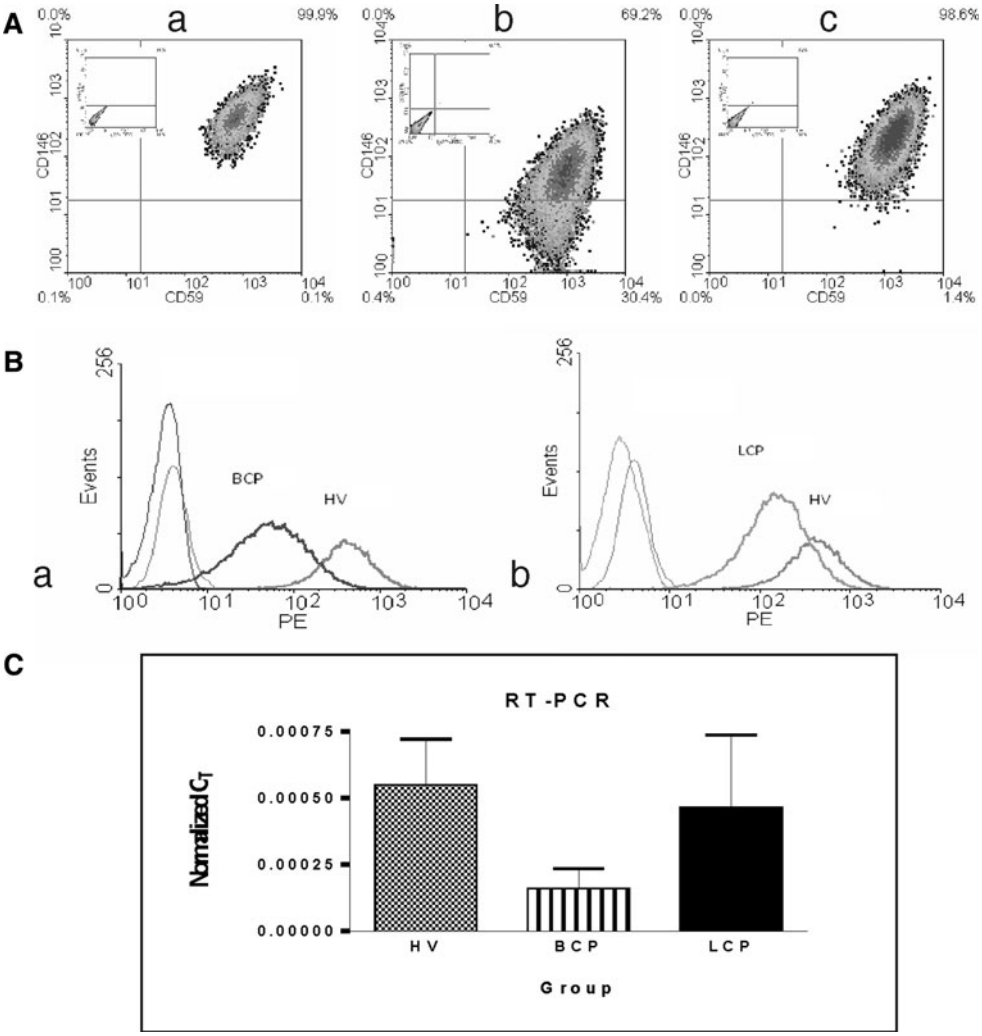


Fig. 6 CD146 analysis: **A** Density plots showing %MSC CD146 + from a healthy volunteer (HV) (*a*), a breast cancer patient (BCP) (*b*) and a lung cancer patient (LCP) (*c*). **B** Histograms comparing BCP (*a*) and LCP (*b*) CD146 relative fluorescence index (RFI) with the HV-RFI. **C** CD146 RNA quantification by real time PCR using normalized threshold cycle (C_T) mean to compare the expression levels per group

MSCs that show triple plasticity strongly express CD146 antigen compared with unipotential MSC, we evaluated the percentage of MSCs that express CD146 antigen (positive in MSCs from the BM vascular niche). We also determined the level of CD146 expression per MSC in the BM samples of these patients and HVs. Finally, we quantified the levels of Dkk-1, an osteogenic inhibitor factor, in PBP of patients and HVs.

We confirm here that BM-MSCs from untreated advanced BCPs and LCPs without bone and BM metastasis have a lower cloning efficiency compared with those from HVs. Moreover, we observed an increase in chondrocyte differentiation resulting in an incomplete differentiation process towards osteogenic and adipogenic lineages (Fig. 8).

Many studies have been performed to investigate the successive events of the differentiation process and the relationship among the three lineages. Some authors have reported that adipocytes are the first to diverge and that a common osteo/chondro precursor gives rise to the other two lineages [53, 54], which may explain the fact that an increase in the chondrocyte lineage leads to a decrease in

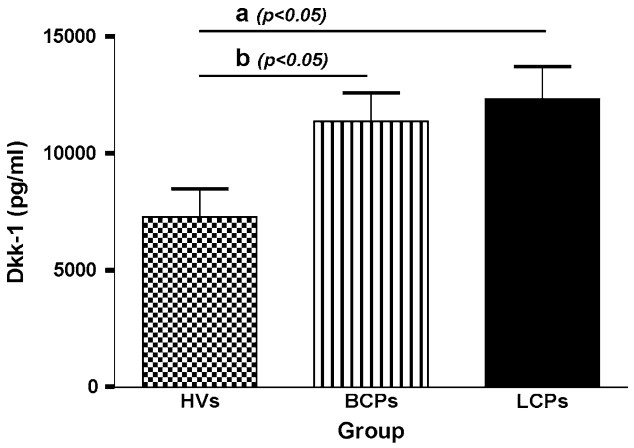


Fig. 7 Dkk-1 peripheral blood plasma levels: Bar graph for untreated advanced breast cancer patients (BCPs), lung cancer patients (LCPs) and healthy volunteers (HVs) groups. Values are expressed as mean ± SE. Statistical analysis: Unpaired *t* test; *a*: $p < 0.05$; *b*: $p < 0.05$

the osteogenic lineage and vice versa, due to an imbalance in the differentiation of the common precursor. Chondrogenesis and osteogenesis occurs simultaneously in vivo, and a common specific transcription factor (Osx) regulates the precursor commitment to osteo or chondrogenic lineages during bone formation [53, 55].

Plasticity may also be due to MSC potentiality, and the presence of different quantities of uni-, bi- or tri-potential BM-MSC may explain, at least in part, the defect observed in MSC plasticity. Recently, Russell et al. [40] reported that multipotential BM-MSCs that are capable of giving rise to at least three differentiated lineages overexpress of CD146 antigen. In addition, they found a correlation between CD146 expression and proliferation through transmembrane signalling. In accordance with those reports, we observed that BM-MSCs from untreated advanced BCPs and LCPs that showed lower capacity to differentiate into osteocytes and adipocytes and lower proliferation capacity also had lower levels of CD146/MSC. Moreover, some authors proposed CD146 as a marker for MSCs from BM that are clonogenic and osteogenic in vivo [56], which makes a more interesting perspective in relation to osteogenic differentiation and its consequent implications for osteolytic bone lesions observed in bone metastasis in advanced BCPs and LCPs. Taken together, these observations suggest that a decrease in CD146 expression indicates that these patients' MSCs have reduced clonogenic and osteogenic capacity and include fewer MSCs from the vascular niche. Vascular niche MSCs are very close to haematopoietic precursors and their regulation [39].

Pinzone et al. [57] have postulated that damaged bone provides a permissive microenvironment for tumour growth and metastatic events. Dkk-1, a Wnt signaling inhibitor, can disrupt the repair of bone. Therefore, Dkk-1 overexpression and secretion lead to the inhibition of osteoblast maturation and, as a result, stimulation of osteoclast differentiation and proliferation [23]. Thus, Dkk-1 secretion by tumours into the bone can lead to irreparable damage to the tissue. Multiple myeloma, osteosarcoma, lung cancer, breast cancer, and some forms of prostate cancer present examples of Dkk-1 mediated damage [23, 24, 58]. It has also been shown that high levels of Dkk-1 in the PBP of individuals in the later stages of lung cancer, breast cancer and multiple myeloma are significantly associated with tumour progression, decreased survival and the presence of osteolytic lesions [23, 24, 58]. Our results show for the first time that PBP-Dkk-1 levels can also be an indicator of bone imbalance in advanced stages of breast and lung cancer before osteolytic bone metastasis. Menezes et al. [59] proposed that Dkk-1 is a critical factor that modulates the bone microenvironment. Based on the location and components of the microenvironment, Dkk-1 will support different outcomes. Dkk-1 inhibits osteoblastic differentiation of BM-MSCs and high circulating levels of it are associated with osteolytic lesions.

In comparing data from previous reports to our results from studies on untreated advanced BCPs and LCPs without BM/bone metastasis, we have found some interesting correlations regarding osteogenesis. For example, Scutt and Bertram [60] found that when MSCs from HVs were cultured in osteogenic medium supplemented with

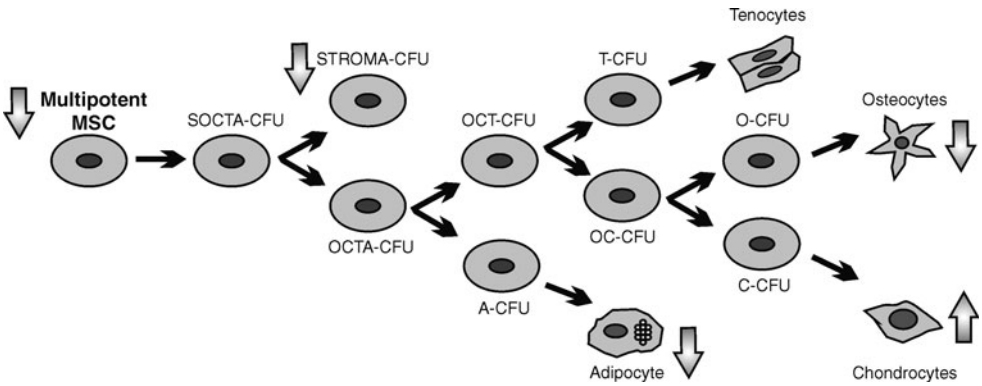


Fig. 8 MSC and plasticity in untreated advanced breast cancer and lung cancer patients (BCPs and LCPs): Colony-forming units (CFU): SOCTA (Stromal-Osteogenic-Chondrogenic-Tenocyte-Adipogenic), OCT (Osteogenic-Chondrogenic-Tenocyte), A (Adipocyte), T (Tenocyte), OC (Osteogenic-Chondrogenic), O (Osteogenic), C (Chondrogenic) and STROMA (stromal)

bFGF, the percentage of osteogenic colonies, collagen synthesis and calcium extracellular deposits increased. Furthermore, Huang et al. [61] found bFGF essential to the osteogenesis differentiation of MSCs. We observed in previous studies that BM-MSCs from untreated advanced BCPs and LCPs without BM/bone metastasis release low levels of bFGF compared to HVs [43]. Therefore, lower levels of bFGF may modify osteogenic differentiation of patients' MSCs and the production of bone mineralised matrix [60]. Regarding chondrogenesis, some authors have found bFGF levels to be correlated with inhibition of chondrogenesis [62, 63]. Thus, observed changes in chondrogenesis could be explained in part by the low levels of bFGF release [43].

In addition, we have previously observed a decrease in the percentage of mesenchymal stromal cells from subconfluent primary cultures expressing TGFβ receptors type I, II and III in both groups of patients and in the percentage of MSCs expressing FGF receptor type I in LCPs, as well as lower expression/MSC of TGFβ and FGF receptors in BCPs and LCPs compared to HVs; those receptors are implicated in osteogenic differentiation of MSCs and in the formation of the bone mineralisation matrix [64]. The development of an adequate mineralisation matrix by osteoblasts and osteocytes is due to the appropriate interactions between β integrins such as $\alpha_2\beta_1$ and matrix components such as collagen type I. Therefore, the reduced bone mineralisation matrix and the random calcium deposits that we found in the osteogenic differentiation cultures of both types of patients could be related to the reduced expression of collagen type I previously observed in MSCs from BCPs and LCPs compared to those from HVs [65].

Recent reports have also shown reactive oxygen species (ROS) and lipid oxidation products to be inhibitors of osteoblast differentiation, and in accordance with those studies, previous data from our group reported higher levels of ROS and lipid oxidation species in PBP and BMP from untreated advanced BCPs and LCPs without BM/bone metastasis when compared with HVs, which might be related to the defect in osteogenic differentiation of patients' MSCs that we found in the present study [66, 67].

Finally, MMP-2 has been described as a proteolytic factor for the cleavage and subsequent activation of IL-1β, which appears to be involved in the inhibition of chondrogenic differentiation [68, 69]. We found lower levels of soluble MMP-2 in BM-MSC primary cultures from untreated advanced BCPs and LCPs without BM/bone metastasis compared with levels in HVs. Therefore, the low levels of MMP-2 could be related to a decrease in IL-1β activation and consequent lack of chondrogenesis inhibition [70]. Furthermore, it is known that MMP-2 can alter migration, proliferation and osteogenic differentiation of BM-MSCs; thus, the low levels of MMP-2 found in these BCP and LCP cultures might be due to the low cloning and osteogenic differentiation capacity of patients' MSCs [71]. Because our present findings are in concordance with our previous observations and because untreated advanced BCPs and LCPs are more likely to develop BM and bone metastasis, we suggest that the failure in the plasticity of MSCs might precede tumour cell invasion into BM/bone, and that these events may favour tumour cell affinity for BM/bone microenvironment.

Because no patient had any bone disorder by the time of this study, we propose that the primary tumour affected somehow MSCs' self-renewal capacity and plasticity to generate osteocytes, and because MSCs are common precursors for adipocytes and chondrocytes, a failure in MSC plasticity would display a defect in every lineage.

Based on the data presented here, we conclude that analyses of BM-MSCs osteogenic differentiation, CD146 expression and Dkk-1 levels in PBP from untreated advanced BCPs and LCPs, without BM and bone metastasis, could be utilised as predictive factors of future BM and bone disorders. Furthermore, it is possible that these studies, in association with biochemical markers of bone turnover, could also be useful for prognosis determination and clinical monitoring.

In conclusion, our data support the concept that the reservoir of BM-MSCs from these patients may be responsible for creating a pre-metastatic niche suitable for the invasion and proliferation of breast and lung cancer cells in BM and bone. Moreover, the reservoir of MSCs in the BM of these patients may not be adequate to achieve regeneration of bone after the tumour cells metastasise to BM/bone, as MSCs also alter the production and activity of mature osteoblasts and osteoclasts. Thus, it is possible that BM-MSCs promote a cycle of tumour cell growth and bone destruction.

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Conflict of interest No competing financial interests exist.

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