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Research Article

Transcriptional profile of fibroblasts obtained from the primary site, lymph node and bone marrow of breast cancer patients

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Abstract

Cancer-associated fibroblasts (CAF) influence tumor development at primary as well as in metastatic sites, but there have been no direct comparisons of the transcriptional profiles of stromal cells from different tumor sites. In this study, we used customized cDNA microarrays to compare the gene expression profile of stromal cells from primary tumor (CAF, n = 4), lymph node metastasis (N+, n = 3) and bone marrow (BM, n = 4) obtained from breast cancer patients. Biological validation was done in another 16 samples by RT-qPCR. Differences between CAF vs N+, CAF vs BM and N+ vs BM were represented by 20, 235 and 245 genes, respectively (SAM test, FDR < 0.01). Functional analysis revealed that genes related to development and morphogenesis were overrepresented. In a biological validation set, NOTCH2 was confirmed to be more expressed in N+ (vs CAF) and ADCY2, HECTD1, HNMT, LOX, MACF1, SLC1A3 and USP16 more expressed in BM (vs CAF). Only small differences were observed in the transcriptional profiles of fibroblasts from the primary tumor and lymph node of breast cancer patients, whereas greater differences were observed between bone marrow stromal cells and the other two sites. These differences may reflect the activities of distinct differentiation programs.

Keywords: bone marrow, breast neoplasm, fibroblast, gene expression profiling, lymph node, mesenchymal stromal cells, tumor microenvironment.

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Introduction

Fibroblasts are mesenchymal cells derived from the mesoderm and are involved in maintaining tissue architecture. These cells secrete a variety of soluble factors that modulate surrounding cell functions.

Cancer-associated fibroblasts (CAF) are the most prominent cell type in the stroma of primary tumors. These CAFs are qualitatively different from normal fibroblasts (Orimo *et al.*, 2005) and heterotypic interactions between cancer cells and stromal cells lead to malignant cell proliferation and metastasis. Indeed, the loss of tumor suppressor genes such as PTEN (Trimboli *et al.*, 2009) or TP53 (Patocs *et al.*, 2007) in stromal fibroblasts results in the activation of pathways leading to epithelial cell carcinogenesis or regional node involvement.

The impact of CAFs on patient prognosis provides insight into their tumor-specific effects (Paulsson and Micke, 2014). The total fibroblast content of breast tumors and the tumor-stroma ratio (< 50% *vs.* > 50% stroma) have an independent impact on recurrence-free and overall survival (de Kruijf, 2011). A higher proportion of αSMA myofibroblasts in breast tumors is also associated with higher proliferation of tumor cells and an unfavorable prognosis (Surowiak *et al.*, 2007). In addition, a metabolic partnership between catabolic fibroblasts and anabolic cancer cells creates a nutrient-rich environment, even under hypoxia, that supports tumor growth (Martinez-Outschoorn *et al.*, 2014).

Several hypotheses have tried to establish the origin of CAFs. First, it is well known that resident fibroblasts, when in contact with malignant cells, become activated myofibroblasts that express specific markers such αSMA

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(alpha smooth muscle actin) (Kalluri and Zeisberg, 2006). Second, epithelial or endothelial cells can trans-differentiate to acquire a mesenchymal phenotype in a process known as epithelial or endothelial mesenchymal transition (Petersen *et al.*, 2003; Zeisberg *et al.*, 2007; Guarino *et al.*, 2009). Third, mesenchymal stem cells (derived from bone marrow or adipose tissue) are recruited to the tumor site to create a favorable microenvironment, in a process similar to wound healing, thereby giving rise to 20-40% of CAFs (Karnoub *et al.*, 2007; Mishra *et al.*, 2008; Shimoda *et al.*, 2010; Quante *et al.*, 2011).

In breast cancer, the involvement of regional lymph nodes is associated with poor prognosis (Kim *et al.*, 2006; Edge and Compton, 2010). Normal lymph nodes contain specific fibroblasts known as reticular cells that are specialized in maintenance of the nodes reticular fiber architecture and the maturation of lymphocytes that express specific chemokines (Vega *et al.*, 2006). However, little is known about the role of fibroblasts in lymph node metastasis. In accordance with the primary tumor site, involved lymph nodes show an altered microenvironment that includes the presence of activated fibroblasts (Yeung *et al.*, 2013). In addition, lymph node fibroblasts may also induce the proliferation and migration of breast cancer cells (Santos *et al.*, 2011).

The origin of fibroblasts in metastatic lymph nodes is speculative, but one possibility is that they originate from the same sources hypothesized for the primary tumor site. Moreover, recent observations suggest that cancer cells carry fibroblasts during their migration to metastatic sites such that these co-traveling cells may facilitate tumor progression in secondary organs (Duda *et al.*, 2010).

Bone marrow (BM) is a common homing organ and a reservoir for disseminated tumor cells in breast cancer (BC). Although only a small fraction of patients present overt BM metastasis, 70% of advanced breast cancer patients develop bone metastasis. Bone marrow is a source of mesenchymal stem cells (MSc) that can give rise to cells of mesodermal lineages (El-Haibi and Karnoub, 2010) such as adipocytes, osteocytes and chondrocytes. Besides playing an important pro-tumorigenic role in the microenvironment of bone metastasis (Martinez *et al.*, 2013), these mesenchymal stem cells can also home to sites of tumorigenesis and integrate into the tumor stroma (El-Haibi and Karnoub, 2010).

It is still unclear whether the transcriptional profile can reveal the cellular origin of CAFs. Given that bone marrow stromal cells are a candidate progenitor cell source, the main goal of this work was to compare the gene expression profiles of stromal cells from the primary tumor, lymph nodes and bone marrow of breast cancer patients.

Material and Methods

Patients

This study was done in accordance with the Declaration of Helsinki (1964) and was approved by the Institutional Ethics Committees (Comitê de Ética do Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo, CAPPesq process nos. 110/02, 368/03, 102/05, 0848/08 and 055/11; and Comitê de Ética do Instituto Brasileiro do Controle do Câncer; approved on 08/02/2002 and 07/27/2008). Twenty-seven breast cancer patients provided signed informed consent and were enrolled in the study. Samples from 11 patients were destined for microarray experiments and from 16 patients were used in biological validation studies. Primary tumor, lymph node metastasis and bone marrow samples were obtained from 11, 8 and 8 patients, respectively (Table 1). The patients received no neoadjuvant chemotherapy or radiotherapy prior to sample collection. All patients were treated at the Instituto Brasileiro de Controle do Câncer (IBCC) in São Paulo city, Brazil.

Primary cell culture

All tumor samples (primary tumor, lymph node and bone marrow) were collected during breast surgery. Involvement of the primary site and lymph node was confirmed histologically. The samples were cut into small pieces and fibroblast primary cultures were established through explant methodology. The mesenchymal origin of the cells was confirmed by the expression of vimentin and lack of expression of pan-cytokeratin and CD45. The culture conditions were as previously described (Rozenchan *et al.*, 2009; Santos *et al.*, 2011; Campos *et al.*, 2013). αSMA expression was detected in all samples from primary tumor and lymph nodes and has been described in previous reports (Rozenchan *et al.*, 2009; Santos *et al.*, 2011; Campos *et al.*, 2013).

Bone marrow aspirates from the sternal area were collected during the breast surgical procedure. Stromal cells were recovered from the mononuclear cell layer by using a Ficoll-Paque^{TM} Plus solution gradient (Amersham Biosciences, Amersham, England) (Sekiya *et al.*, 2002) and plated in 75 cm^2 culture flasks. The cultures were maintained in Iscove's Modified Dulbecco's Medium (IMDM) (Gibco, New York, USA), supplemented with HEPES (25 mM), ampicillin (100 μg/mL), streptomycin (100 μg/mL) and 10% fetal bovine serum (Gibco). One-half of the supernatant with non-adherent cells was removed weekly and replaced with fresh medium until a confluent layer was obtained. After three cell passages, morphologically homogeneous cultures containing only fibroblastoid cells were obtained and used for further studies. The mesenchymal origin was confirmed by the expression of human vimentin (clone Vim 3b4, 1:100; Dako Corporation, Carpinteria, USA) and lack of expression of CD-45 (clone 2B11/PD7/26, 1:500; Dako) and cytokeratin (AE1/AE3, 1:50; Dako).

Detection of occult tumor cells

Occult tumor cells were detected in bone marrow aspirates via nested-PCR that detected the expression of cyto-

Table 1 - Patients characteristics.

Sample	Age	Clinical stage	Histological type	Lymph node	ER	PR	ErbB2
* CAF_1	52	II	IDC	-	-	-	+
* CAF_2	44	II	IDC	-	-	+	-
* CAF_3	53	II	ILC	-	-	-	-
* CAF_4	79	II	IDC	-	+	+	-
* N+_1	59	III	ILC	+	-	+	-
* N+_2	41	III	IDC	+	-	+	-
* N+_3	49	III	IDC	+	+	+	+
* BMMC_1	54	I	IDC	-	+	+	+
* BMMC_2	69	I	IDC	-	+	+	-
* BMMC_3	76	III	IDC	+	+	+	-
* BMMC_4	57	II	IDC	+	+	+	-
† CAF_5	57	III	IDC	+	+	+	+
† CAF_6	63	III	IDC	+	ND	ND	ND
† CAF_7	50	I	IDC	-	+	+	-
† CAF_8	66	II	IDC	+	+	+	+
† CAF_9	57	II	IDC	-	+	+	+
† CAF_10	41	II	IDC	-	+	+	+
† CAF_11	82	III	IDC	+	-	-	+
† N+_4	47	II	IDC	+	+	+	+
† N+_5	40	II	IDC	+	+	+	-
† N+_6	65	II	IDC	+	-	-	-
† N+_7	81	III	IDC	+	+	+	-
† N+_8	43	II	ILC	+	ND	ND	ND
† BMMC_5	51	III	IDC	+	+	+	-
† BMMC_6	63	II	IDC	-	-	-	+
† BMMC_7	41	III	IDC	+	+	+	-
† BMMC_8	50	II	IDC	-	+	+	-

*Samples used in microarray experiments. †Samples used in biological validation set in RT-qPCR experiments. CAF – fibroblasts from primary tumor; N+ - fibroblasts from lymph node; BMMC – bone marrow mesenchymal cells. IDC – invasive ductal carcinoma; ILC – invasive lobular carcinoma; (+) positive or (-) negative immune-expression for ER – estrogen receptor; PR – progesterone receptor. ND – not determined.

keratin 19 (KRT19) in cDNA samples (Table S1). The sensitivity was determined by serially diluting MDA-MB231 cells that express KRT19 in peripheral blood mononuclear cells from a healthy donor; with this approach it was possible to detect one epithelial tumor cell in a million. An external amplicon of 798 bp was generated by 35 cycles in the first reaction of the nested-PCR. The product of the first reaction was then subjected to a second amplification of 30 cycles that generated a final product of 461 bp in bone marrow aspirates containing occult tumor cells.

RNA extraction and reverse transcriptase reaction

Total RNA was extracted using TRIZOL® reagent (Invitrogen); purity was assessed by measuring the absorbance at 260 and 280 nm (260/280 ratio > 1.7) and intactness was assessed by visualizing the 28S and 18S bands (28S/18S ratio > 1.5) after electrophoresis in 1.5% agarose gels. cDNAs for microarray validation were reverse transcribed from 2 μg of total RNA in a 20 μL reaction, using 2 μL of random hexamers (0.5 μg/μL) and 200 U of reverse transcriptase (Superscript II, Invitrogen).

cDNA microarray assembly, hybridization and analysis

Total RNA from stromal cell primary cultures was processed through a two-round RNA amplification procedure done by combining antisense RNA (aRNA) amplification with a template-switching effect, according to a previously described protocol (Wang *et al.*, 2000; Baugh *et al.*, 2001). Three to five micrograms of aRNA was then used in a reverse transcriptase reaction in the presence of Cy3- or Cy5-labeled dCTP (GE Heathcare, Piscataway, NJ, USA) and SuperScript II (Invitrogen). The Hb4A normal epithelial mammary cell line was used as a reference. Hybridizations were done on customized cDNA microarray

slides containing 4608 human genes (Brentani *et al.*, 2005) (GEO accession number GPL1930) in a GeneTac Hybridization Station (Genomic Solutions). Dye swap was done for each sample analyzed to control for dye bias. Hybridization was done at 65 °C. Hybridized arrays were scanned with a confocal laser scanner (Arrayexpress, PerkinElmer Life and Analytical Science Inc., Boston, MA, USA) and the data were recovered with Quantarray software (PerkinElmer). Quantified signals were then submitted to log transformation and to Lowess normalization.

Microarray data analysis

To identify differences among stromal cells from primary tumor, lymph node and bone marrow, data obtained from the three sites were normalized together and only genes present in 100% of the samples were submitted to SAM (significance analysis of microarray) multiclass statistical test using FDR < 0.01. Subsequently, to identify specific differences, differentially expressed genes were further analyzed with the SAM two class test, with FDR < 0.01. Hierarchical clustering based on Euclidian distances and complete linkage was done using the differentially expressed genes, with reliability assessed by the bootstrap technique (TMEV software). Functional analysis was done using the DAVID (Database for Annotation, Visualization and Integrated Discovery) v.6.7 and IPA (Ingenuity Pathway Analysis©, Ingenuity Systems, Inc.). To identify differences in bone marrow according to the presence of occult cells, we used the HT self-self statistical test for low sample numbers (Vêncio and Koide, 2005).

RT-qPCR

Primer sets were designed using Primer Blast (Table S1). Sequences present in different exons were selected. Quantitative RT-PCR was done using a Power Sybr Green PCR master mix (Applied Biosystems) in an ABI HT 7900 thermo cycler (Applied Biosystems). All samples were tested in duplicate and average values were used for quantification. Relative expression was calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The gene PPIA was selected as a reference gene and the Ct average (for the same transcript) of all samples was used as a reference.

Results

Patients

Twenty-seven patients provided written informed consent and were enrolled in the study. Patient age ranged from 40 to 82 years (median: 54 years). According to the clinical stage, the majority (17 patients) were stage II, ten patients were stage III and three were stage I. Sixteen patients had lymph node metastasis at surgery. Immunohistochemical evaluation of the tumors indicated that five patients were negative for hormonal receptors (estrogen or progesterone receptors), whereas 20 patients were positive for at least one hormonal receptor, 15 patients were negative and ten patients were positive for the ErbB2 receptor; it was not possible to evaluate the receptor status of two patients (Table 1).

Influence of occult tumor cells in primary cultures of bone marrow mesenchymal cells

As bone marrow may be a homing organ and a reservoir for disseminated tumor cells in breast cancer, we used nested-PCR to screen bone marrow aspirates for cytokeratin 19 expression, a marker of occult tumor cells. Among eight patients who had bone marrow aspirates collected during surgery, three tested positive for cytokeratin 19 expression (BMMC_3, BMMC_4, BMMC_6, Table 1).

We then examined whether bone marrow mesenchymal cell gene expression was associated with the presence of occult malignant cells in bone marrow. After establishing primary cultures, samples from two patients with lymph node involvement and the presence of occult tumor cells in bone marrow and samples from two patients with no lymph node involvement and a lack of occult tumor cells were selected. Twenty-one differentially expressed genes with a 1.38- to 7.20-fold change in expression were identified and included nine upregulated (PTHLH, TLOC1, NCOA6, C17orf57, ANAPC11, MAST4, POLR3E, CPNE1 and B4GALT5) and 12 downregulated (MRPL2, NAT10, DAP, RNF2, FLOT2, FKBP10, SLIT3, EBNA1BP2, SLC35B2, MICAL2, GPR3 and TSPAN17) genes in bone marrow mesenchymal cells from patients with occult tumor cells. However, none of the selected genes were confirmed as differentially expressed when evaluated by RT-qPCR (data not shown). These results indicate that the presence of occult malignant cells does not significantly affect the molecular characteristics of bone marrow stromal cells.

Gene expression profile of mesenchymal cells from bone marrow, primary tumor and lymph node

Gene expression was initially evaluated using all of the genes represented in the microarray. Comparison of the expression profile of stromal cells from the primary tumor, lymph node and bone marrow of breast cancer patients identified 296 genes that were differentially expressed (SAM multiclass test) (Figure S1). However, since some samples had genes that gave no signal (probably because of methodological errors rather than biological issues) we excluded these genes and reanalyzed the samples. This reevaluation included only genes present in all of the samples and revealed 267 differentially expressed genes among fibroblasts from different origins. (Table S2, Figure 1A). These genes clustered the samples according to their origin, indicating that differences reflecting the samples origin were more important than differences among individual patients (Figure 1B). To detect specific differences related to

the cell origin, a second analysis was done by comparing two groups at a time using the SAM two-class test.

Comparison of fibroblasts obtained from the primary tumor and lymph node identified 20 differentially expressed genes (eight less expressed and 12 more expressed in the primary tumor). When fibroblasts from the primary tumor and bone marrow were compared, 235 differentially expressed genes were observed (101 less expressed and 134 more expressed in bone marrow), and when fibroblasts from lymph node and bone marrow were compared, 245 differentially expressed genes were identified (105 less expressed and 140 more expressed in bone marrow stromal cells) (Table S2). These findings showed that (1) the gene expression profiles of CAFs and N+ were quite similar, (2) the majority of genes were differentially expressed in comparisons of stromal cells from each of these two tumor sites (CAFs and N+) with bone marrow mesenchymal cells, and (3) bone marrow stromal cells had the most differential profile among the three sites (Table S2).

Functional annotation and selection of candidate genes

An important issue when analyzing gene lists is to detect enriched functions. For this analysis, the 267 genes differentially expressed among bone marrow mesenchymal cells, CAFs and N+, were grouped as more expressed in bone marrow stromal cells and more expressed in CAFs and N+. These genes were then used as input data and biological processes from Gene Ontology were screened for enrichment. Functions enriched among genes less expressed in bone marrow mesenchymal cells (and more expressed in CAFs and N+) included negative regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic processes, negative regulation of transcription and apoptosis (Table S3) and functions enriched among genes more expressed in bone marrow mesenchymal cells (as compared with CAFs or N+) included mesoderm formation, response to wounding, regeneration and embryonic morphogenesis, with the latter including only a few genes such as NOTCH2, EYA2, MACF1, TXNRD1, C17ORF28 and HECTD1 (Table S4). In agreement with these findings, Ingenuity Pathway Analysis (IPA) indicated a high score for networks composed of genes related to skeletal and muscular system development (Figure 2A), connective tissue disorders (Figure 2B) and cellular development (Figure 2C), as well as other networks related to development (Table S5).

Expression of candidate genes in another series of samples

The expression of some genes (ADCY2, ANTXR1, CYR61, DNAH1, DUSP1, FGFR1, HECTD1, HNMT, KLF5, LOX, MACF1, MYH9, NOTCH2, SLC1A3, TACC1, TXNRD1 and USP16) was also evaluated by another methodology (RT-qPCR) in some samples not ana-

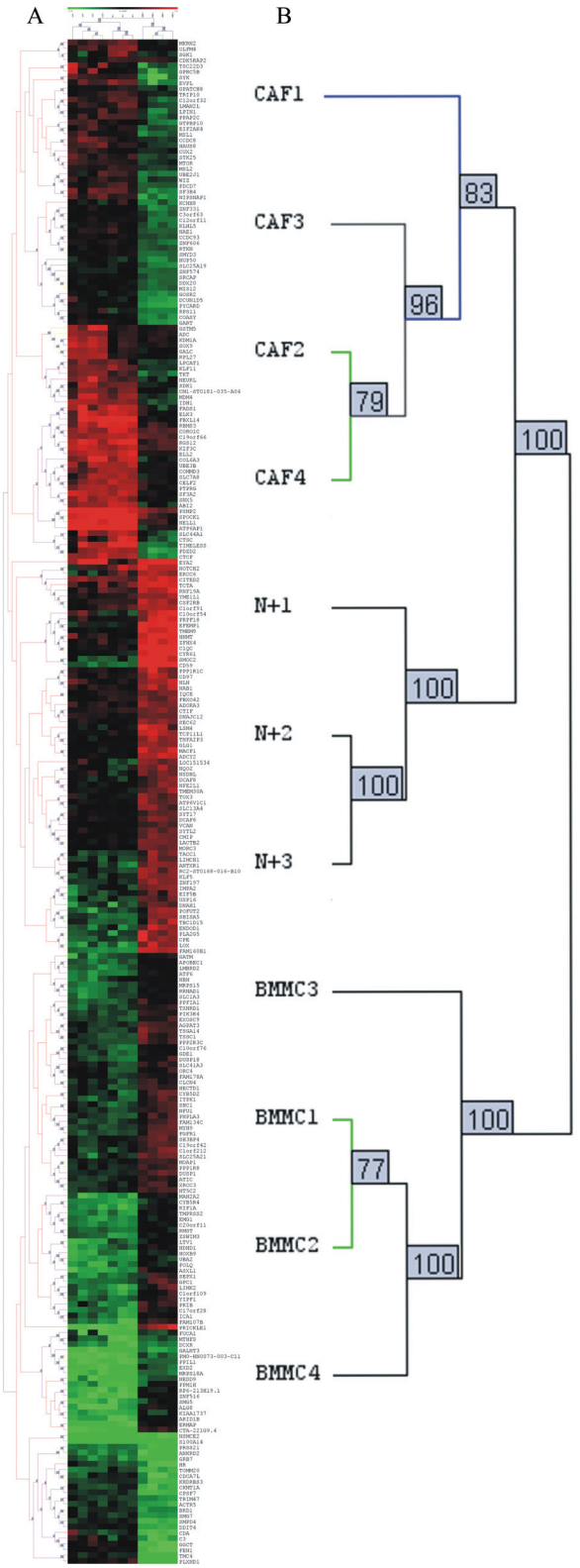

Figure 1 - Fibroblast analysis. (A) Fibroblast clustering according to cell origin. (B) Detail of the hierarchical clustering of fibroblasts from different origins based on the Euclidian distance of 267 genes differentially expressed in fibroblasts obtained from breast primary tumor (CAF), lymph node (N+) and bone marrow mesenchymal cells (BMMC). The reliability values were determined by the bootstrap technique and are shown in the gray boxes in the dendrogram. These genes correctly clustered the samples according to their origins.

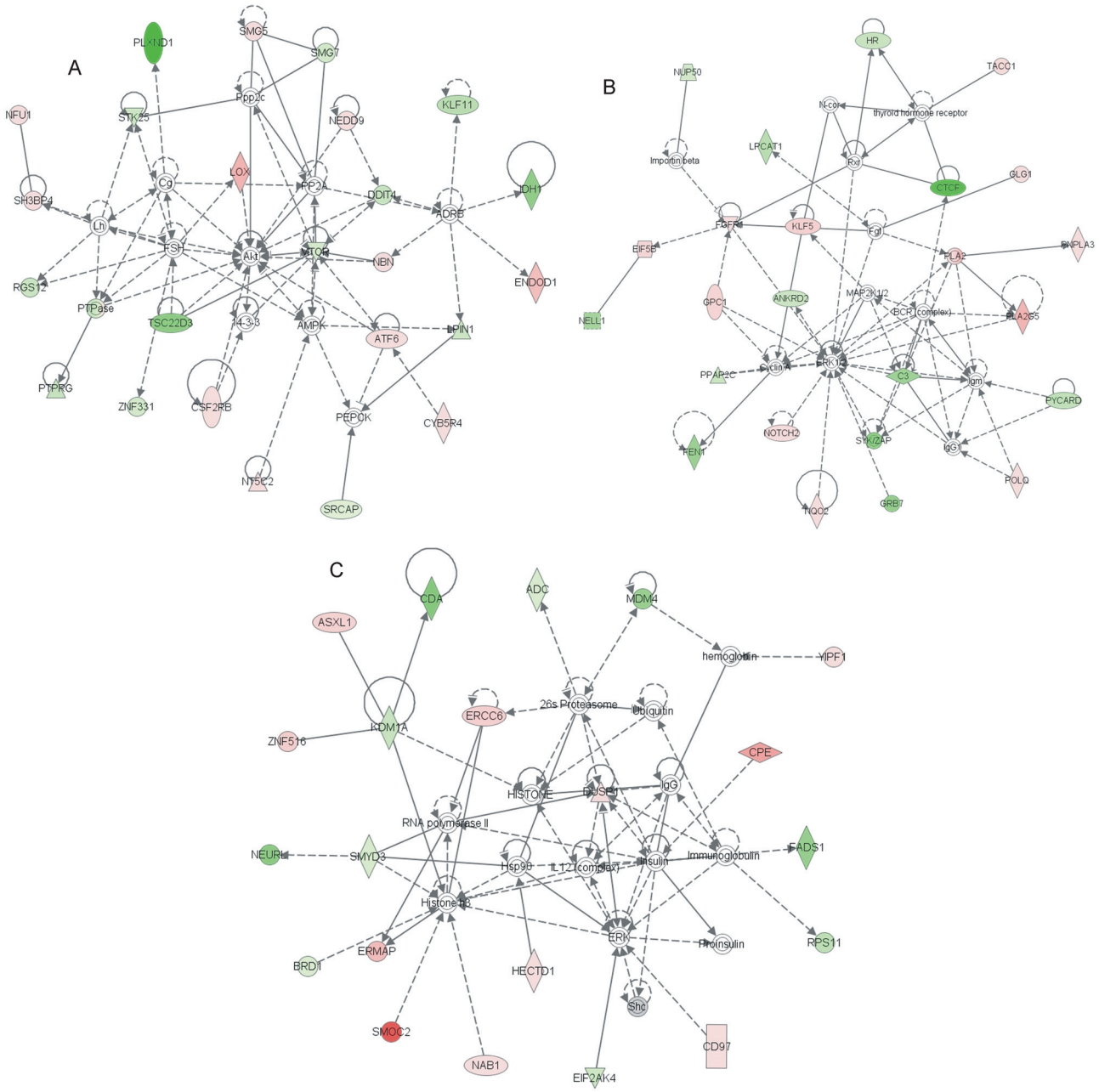

Figure 2 - Top networks observed in Ingenuity Pathway Analysis (IPA). (A) Network based on skeletal and muscular system development and function, cellular assembly and organization, cellular growth and proliferation. (B) Network based on connective tissue disorders, inflammatory disease, skeletal and muscular disorders. (C) Network based on cellular development, digestive system development and function, and embryonic development. The 267 genes differentially expressed among CAF, N+ and BMMC, as well as the fold change of CAF/BMMC, were used as input data for this analysis. Small circles identify proteins that act as hubs to interconnect groups of proteins to create protein clusters in pathways. Proteins in red were more expressed in bone marrow mesenchymal cells, whereas proteins in green were less expressed in bone marrow mesenchymal cells. Solid lines represent direct relationships. Lines connecting the proteins indicate known interrelationships obtained from the IPA database.

lyzed by microarray (biological validation set, indicated in Table 1). The selection of these genes was based on their relation to morphogenesis and differentiation, or on a fold-change in expression > 2. In this analysis, NOTCH2 and SLC1A3 were significantly more expressed in fibroblasts from lymph nodes (N+) when compared to primary tumor samples (CAF) (Figure 3A); ADCY2 and SLC1A3 were confirmed more expressed in bone marrow (BMMC) than in primary tumor fibroblasts (CAF) (Figure 3B) and ADCY2 was significantly more expressed in bone marrow stromal cells (BMMC) when compared to lymph node fibroblasts (N+) (Figure 3C).

Since there was major agreement in the expression data obtained by microarray and RT-qPCR in the compari-

son between primary tumor and bone marrow stromal cells, the expression of other genes was further tested in samples of the biological validation set from these tumor sites. This analysis confirmed that HECTD1, HNMT, LOX, MACF1 and USP16 were more expressed in bone marrow stromal cells (Figure 3D).

Discussion

The main goal of this work was to compare the gene expression profiles of stromal cells from the primary tumor, lymph node and bone marrow of breast cancer patients. In this analysis, 267 genes were differentially expressed among fibroblasts from primary tumor, lymph node and bone marrow. These genes represented ~8% of the genes analyzed and appropriately discriminated among fibroblasts according to their origin. In contrast to small transcriptional differences between fibroblasts from the primary tumor and lymph nodes, a marked difference was observed when fibroblasts from these two sites were compared with bone marrow mesenchymal cells.

Transcriptional profile analysis has shown that fibroblasts from different anatomic sites and distinct donors exhibit topographic differentiation (Chang *et al.*, 2002). Moreover, the fibroblast gene expression program follows a specific anatomic segmentation pattern that is homogeneous among samples, regardless of differences among host tissue donors (Rinn *et al.*, 2006). These observations indicate that the positional memory of fibroblasts may be determined during embryogenesis. In the case of lymph node reticular cells, transcriptional differences were also observed in early passages, when compared to dermal fibroblasts; such differences can affect nearly 8% of analyzed genes (Vega *et al.*, 2006). In addition, differences in the transcription profile of reticular cells from lymph nodes obtained from different organs have also been observed (Fletcher *et al.*, 2011). In this case, topographic differences, which were greater than differences among tissue donors, probably involved the HOX gene family (Chang *et al.*, 2002; Rinn *et al.*, 2006).

Unexpectedly, only a small transcriptional difference (~0.6% of the arrayed genes) was observed as differentially expressed between fibroblasts from the primary tumor and lymph node compared to a more striking difference between bone marrow stromal cells and fibroblasts from the other two sites. Similarity between the gene expression profiles of fibroblasts from these two sites could be explained by the presence of tumor cells that modulate their microenvironment in a similar way in the primary tumor and lymph node. Cancer cells are known to release growth factors, *e.g.*, bFGF, VEGF, PDGF, EGF, TGFB and others, that modulate fibroblast functions such as proliferation and activation (Mueller and Fusenig, 2004; Kalluri and Zeisberg, 2006). In addition, malignant cells may markedly influence the CAF gene expression pattern (Rozenchan *et al.*, 2009; Santos *et al.*, 2011) and their metabolism (Pavlides *et al.*, 2009; Sotgia *et al.*, 2012).

However, this similarity may also be explained by the fibroblasts origin that involves a common source, including

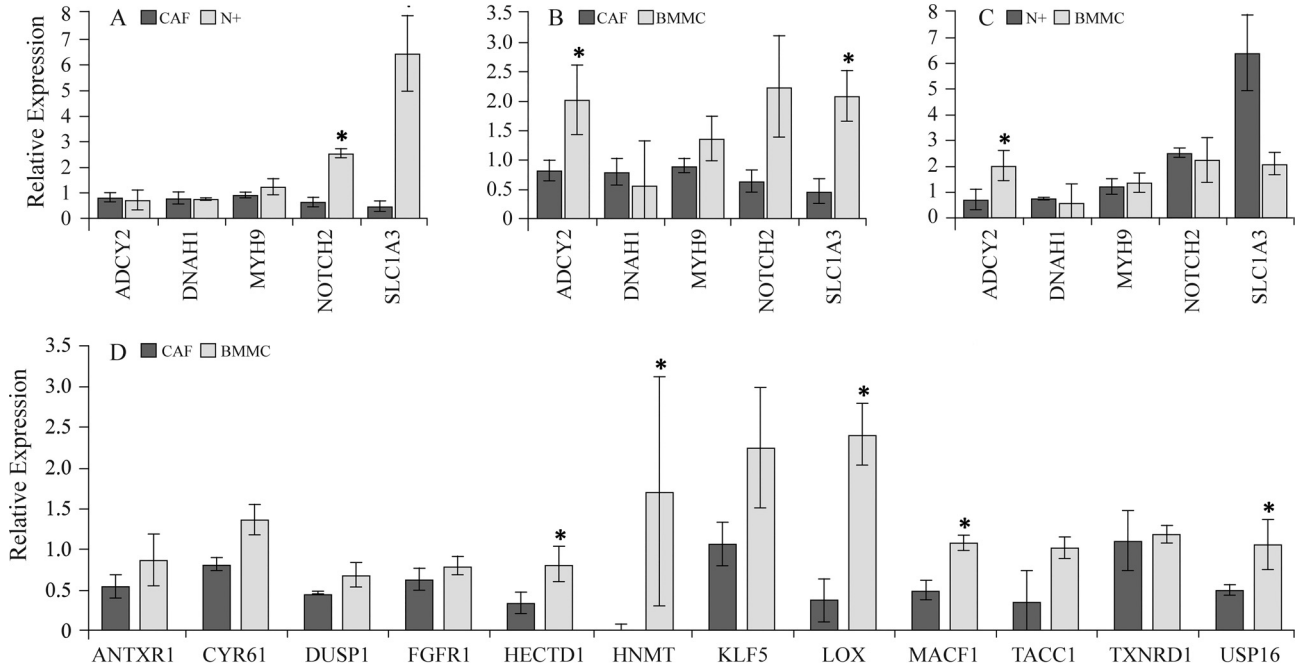

Figure 3 - Gene expression of fibroblast samples. (A) Primary tumor (CAF, n = 6 or 7) *vs.* lymph node (N+, n = 3 or 4). (B) Primary tumor (CAF, n = 6 or 7) *vs.* bone marrow (BMMC, n = 3 or 4). (C) Lymph node (N+, n = 3 or 4) *vs.* bone marrow (BMMC, n = 3 or 4). (D) Primary tumor (CAF, n = 3) *vs.* bone marrow (BMMC, n = 3). Relative expression based on $2^{-\Delta\Delta Ct}$. PPIA was used as a reference gene, and average Ct values for the same transcript were used as the sample reference. Bars represent mean ± SE. *p ≤ 0.05 (Mann-Whitney test).

the epithelial-mesenchymal transition, the co-migration of CAFs and cancer cells to metastatic sites, and the migration and differentiation of mesenchymal stem cells from the bone marrow to tumor sites. None of these is necessarily exclusive, but may occur simultaneously, in different proportions, during tumor-stroma co-evolution (Hanahan and Weinberg, 2011).

Up to 20% of tumor-associated fibroblasts may originate from bone marrow mesenchymal stem cells (Quante *et al.*, 2011). By using a mouse model of gastric cancer and the tracking of bone marrow mesenchymal stem cells with a reporter gene that allows their discrimination from resident cells in the tumor microenvironment, it was shown that only myofibroblasts derived from bone marrow mesenchymal stem cells could survive more than four passages in culture; resident myofibroblasts could not survive beyond the fourth passage (Quante *et al.*, 2011)**.** In addition, conditioned media from cancer cells can influence the differentiation of mesenchymal stem cells into CAFs (Mishra *et al.*, 2008).

In agreement with these findings, in the present work the expression of some genes involved in mesoderm formation, response to wounding, regeneration and embryonic morphogenesis was enhanced in bone marrow mesenchymal cells when compared with CAF or N+. These genes include HECTD1 (HECT domain containing E3 ubiquitin protein ligase 1), involved in negative regulation of the Wnt-Axin pathway (Tran *et al.*, 2013), MACF1 (microtubule-actin crosslinking factor 1) involved in the regulation of cell migration after ErbB2 stimulation (Benseddik *et al.*, 2013), LOX (lysyl oxidase) involved in creating a fibrotic environment favorable to tumor cell colonization (Cox *et al.*, 2013) and SLC1A3 (solute carrier family 1 [glial high affinity glutamate transporter], member 3) involved in the response to wounding. The genes ADCY2 (adenylate cyclase 2 [brain]) involved in signal transduction from membrane receptors, HNMT (histamine N-methyltransferase) involved in histamine metabolism and USP16 (ubiquitin specific peptidase 16) involved in histone deubiquitination and cell cycle were also found to be more expressed in bone marrow mesenchymal cells.

This work represents the first attempt to characterize the transcriptional profiles of fibroblasts from different sites in breast cancer patients, including primary cultures of stromal cells from bone marrow and lymph node, which are very difficult to establish and maintain in culture. The majority of studies that have investigated the origin of cancer fibroblasts have used mice (Quante *et al.*, 2011; Kidd *et al.*, 2012) and their findings are sometimes difficult to translate to humans. On the other hand, studies that have examined the differentiation of bone marrow mesenchymal cells to CAFs have used mainly bone marrow from health donors and have not considered the influence of the tumor on this site (Mishra *et al.*, 2008). One of the shortcomings of the present study was the incomplete agreement between the expression data obtained by microarray and RT-qPCR. One possible explanation for this discrepancy is that fibroblast primary cultures change their transcriptional pattern over time, especially lymph node stromal cells (Vega *et al.*, 2006), and the RT-qPCR expression data were obtained from fibroblasts in later passages than those used for the microarray. Although unpaired samples were used in this work, it was previously shown that samples obtained from different sites of the same individual, or subjected to differential experimental conditions, may cluster together (50% of the samples) or in different branches (another 50%) (Folgueira *et al.*, 2005; Perou *et al.*, 2000), thereby blurring transcriptional differences attributed to tissue specificities. In the present work, the analysis of unpaired tissue samples allowed the identification of differentially expressed genes that were homogeneously expressed within the groups.

In summary, only small differences were observed in the transcriptional profiles of fibroblasts from the primary tumor and lymph node of breast cancer patients. In contrast, the difference in gene expression between these two tissues and bone marrow stromal cells was much greater. The differentially expressed genes identified here may reflect a distinct differentiation profile or adaptations that bone marrow mesenchymal cells may undergo to become CAFs. However, functional studies are needed to confirm the influence of these genes in the differentiation of bone marrow mesenchymal cells into CAFs.

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Internet Resources

Primer Blast, http://www.ncbi.nlm.nih.gov/tools/primer-blast.

DAVID (Database for Annotation, Visualization and Integrated Discovery) v.6.7, http://david.abcc.ncifcrf.gov/tools.jsp (access date - Sept 14, 2013).

Supplementary Material

The following online material is available for this article:

Table S1 - Primers used in this work.

Table S2 - Differentially expressed genes.

Table S3 - Functional annotation of biological processes of less expressed genes in stromal cells derived from bone marrow.

Table S4 - Functional annotation of biological processes of more expressed genes in stromal cells derived from bone marrow.

Table S5 - Top networks observed in Ingenuity Pathway Analysis (IPA).

Figure S1 - Heat map of differentially expressed genes that gave no signal.

This material is available as part of the online article from http://www.scielo.br/gmb.

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