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Cell-Cell Communication Networks Propose a Modulation of the Hematopoietic Stem Cell Niche by Invading Breast Carcinoma Cells

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Abstract

Background: The human bone marrow can become a target of disseminated tumor cells in a relevant proportion of breast cancer patients. However, the underlying pathophysiology is incompletely understood. This study aims to identify and characterize potential mechanisms modulating the bone marrow hematopoietic microenvironment by invading breast cancer cells (BCC) as a basis for experimental evaluation.

Methods: Static cell-cell communication networks, representing the integrated signaling among breast carcinoma cell lines (MCF-7 or MDA-MB-231), bone marrow-derived mesenchymal stromal cells (MSC) and hematopoietic stem and progenitor cells (HSPC), were constructed *in-silico* by combining differentially overexpressed genes of the involved cell populations with known ligand-receptor interactions. Using the networks as guidance, pathophysiological relevance of the analyzed populations to breast cancer-initiated hematologic abnormalities was appraised by systematic literature mining. *In-vitro* co-culture modeling was performed to evaluate the paracrine effects of BCC on MSC-HSPC signaling and to validate main implications of the exposed signaling network.

Results: Breast cancer cells exhibited intensive bidirectional intercellular signaling with MSC and to a lesser extent with HSPC. BCC-derived signals were reported to recruit MSC to sites of breast cancer, activate tumor associated fibroblasts (TAF) and modify MSC differentiation. Hematopoietic microenvironment-derived signals were predominantly associated with BCC attraction and metastatic progression. Potential ligands that protect from metastases were exclusively HSPC-derived. *In-vitro* co-culture modeling revealed that BCC mediated loss of the niche-derived hematopoiesis-supporting factor SDF-1 and the emergence of FGF-2 in the MSC-HSPC interaction.

Conclusion: We propose a modulation of MSC by BCC, inter alia via the FGF-2/FGFR1 pathway, resulting in activation of TAF, generation of a vascularized tumor stroma, breast cancer progression and consequential impairment of hematopoiesis by reduction of SDF-1 levels. Those indirect changes in the HSC niche upon BCC invasion might increase the vulnerability for bone metastasis in breast cancer patients.

Keywords: Hematopoietic stem cell niche; Hematopoietic microenvironment; Tumor associated fibroblasts; Mesenchymal stem cells; Hematopoietic stem cells; Breast cancer cells; Bone metastasis; Cell-cell communication networks; Coculture models; Signaling

Abbreviations: BC: Breast Cancer; BCC: Breast Cancer Cell; CAR cell: CXCL12-Abundant Reticular cell; CI: Control Index; CMP: Common Myeloid Progenitor; DMEM: Dulbecco's Modified Eagle's Medium; DTC: Disseminated Tumor Cell; FBS: Fetal Bovine Serum; FGF: Fibroblast Growth Factor; FGFR: Fibroblast Growth Factor Receptor; GMP: Granulocyte and Monocyte Progenitor; HER2: Human Epidermal growth factor Receptor 2; HSC: Hematopoietic Stem Cell; HSPC: Hematopoietic Stem and Progenitor Cell; MBEI: Model-Based Expression Index; MEP: Megakaryocytic and Erythroid Progenitor; MLP: Multi Lymphoid Progenitor; MPP: Multipotent Progenitor; MSC: Mesenchymal Stromal Cell; PDGFR: Platelet Derived Growth Factor Receptor; SDF-1: Stromal cell-Derived Factor 1; SMC: Smooth Muscle Cell; TAF: Tumor Associated Fibroblast; TAM: Tumor Associated Macrophage; VEGF: Vascular Endothelial Growth Factor

Introduction

Breast cancer is the most common malignancy in females and associated with a high frequency of disseminated tumor cells (DTC) detectable in the bone marrow at early disease stages [1]. Despite a great progress in diagnosis and treatment of breast cancer in recent years, bone metastases continue to be related with very high morbidity and mortality [2,3]. Tissue invasion and metastasis is one important hallmark of cancer that relies on a complex system of communication with cell types from the original site of cancer and the potential metastatic niche [4,5]. As an example, the tropism of breast cancer cells to bone marrow was recently reported to rely on the selection of bone metastatic cells with affinity for specific stromal signals by the primary tumor stroma [6]. Clinical observations also suggest distinct paracrine bone marrow alterations by neoplastic breast cells [7].

For pathophysiological investigation of those observations, it

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is important to consider the composition and spatial organization of the bone marrow microenvironment. The conceptual "stem cell niche" model assumes that every adult stem cell depends on a very unique microenvironment *in-vivo*, which is defined both as physical compartment and the presence of certain environmental conditions [8]. This microenvironment is necessary for maintenance of the stem cell pool. Moreover, the communication between the niche and its hosted stem cells is essential for synchronization with physiological demands [9]. Although the complexity of the niche for hematopoietic stem cells (HSC) has not been revealed entirely, several cellular and extracellular components, including even inorganic factors, have been identified [10]. Intercellular communication is essential for the hematopoietic microenvironment and to a large extent provided by paracrine signals such as stromal-derived factor 1 (SDF-1) and growth factors (Figure 1).

Initially, bone-lining osteoblasts that are in vicinity to the endosteum were found to support hematopoiesis [11,12]. Further studies specified spindle-shaped, N-cadherin$^+$ CD45$^-$ osteoblasts as potent cells for quiescence and maintenance of HSC, predominantly by cell-contact dependent mechanisms [13]. Sinusoidal endothelium, comprised by endothelial cells and pericytes, was found to promote the proliferation and significantly regulate the recruitment of HSC as well as the transendothelial migration from the bone marrow niche into the circulation, i.e. mobilization and the reverse process, called homing [14-16]. Those observations suggest a division of the hematopoietic stem cell niche in an "endosteal" compartment, which facilitates quiescence and maintenance of HSC and a "vascular" compartment regulating mobilization and homing [16] (Figure 1). However, both compartments are interconnected and should not be regarded as strictly separated [17]. Mesenchymal stromal cells (MSC) are a decisive component in the hematopoietic stem cell niche, as several MSC-secreted cytokines are important for HSC functioning and MSC are a source of multipotent stem cells, which generate the hematopoiesis-supporting cell populations described above. Moreover, MSC-subpopulations,

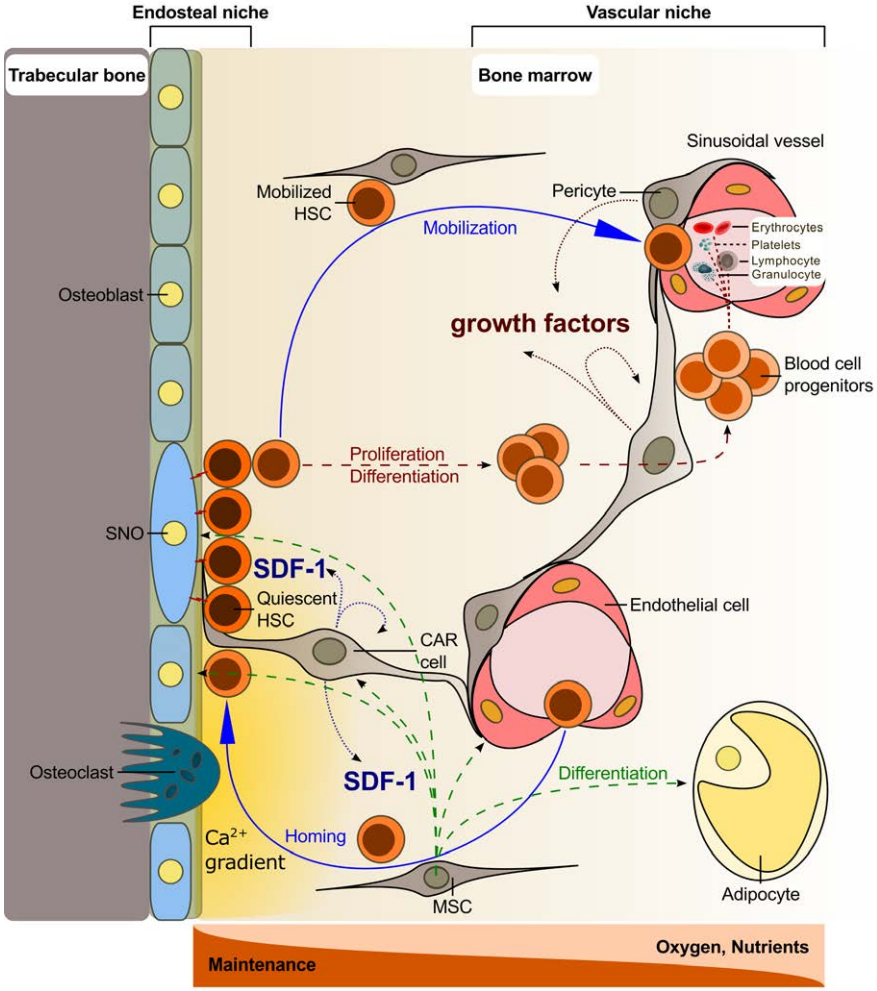

Figure 1: Composition of the hematopoietic microenvironment in bone. Based on [16,103,104] HSC quiescence and maintenance at the endosteal niche is facilitated by spindle-shaped, N-cadherin$^+$ CD45$^-$ osteoblasts, predominantly in a cell-contact dependent manner [13]. Mobilization and homing of HSC occurs via the vascular niche, which is comprised by endothelial cells and pericytes. Both niches are interconnected. The concentration of calcium decreases and the concentrations of oxygen and nutrients increase, as HSC migrate towards the vasculature. This is associated with proliferation and differentiation. Distribution and support of HSC and progenitors are significantly regulated by cytokines. SDF-1 is among the most important of those. Growth factors attract HSC to the vascular niche and therefore facilitate mobilization. MSC-subpopulations, such as CAR and nestin-expressing pericytes, support HSC via cytokine secretion and additionally in a cell-contact dependent manner [18,19,22]. MSC have the potential to differentiate into most of the relevant niche cell populations (osteoblasts, endothelial cells, CAR cells, pericytes) [29,30].

such as CXCL12-abundant reticular (CAR) cells and nestin-expressing pericytes provide significant support to hematopoietic stem and progenitor cells (HSPC) [18-20]. The interplay between MSC-derived SDF-1 and its receptor CXCR4 expressed on HSC has a crucial role in regulating the homing and mobilization of HSPC to and from the niche [21]. Naturally, MSC fates such as proliferation and differentiation are also markedly influenced by soluble factors [22].

Emerging evidence suggests a reciprocal interaction between DTC and the bone microenvironment [4,23]. However, most reports focus on the impact of MSC on cancer cells, either in terms of building a supportive tumor stroma or as potential vehicles in targeted therapies [22]. Observations from *in-vivo* experiments confirm that invasion of bone marrow by prostate cancer cells results in dysregulation of bone metabolism by affecting the paracrine signaling of HSC and MSC in bone marrow [24]. Although it can be assumed that paracrine communication between breast cancer cells (BCC) and bone marrow cells also substantially contributes to morbidity and mortality of cancer patients, there is very limited knowledge about the influence of breast cancer cell-derived signals on bone marrow function.

The combination of *in-vitro* coculture modeling and computational analysis of gene expression data is proposed as a promising method to analyze the tumor microenvironment and draw conclusions for the *in-vivo* situation [25]. Gene expression-based cell-cell communication networks, which were used in this study, have been demonstrated as valuable tool to dissect principles of paracrine interaction within the complex hematopoietic system and identify key signals that regulate the fate of hematopoietic stem cells [26-28]. For the first time, we extended this tool to non-hematopoietic cells and systematically examined the potential cell-cell communication between BCC, MSC and HSPC *in-silico* with special respect to metastatic progression and modulation of bone marrow function. *In-vitro* coculture modeling provided further information about the influence of BCC on the communication between HSPC and MSC. We finally identified and characterized a complex system of potential BCC-related modifications of the bone marrow hematopoietic microenvironment for further *in-vitro* and *in-vivo* validation.

Materials and Methods

Cell culture

All cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 on appropriate tissue-culture treated plastic surfaces and cultured in D-MEM GlutaMAX (Invitrogen, Darmstadt, Germany) supplemented with 10% fetal bovine serum (FBS, Biochrom, Berlin, Germany), unless stated otherwise.

Primary MSC were isolated from human bone marrow aspirates of healthy donors as described previously [29], after informed consent and approval by the local ethics committee. All MSC included in the study met the minimal criteria according to the International Society for Cellular Therapy [30]. CD73, CDw90, CD105 and CD166 were present in at least 95% of the cells, while CD34 and CD45 were absent. MSC were seeded at 5×10^3 to 10^4 cells/cm^2, cultured as adherent monolayers and grown to 95% confluence with a total media exchange every third day. Human MSC were characterized for surface marker expression pattern (CD73, CD90, CD105, CD146, CD166, CD14, CD34 and CD45) by flow cytometry and the potential to differentiate along osteogenic and adipogenic lineages using standard differentiation media. Primary MSC were utilized within passages 3 to 5.

The human cell line SCP-1 was kindly provided by Professor Dr. Matthias Schieker at Klinikum der Universität München. These cells have been generated by lentiviral transduction of hTERT into bone marrow-derived human MSC, which were originally purchased from Cambrex Corporation [31]. The human cell lines MCF-7 and MDA-MB231 were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany) and the American Type Culture Collection (ATCC, Manassas, USA), respectively. For control, we also evaluated the effect of supernatant transfer from normal breast epithelial MCF-10A cells (obtained from ATCC, Manassas, USA) in comparison with supernatant transfer from MCF-7 and MDA-MB231. Conditioned media of this control study have been generated using serum-free aMEM.

Co-culture models

To discriminate the effects of carcinoma cells on MSC that are strictly based on soluble factors from effects that are cellcontact-dependent, different coculture models between MSC (i.e. primary MSC and SCP-1) and the breast cell lines (i.e. MCF-7, MDA-MB231 and MCF-10A) were applied.

For direct coculture, MSC were seeded at a density of 5×10^3 cells/cm^2 and grown for 3 days to 95% confluence. Thereafter, a total media exchange was combined with addition of breast cancer cells at 2.5×10^3 cells/cm^2. At indicated time points, test media were collected and stored at -80°C until evaluation of SDF-1 content.

For supernatant transfer, breast cell lines were seeded at 2.5×10^4 cells/cm^2 into a T-75 tissue culture flask containing 20 ml of serum-free culture medium. After 72 h, conditioned media were filtered (0.2 µm) and stored at -80°C until the day of use. After thawing and prior to use on MSC, 10% FBS was added to both conditioned media and fresh culture medium as appropriate, to serve as control.

For permanent indirect coculture, MSC and breast cancer cells were cultured simultaneously in the same vessel, but separated by a porous membrane. This was achieved by introducing Millicell hanging cell culture inserts (Millipore, Schwalbach/Taunus, Germany) with a pore size of 1 µm onto subconfluent layers of MSC in 6-well plates and seeding of breast cancer cells at 5×10^3 cells/cm^2 on the porous membrane. At indicated time points, each 500 µl of supernatant from the upper and the lower chamber were collected, pooled and stored in -80°C for later analysis of cytokine content. The amount of collected sample medium was immediately replaced by control medium to ensure a constant culture volume.

CXCL12/SDF-1 ELISA

To measure SDF-1 secreted by MSCs either cultured with tumor cells directly or with conditioned medium, an antibody sandwich ELISA (R&D Systems, Wiesbaden, Germany) was used following the manufacturer's instructions.

Microarray datasets

Affymetrix data for bone marrow-derived MSC and BCC were obtained from Gene Expression Omnibus [32]. Illumina data of human cord-blood derived hematopoietic stem and progenitor cells that had been sorted into distinct progenitor classes by flow cytometric cell sorting were kindly provided by Laurenti et al. [33]. Normalized gene expression data of SCP-1 cells after 3 days of incubation with control medium versus MCF-7 conditioned medium were obtained from the Miltenyi analysis service (Miltenyi Biotec, Bergisch Gladbach, Germany). Please refer to Table 1 for accession numbers and date of

Population	GEO accession number / Source / Date of retrieval	Array type
MSC	GSE21511 / [87] / Feb 22, 2011	GPL5175
SCP1	GSE49858	GPL6480
MCF7	GSE19154 / [88] / Mar 12, 2011	GPL5188
MDA	GSE16732 / [89] / May 06, 2011	GPL5188
HSPC	GSE42414 / [33]	GPL14951

Gene Expression Omnibus (GEO), Human mesenchymal stromal cells (MSC), human cell line SCP-1, human breast cancer cell line MCF-7, human breast cancer cell line MDA-MB231 (MDA), human hematopoietic stem and progenitor cells (HSPC), Affymetrix Human Exon 1.0 ST Array [transcript (gene) version] (GPL5175), Agilent Whole Human Genome Oligo Microarray 4x44K (GPL6480), Affymetrix Human Exon 1.0 ST Array [probe set (exon) version] (GPL5188), Illumina HumanHT-12 V4.0 expression beadchip (GPL14951).

Table 1: Sources of gene expression data used for construction of cell-cell communication networks.

retrieval. The surface expression phenotypes of blood progenitors were as follows:

- Hematopoietic stem cells (HSC): CD34+CD38-CD90+CD45RA-Flt3+CD10-;
- Multipotent progenitors (MPP): CD34+CD38-CD90-CD45RA-Flt3+CD10-;
- Multilymphoid progenitors (MLP): CD34+CD38-CD90^{neg-lo}CD45RA+Flt3+CD10+;
- Common myeloid progenitors (CMP): CD34+CD38+CD90-CD45RA-Flt3+CD10-;
- Granulocyte and monocyte progenitors (GMP): CD34+CD38+CD90- CD45RA+ Flt3+CD10-;
- Megakaryocytic and erythroid progenitors (MEP): CD34+CD38+CD90-Flt3-CD45RA-CD10-;
- Mature: All blood progenitors being CD34+CD38+.

Although differences in the communication with the microenvironment between HSPC from cord blood and bone marrow cannot be excluded, it is shown that both hematopoietic populations have many similarities, including a stromal environment [34]. Previous studies have also proofed that HSPC from cord blood and bone marrow exhibit very similar gene expression profiles. In fact, cord blood and bone marrow hematopoietic progenitors exhibit more similar gene expression profiles than to hematopoietic progenitors from peripheral blood [35]. In addition, it is reported that 51 out of 12600 genes were differentially expressed between cord blood HSPC and bone marrow CD34+ cells [36]. Only 8 of the 51 differentially expressed genes are belonging to the ligand/receptor category.

Microarray processing

Raw gene expression values were obtained using the dCHIP software package [37]. Normalization within the Affymetrix and Illumina arrays was performed using the invariant set method. Expression values were calculated as Model-Based Expression Indices (MBEIs) with PM-only model, followed by outlier detection [38]. Annotation and matching of the array probeset identification numbers (IDs) to the respective entrez gene IDs allowed for merging of data from the different array types. This was performed by facilitating the ensembl database as at March 2011 (biomaRt package, R 2.12.1 statistical environment) [39]. The expression value for each gene was calculated by the mean of all its respective MBEIs.

Identification of differentially over-expressed genes

The non-parametric rank product comparison (100 permutations, cutoff p-value = 0.05; RankProd package, R 2.12.1 statistical environment) [40] was performed between the gene expression profiles of the cell-type of interest and the profiles of each of the other cell types in the respective network. The robustness of this test should allow for the combination of data from different laboratories and array types [41].

Network construction and visualization

The network construction is described in detail by Qiao et al. [26]. Briefly, potential ligand-receptor interactions were determined based on a list (S1 Table) that was compiled by interrogating public protein interaction databases and literature mining. At time of construction, this list comprised a set of 344 ligand-receptor gene pairs. Ligands and receptors representing differentially over-expressed genes among all cell types in the respective networks were connected based on the list of potential ligand-receptor interactions. Other influences on cytokine secretion and surface expression, such as posttranscriptional regulation, differential RNA transport and translation as well as different genomic and proteomic degradation kinetics or internalization of receptors, were not included into analysis. Ligands have been termed as 'autocrine', if both ligands and receptors are expressed by the same cell type, and "paracrine" otherwise. If different cell types in one network express identical autocrine signals, this was also considered as potential cell-cell communication and the respective nodes were connected. Networks were built in R and visualized (Figures S1-S4) using the open source software Cytoscape (v2.8.3) [42].

The control index (CI) was defined as the ratio of efferent paracrine signals n_e for each cell type and afferent paracrine signals n_a ($CI = n_e / n_a$). In combination with the absolute number of paracrine signals ($n_t = n_e + n_a$), this measure was used for an estimation to which extent a cell type is susceptible to the pool of signals in the network ($CI < 1$) or modulating the network properties ($CI > 1$).

Literature mining

All network interactions which involved MSC or carcinoma cells were evaluated for reported and potential functional relevance by literature mining using PubMed as of 14th july, 2013. Articles were searched by combining MeSH-terms of the respective ligands, receptors and interacting cell types. Publications describing functional analysis of the interactions to be examined most closely were selected for detailed reading. In case of excessive literature, literature mining was stopped, when sufficient information was gathered to make a comprehensive statement.

Statistical analysis

ELISA data were normalized to internal control conditions and expressed as median [interquartile range 25 – 75%]. A minimum of 4 biological replicates were sampled in duplicate or triplicate. Probability values were estimated using the nonparametric Wilcoxon signed rank test. Probability levels <0.05 were accepted as significant.

Results and Discussion

Two BCC lines (MCF-7 and MDA-MB231) representing different kinds of breast cancer disease, were used in the present study. MCF-7 are reported to have a predominantly epithelial phenotype and low metastatic potential, while MDA-MB231 show a mesenchymal phenotype and are highly invasive [43]. The potential interactions of those BCC lines with an artificial HSC niche were systematically analyzed by construction of static cell-cell communication networks and subsequent literature mining. Supernatant transfer of MCF-7 and

the MSC line SCP-1 provided an *in-vitro* simulation for the paracrine modification of bone marrow function in breast cancer patients. Further in-vitro coculture modeling was performed to validate implications of the network analysis.

Signaling in the artificial bone marrow microenvironment

Cell-cell communication networks exhibited paracrine communication among all included cell populations (Figures 2, S1-S4), i.e. BCC lines (MDA-MB231 or MCF-7) and cell populations representing the artificial hematopoietic microenvironment (HSPC and MSC).

As qualitative measures for the susceptibility towards network signals, CI, i.e. the ratio of efferent and afferent signals, suggested that

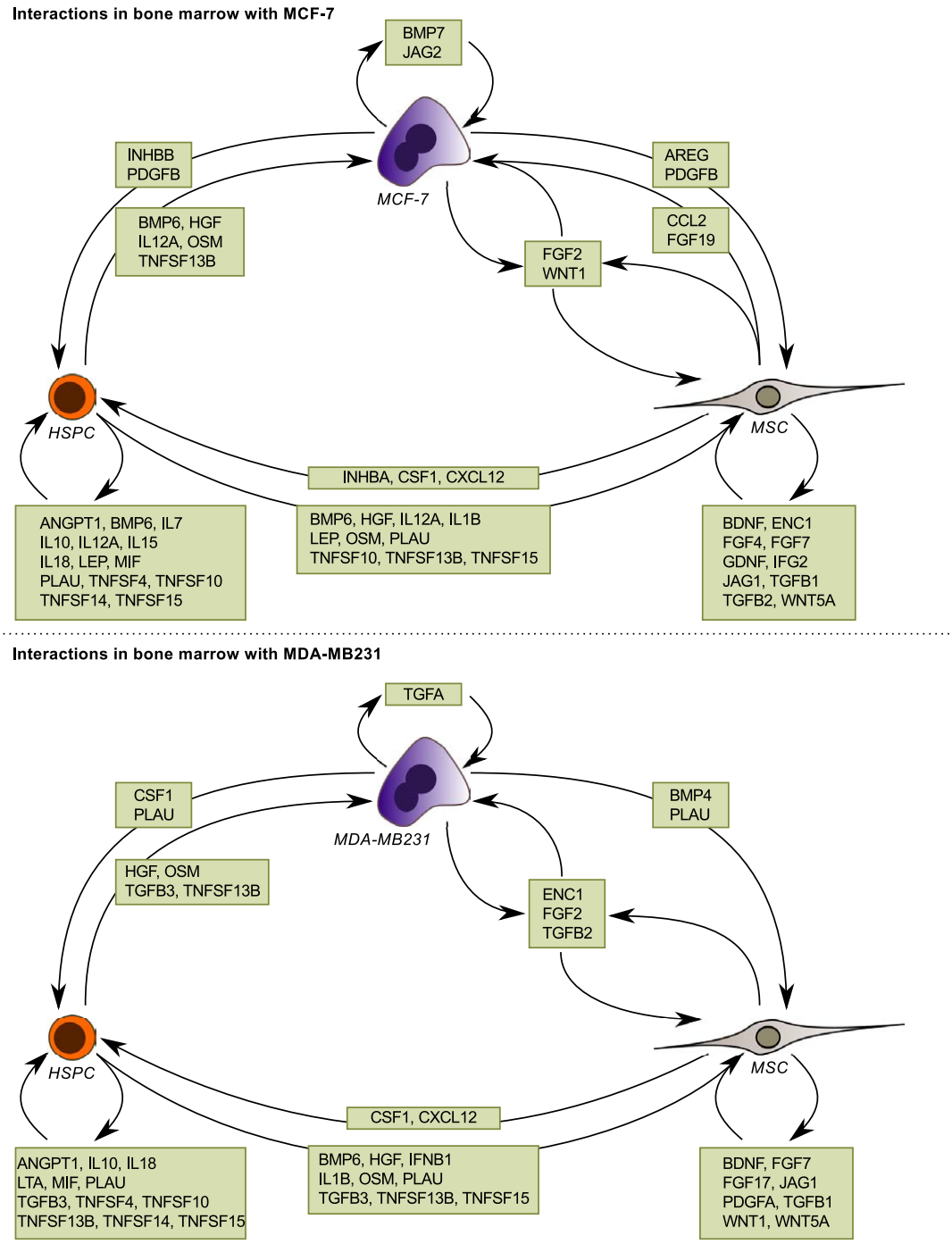

Figure 2: Schematic of intercellular signaling between BCC and bone marrow cells. Schematic visualization of cell-cell-communication network between breast cancer cell lines (MCF-7, MDA-MB231), MSC and HSPC. Gene symbols of upregulated ligand genes matched to upregulated receptor genes on the same cell population (autocrine signaling) or other cell populations (paracrine signaling) are grouped to clarify the principles of intercellular communication among the examined cell populations.

the fate of immature cells (MSC and HSC) appears to be markedly determined by the microenvironment (Table 2). In contrast, progenitors (MPP, MLP, CMP, GMP and MEP) exhibited high CI values, suggesting less susceptibility to external signals. This observation supports the concept of feedback signaling from blood progenitors to HSC [26-28]. Most of the ligands among HSC and progenitors are already reported to affect HSPC fate. BCC exhibited higher CI than stem cells but lower than progenitor cells (Table 2).

A major characteristic of the primary MSC was autocrine signaling (Table 3) as shown in both MCF-7 and MDA-MB231 networks (Figure 2). Those autocrine signals either promoted the proliferation or regulated the differentiation of MSC *in-vitro* (Table 3). This is in line with the hypothesis that MSC provide their own niche [17].

Overall, cell-cell communication networks presented MSC as a crucial part of the HSC niche, since the factors provided from MSC to HSPC (INHBA/Inhibin-βA, CSF1/M-CSF, CXCL12/SDF-1) are reported to affect key features that regulate the stem cell pool such as proliferation, differentiation, mobilization and homing of HSPC [9,16,18,21,44-49].

Almost all signals we identified by our network analysis with relevance to the BCC-MSC interaction were soluble. We therefore postulate that the modulation of MSC by breast cancer cells is substantially mediated by paracrine signaling, rather than by direct cell-cell contact. Supernatant transfer allowed to specifically study this paracrine modulation of MSC by BCC, without confounding contact-dependent signaling and physical displacement effects.

Mechanisms of network modulation by BCC

BCC exhibited particularly intensive intercellular signaling with MSC (Figure 2). Most of the signals involved are associated with metastatic breast cancer disease (Tables 4-6). Specifically, our network supports the idea that invading BCC in the bone marrow benefit from the hematopoietic microenvironment in a parasitizing fashion [9] and moreover, actively modify this microenvironment [22]. In our network analysis, this modulation occurred via two main mechanisms:

Secretion of ligands: As depicted in Figure 2, BCC lines secreted ligands either not present in the physiological niche (AREG/Amphiregulin, BMP4/BMP-4, INHBB/Inhibin-βB, PDGFB/PDGF-BB) or ligands already employed in the intercellular communication of HSPC with MSC (CSF1/M-CSF, PLAU/uPA) and in the autocrine signaling of MSC (FGF2/FGF-2, WNT1/Wnt-1, ENC1/CCL28, TGFB2/TGF-β2).

	MCF7		MDA	
Cell Type	**CI**	**n_t**	**CI**	**n_t**
MSC	0.43	33	0.30	26
Tumor Cells	0.50	15	1.17	13
HSC	0.21	23	0.20	18
MPP	1.20	33	2.00	6
MLP	1.50	20	1.57	18
CMP	13.00	14	9.00	10
GMP	1.71	19	1.67	16
MEP	3.33	13	2.00	6
Mature	1.33	14	1.57	18

Total count ($n_t = n_e + n_a$) of signals connecting the human breast cancer cell lines MCF-7 (MCF) or MDA-MB231 (MDA) with other network populations and the respective ratio of efferent and afferent signals (i.e. control indices, $CI = n_e/n_a$).

Table 2: Control indices and total count of breast cancer cell network signals.

Gene	Alias	Functions
BDNF	BDNF	Neural differentiation [90]
ENC1	CCL28	Chondrogenesis [91]
FGF2	FGF-2	Osteogenesis and proliferation [92,93]
FGF4	FGF-4	Proliferation of MSC without loss of pluripotency [94]
FGF7	FGF-7	Osteogenesis and proliferation [93]
FGF17	FGF-17	Expression in developing bone [95]
GDNF	GDNF	Neural differentiation [96]
IGF2	IGF-2	Osteogenesis [97]
JAG1	Jagged-1	Chondrogenesis [98]
TGFB1	TGF-β1	Chondrogenesis [99]
TGFB2	TGF-β2	Chondrogenesis [91]
WNT1	Wnt-1	Osteogenesis [100]
WNT5A	Wnt-5a	Osteogenesis, no influence on proliferation [101,102]

HGNC gene symbol (Gene), usual name (Alias) and reported functions in MSC based on literature mining (Functions).

Table 3: Potential functions of autocrine signaling among MSC.

Gene	Source	Alias	Recruitment	TAF activation	SMC differentiation	Proliferation	Attraction of BCC	Progression of BC	Osteogenesis	Osteolysis	Adipogenesis	Chondrogenesis	Angiogenesis
AREG	MCF7	Amphiregulin	x				x	x					x
PDGFB	MCF7	PDGF-BB	x	x	x	x		x	x				x
WNT1	MCF7	Wnt-1	x					x	x				
FGF2	MCF7	FGF-2	x	x		x		x	x				x
FGF2	MDA	FGF-2	x	x		x		x	x				x
BMP4	MDA	BMP-4									x	x	
ENC1	MDA	CCL28	x					x		x		x	
PLAU	MDA	uPA	x	x	x						x		
TGFB2	MDA	TGF-β2	x	x				x		x		x	

Human breast cancer cell line MCF-7 (MCF), human breast cancer cell line MDA-MB231 (MDA), HGNC gene symbol (Gene), tumor associated fibroblasts (TAF), smooth muscle cell (SMC), BCC (BCC), breast cancer (BC).

Table 4: Potential effects of BCC-derived signals on MSC.

Gene	Source	Alias	TAF-derived	Attraction of BCC	Recruitment of TAM	Invasiveness	Progression	Resistance	Inhibition	Destruction
BMP6	MPP	BMP-6							x	
CCL2	MSC	MCP-1	x	x	x					
FGF19	MSC	FGF-19					x			
FGF2	MSC	FGF-2	x				x	x		
HGF	GMP	HGF				x	x			
IL12A	MLP	IL-12A							x	x
OSM	GMP	Oncostatin-M				c	c		c	
TNFSF13B	CMP	BAFF				x	x			
TNFSF13B	GMP	BAFF				x	x			
WNT1	MSC	Wnt-1	x				x			

HGNC gene symbol (Gene), BCC (BCC), tumor associated fibroblasts (TAF), tumor associated macrophages (TAM). "c" indicates conflicting reports with respect to promotion or inhibition of metastasis.

Table 5: Potential effects of niche-derived signals on MCF-7.

Gene	Source	Alias	TAF-derived	Attraction of BCC	Recruitment of TAM	Invasiveness	Progression	Resistance	Inhibition	Destruction
ENC1	MSC	CCL28					x			
FGF2	MSC	FGF-2	x				x	x		
HGF	GMP	HGF				x	x			
OSM	GMP	Oncostatin-M				c	c		c	
TGFB2	MSC	TGF-β2	x				x			
TGFB3	Mature	TGF-β3	x				x			
TGFB3	MLP	TGF-β3	x				x			
TNFSF13B	CMP	BAFF				x	x			
TNFSF13B	GMP	BAFF				x	x			

HGNC gene symbol (Gene), BCC (BCC), tumor associated fibroblasts (TAF), tumor associated macrophages (TAM). "c" indicates conflicting reports with respect to promotion or inhibition of metastasis.

Table 6: Potential effects of niche-derived signals on MDA-MB231.

Receiving signals from the niche: BCC captured signals originating from MSC (CCL2/MCP-1, ENC1/CCL28, FGF2/FGF-2, FGF19/FGF-19, TGFB2/TGF-β2, WNT1/Wnt-1) and from HSPC (TNFSF13B/BAFF, BMP6/BMP-6, HGF, IL12A/IL12-A, OSM/Oncostatin-M, TGFB3/TGF-β3). Most (10/12) captured signals were already present in the intercellular communication between HSPC and MSC. Only MCP-1 and FGF-19 were exclusively delivered to MCF-7 cells.

Based on existing literature, the functions of those network signals directed from BCC to MSC were potentially sufficient to promote the generation of a vascularized tumor stroma (Table 4). This was reported to be mediated by MSC recruitment, transition into tumor associated fibroblasts as well as modulation of proliferation and differentiation. INHBB/Inhibin-βB, CSF1/M-CSF, PDGFB/PDGF-BB and PLAU/uPA were directed from BCC to HSPC (Figure 2). All those signals have the potential to modulate the physiological differentiation and proliferation of HSPC or are associated with the induction of myeloproliferative disease [44-47,50-54]. Moreover, M-CSF is reported to induce tumor associated macrophages [55].

The majority of network signals captured by MCF-7 and MDA-MB231 were reported to promote cancer cell invasion and metastatic colonization. Among those ligands, MSC-derived ones were consistently reported to promote osseous metastasis, while some HSPC-derived ones were also reported to inhibit cancer progression (Tables 5 and 6). In contrast to MCF-7, MDA-MB231 did not receive those potential inhibitory signals (BMP-6, IL-12A), suggesting that MDA-MB231 would be more efficient in invading the bone marrow niche.

It is described that BCC establish reciprocal communication with surrounding normal cells to stimulate growth factor supply [4]. For example, BCC activate MSC in a contact-dependent manner to secrete CCL5, which feeds back and stimulates BCC proliferation [56]. Though the interactions between BCC and bone marrow cells depicted in Figure 2 are based on potential connections in a static system and cannot account for dynamic processes, they resemble several of the proposed reciprocal interactions of cancer cells with the tumor microenvironment [4].

It was recently proposed that invading (prostate) cancer cells and HSC compete for the same niche [57,58]. Our analysis of potential BCC signaling in the bone marrow microenvironment indicates that MSC fate regulation could be an intermediate step of this process, as BCC did not immediately capture the MSC-derived hematopoiesis-supporting factors (Activin A, M-CSF, SDF-1). Moreover, the higher vulnerability of epithelial-like MCF-7 towards HSPC-derived potential cancer-inhibiting signals supports the assumption of a greater efficiency in the initiation of bone metastases by mesenchymal-like BCC such as MDA-MB231 [43]. Our networks characterized both types of BCC by the recruitment of stromal cells to their local environment and by the generation of a premetastatic niche via paracrine modification of the bone marrow microenvironment.

Potential effects of BCC signaling on the hematopoietic microenvironment

Tumor associated fibroblasts (TAF) can be generated from MSC [59]. Intriguingly, most identified paracrine signals directed from BCC towards MSC (PDGF-BB, FGF-2, uPA and the TGF-β family) are widely reported to activate wound healing programs which could result in transition of stromal cells to TAF [60]. TAF are reported to promote dissemination, invasion, proliferation and survival of cancer cells by secretion of soluble factors, angiogenesis, support of tumor metabolism, remodeling of extracellular matrix, generation of an immunosuppressive microenvironment and tumor-associated macrophages, induction of epithelial-mesenchymal transition in neighboring cells and generation of cancer stem cells as well as the establishment of a premetastatic niche [2,60,61]. Some autocrine signals in the cell-cell communication networks were similar for BCC and primary MSC (FGF2/FGF-2, WNT1/Wnt-1, ENC1/CCL28, TGFB2/TGF-β2). All of those autocrine signals are reported as TAF-derived growth factors (Tables 5 and 6). These findings support the idea that BCC have an inherent potential to utilize their stromal microenvironment for further progression [6].

A main goal of our current study was to further delineate the potential modulation of the stromal environment by breast cancer cells. For this purpose, we established an *in-vitro* model comprised of HSPC, MSC and BCC. We assumed that the usage of the non-invasive BCC line MCF-7, rather than MDA-MB231, would be a better representation of the premetastatic situation in this model. First, we stimulated the MSC cell line SCP-1 with BCC line MCF-7 supernatant, following by constructing a cell-cell communication network using the gene expression data of HSPC, stimulated SCP-1 and that of MCF-7 (Figures S1-S4). This network was then compared to that constructed using the gene expression data of HSPC, non-stimulated SCP-1 and that of MCF-7 (Figures S1-S4). Stimulation of SCP-1 with MCF-7 supernatant modified the efferent signaling (loss of CXCL12/SDF-1 and INHBA/Inhibin-βA, addition of FGF2/FGF-2) and increased the number of afferent signals to SCP-1 (PLAU/u-PA). This was associated with a lower SCP-1 control index after supernatant transfer when compared to control (0.67 vs. 1.5), suggesting that paracrine breast cancer signaling diminishes the paracrine impact of MSC in the bone marrow niche and increases the susceptibility of bone marrow-derived MSC to external signals.

Although SCP-1 has been generated from bone marrow-derived MSC, it should be noticed that genetic modification of these cells harbors the potential risk of transformation and consecutive different signaling features. Our rationale for substituting primary MSC by SCP-1 was the advantage of a better standardization, as we observed that primary MSC are very heterogeneous in terms of in-vitro expansion capacity, differentiation into certain lineages and hematopoietic support *in vitro* (data not shown). SCP-1 have a normal karyotype

with no signs of malignant transformation and key features of SCP-1 are similar to primary MSC, including the capacity of multilineage differentiation [31,62] and establishment of cell-cell contact with hematopoietic cells [63]. Moreover, important signaling pathways that appeared in our network between MSC and HSPC, e.g. SDF-1/CXCR4, were also present in the network between SCP-1 (control conditions) and HSPC. The loss of SDF-1 in the cell-cell communication networks after supernatant transfer from MCF-7 is consistent with a potential impairment of hematopoiesis by paracrine breast cancer-derived signals, as SDF-1 is the major regulator of HSPC homing and mobilization [9,16,48]. Oscillations of bone marrow SDF-1 levels are subject to physiological regulation by the sympathetic nervous system [49] and substrate of conventional and novel HSC mobilization regimes [21]. Moreover, SDF-1 is reported to support the maintenance of hematopoiesis by promoting survival, long-term self-renewal capability and quiescence of HSPC [17,18].

Next, we performed ELISA to evaluate SDF-1 content in the supernatant of mesenchymal stromal cells from the bone marrow of healthy donors in three different experimental conditions: a) when directly cocultured with MCF-7 or MDA-MB231 breast cancer cell lines (direct coculture), b) when cultured with conditioned medium of those cell lines (supernatant transfer) or c) when cultured simultaneously in the same vessel with MCF-7 or MDA-MB231, but separated by a porous membrane (permanent indirect coculture). Relative to control conditions, SDF-1 secretion by MSC was significantly reduced in all evaluated models with MCF-7 and MDA-MB231. Supernatant transfer had the strongest effect on SDF-1 reduction (0.38 [0.34 – 0.45], $p<0.001$ and 0.36 [0.29 – 0.41], $p<0.001$), followed by permanent indirect coculture (0.61 [0.54 – 0.66], $p<0.01$ and 0.58 [0.44 – 0.65], $p<0.01$) and direct coculture (0.81 [0.69 – 0.86], $p=0.001$ and 0.82 [0.75 – 0.91], $p<0.05$). These data support our assumption that SDF-1 downregulation in MSC is mediated by paracrine BCC-signaling, and supports the supernatant transfer model as appropriate approach for studying paracrine signaling. We performed a validation study and included the non-malignant epithelial breast cell line MCF-10A. Again, SDF-1 secretion by MSC was significantly reduced after supernatant transfer from MCF-7 and MDA-MB231 relative to control conditions (0.56 [0.40 – 0.63], $p<0.001$ and 0.41 [0.24 – 0.57], $p<0.001$), while there was no change after supernatant transfer from MCF-10A (1.08 [0.66 – 1.29]). This indicates, that the paracrine factors which impair the SDF-1 secretion in MSC, are specific for epithelial breast cells after malignant transformation.

Our group recently confirmed a reduction of SDF-1 mRNA and protein levels as well as a reduced SDF-1 promoter activity in MSC after supernatant transfer from breast cancer cells [64]. As potential mediators of this, we identified an enhanced expression of the TGF-β1 cytokine, downregulation of the SP1 transcription factor as well as increased levels of the posttranscriptional regulator miR23-a [64,65]. Another potential mediator of paracrine MSC modulation by BCC is FGF-2, which was apparently upregulated in SCP-1 after MCF-7 supernatant transfer and is reported to inhibit SDF-1 expression in bone marrow stromal cells [66]. FGF-2 is further described as both inductor of TAF and as TAF-derived growth factor [60]. This is in line with experimental findings that suggest FGF-2 as an autocrine growth factor for breast cancer cells via the MAPK/ERK pathway [67]. Therefore, we propose the FGF-2/FGFR1 axis as important pathway in the reciprocal signaling between breast cancer cells and MSC.

It is important to recognize that the SDF-1/CXCR4-axis has also been reported to significantly contribute to metastasis and cancer progression by many potential mechanisms including promoted invasion, proliferation, survival and angiogenesis [68]. It is postulated that the physiologic trafficking of stem cells is mimicked by cancer stem cells and hence, the interaction of MSC-derived SDF-1 and CXCR4 on cancer stem cells is potentially the most important "axis of evil" with respect to metastasis [69]. In our network analysis, we found that other breast cancer-attracting factors, such as MCP-1, have the potential to act in a similar capacity, perhaps substituting for the SDF-1/CXCR4-axis in late stages of the disease (Figure 3).

In summary, our network analysis suggests that

(a) The hematopoietic microenvironment comprises a complex system of paracrine factors connecting HSPC and MSC,

(b) BCC capture factors of this putative HSC niche and potentially affect the MSC fate via reciprocal signaling,

(c) This could result in TAF activation, breast cancer progression and hematopoietic failure.

Correlation with clinical data

Our group recently showed significantly elevated numbers of CFU-GM after 14 days methylcellulose culture of mononuclear cells derived from the peripheral blood of treatment-naive breast cancer patients (13±3) when compared to an age matched healthy control group (5±2) [64]. This is in tune with an increased mobilization and impaired homing of immature blood cells in those patients.

A clinical aspect pointing towards a qualitative impairment of hematopoiesis due to breast cancer signaling is a significantly increased red blood cell distribution width, that is reported both in presence and without bone marrow infiltration by the BCC [70,71]. Furthermore, a high change of the hemoglobin level after diagnosis, which could in part represent impaired hematopoiesis, is associated with poor overall survival of breast cancer patients [72].

Based on gene expression analysis, our cell-cell communication networks identified the FGF-2/FGFR1 axis as one important pathway in the reciprocal signaling between breast cancer cells and MSC. Indeed, immunohistochemical analysis show expression of FGF-2 protein in approximately 70% of breast cancer tissues, while surrounding benign breast tissue shows no signal [73]. ELISA and western blot analysis confirm a highly elevated concentration of FGF-2 protein in breast cancer tissue in comparison to non-malignant breast tissue [74]. FGF-2 measurement was even proposed as biomarker for breast cancer due to increased levels of FGF-2 in patient's nipple fluids and serum [75-77], supporting the idea of high FGF-2 protein levels due to reciprocal and autocrine signaling between BCC and MSC. FGFR1 gene amplification occurs in approximately 10% of all breast cancer patients [78], with higher frequency in unfavorable subtypes such as triple negative breast cancer [79]. It is associated with early relapse, poor survival and resistance to endocrine therapy in estrogen-receptor positive breast cancer [80]. With regard to the growing body of research indicating that the FGF signaling pathway is involved in the pathogenesis of many malignancies, several more or less selective small molecule tyrosine kinase inhibitors targeting this pathway as well as monoclonal antibodies against FGF have been generated [67,81-85]. Results of a phase II clinical trial on HER2-negative metastatic breast cancer patients confirm an antitumor activity of the broad spectrum tyrosine kinase inhibitor dovitinib, which targets FGFR1–3, VEGFR1–3 and PDGFR, in advanced breast cancer [86].

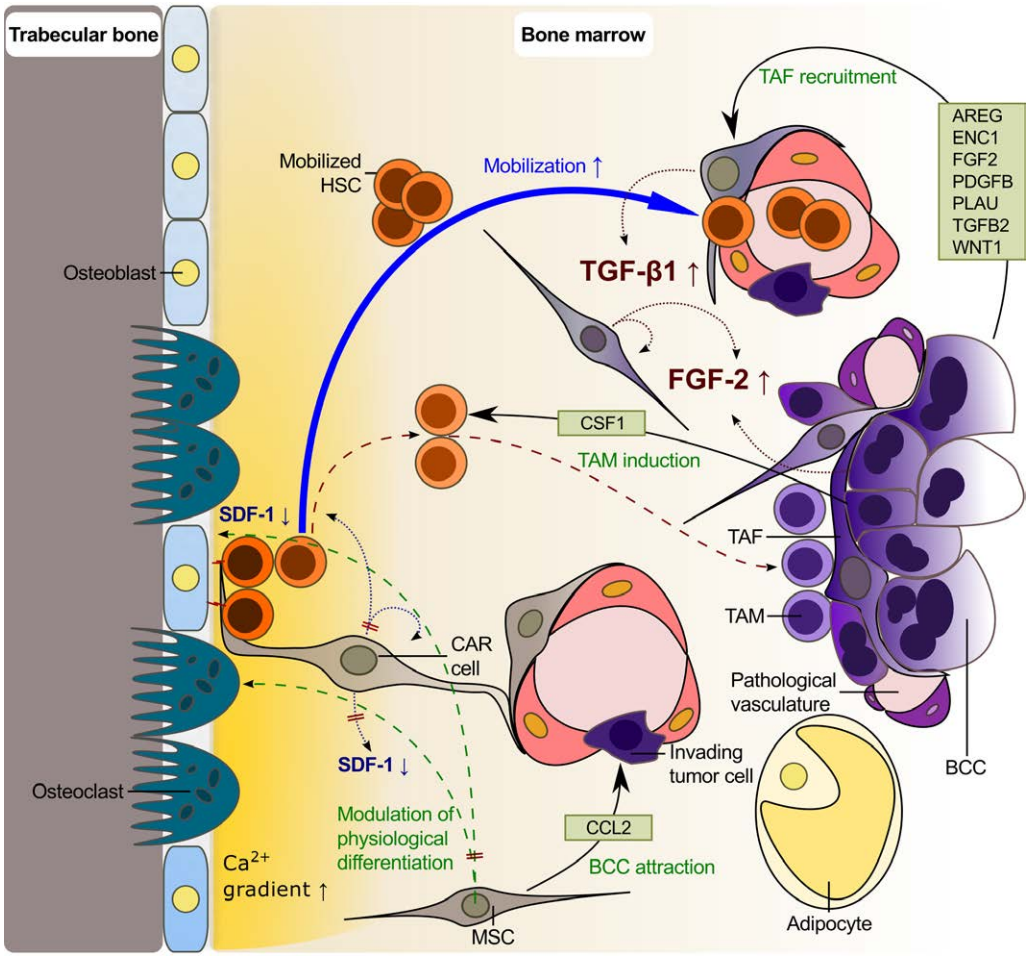

Figure 3: Potential modulation of the hematopoietic microenvironment by invading BCC. Based on existing literature, the interactions identified in the cell-cell-communication networks potentially result in attraction of BCC to the bone marrow (CCL2/MCP-1), recruitment of MSC to the tumor stroma, transition of MSC to TAF and generation of a vascularized tumor stroma (AREG/Amphiregulin, ENC1/CCL28, INHBB/Inhibin-βB, FGF2/FGF-2, PDGFB/PDGF-BB, PLAU/uPA, TGFB2/TGF-β2, WNT1/Wnt-1). Hematopoietic progenitors might be induced to calcium-resorbing osteoclasts and TAM by cancer-derived M-CSF (CSF1). Consistent with experimental data of other groups, FGF-2 appeared as autocrine growth factor in the reciprocal interaction between BCC and MSC in the networks [66,67]. Our group recently identified transforming growth factor β1 (TGF- β1) as potential BCC-induced cytokine which results in impaired SDF-1 secretion by MSC [64]. This data suggests that BCC-induced modification of MSC might result in deregulated localization of HSC with consecutive mobilization and impaired homing. Elevated levels of clonogenic hematopoietic progenitors in the peripheral blood of breast cancer patients *in-vivo* [64] support this idea.

Conclusions

For the first time, we applied the promising method of *in-silico* cell-cell communication network analysis to a system consisting of breast cancer cells, bone marrow derived MSC and hematopoietic cells. This revealed a comprehensive potential paracrine signaling network between BCC and mesenchymal stromal cells (MSC) in an artificial bone marrow microenvironment. Several BCC-derived signals with the potential to modulate MSC and stromal-derived signals with the potential to promote breast cancer bone metastases were identified (Figure 3).

MSC are a key component in the hematopoietic stem cell niche and the cell-cell communication networks suggested SDF-1 as one of the most important MSC-derived paracrine factors regulating HSPC localization and hematopoiesis. Simulation of paracrine breast cancer signaling by *in-vitro* supernatant transfer from BCC line MCF-7 to MSC cell line SCP-1 suggested that the modulation of MSC fate by BCC might reduce the presence of niche-derived factors (e.g. SDF-1) in the bone marrow. This was confirmed by in-vitro coculture modeling. The analysis of network communication further suggested that functional hematopoiesis is a protective factor with respect to bone marrow metastases.

The paracrine communication exposed in this study is therefore illustrating an association between breast cancer progression and impaired hematopoiesis via reciprocal signaling between MSC and BCC. Main implications of the exposed signaling network are validated by *in-vitro* studies of our and other groups as well as by reported clinical data. Re-analysis and combination of gene expression data by systematical examination of cell-cell communication networks is thus able to dissect pathophysiological mechanisms of clinical relevance in a complex system like the bone marrow niche.

Our work provides a foundation for further *in-vitro* and *in-vivo* studies with focus on the potential pathways highlighted in our signaling network. Specifically, we propose the FGF-2/FGFR1-axis, which has already been considered a new therapeutic opportunity in

several malignancies including breast cancer, as important pathway in the reciprocal signaling between breast cancer cells and MSC.

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