Genes to Cells

Molecular profiling of functional interactions between pre-osteoblastic and breast carcinoma cells

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The relationships between cancer cells and the microenvironment play a critical role in cancer growth and development. The bone stroma consists of mesenchymal stem cells and mature osteoblasts that promote cancer growth. Yet it is not completely understood what are the molecular processes guiding cancer cells progression to the bone. In this study, a coculture assay and subsequent gene profiling arrays were used to compare the gene expression profile of a pre-osteoblastic (PO) cell line (MBA-15) with that of a mammary adenocarcinoma (DA3) cells. After coculture, cells were separated by magnetic beads based on the expression of CD326 antigen. RNA was purified and hybridized on gene expression array. The gene expression pattern changes were followed by qRT-PCR. We demonstrate that cocultured DA3 cells express elevated levels of genes that regulate growth and responses to both hormonal stimulus and wounding, as well as reduced expression of genes related to lipid metabolism. Also, cocultured PO cells showed reduced expression of cell junction genes. The study presents a simplified model system, composed of PO and mammary cancer cells, that potentially mimics the molecular interactions in the tumor microenvironment which contribute to tumor progression.

Introduction

The biological properties of cancer cells and the environment at the metastatic site determine the dissemination of cancer metastases to distant organs (Mundy 1997). It has been well established that the surrounding stromal cells interact with tumor cells, thus influencing one another (Bissell & Radisky 2001; Chambers *et al.* 2002; Fidler 2002). Understanding the underlying molecular and cellular mechanisms of cancer progression and the spreading to a specific target organ remain the interest of various studies (Radisky *et al.* 2005). Boyce *et al.* (1999) reviewed the notion that cancer cells that metastasize to the bone possess distinct characteristics, which distinguish them from those that do not spread there. These properties include expression of proteolytic enzymes, angiogenic factors, growth-stimulating factors and their receptors, expression of cell adhesion molecules (CAMs) and resistance to immune surveillance (Boyce *et al.* 1999). Tumor metastasis is a complicated mixture of changes, which involve remodeling of cell–cell and cell–matrix contacts, and cell locomotion. Cell locomotion is mediated by the balance between adhesion and detachment of cells through integrins and remodeling of the extracellular matrix (ECM) (Holly *et al.* 2000; Hornebeck *et al.* 2002). A suggested hypothesis correlates adhesive properties of cells or the matrix with poorly tumorigenic and metastatic properties (Sikes *et al.* 2004). CAMs, for example, mediate cell–cell communication and interactions between cells and the ECM. CAM-mediated interactions initiate the activation of signaling cascades that lead to various cell responses (Patel *et al.* 2002; Shur *et al.* 2002). The expression of CAMs is strictly regulated by soluble growth factors, cytokines, and insoluble proteins composing the ECM (Haraldsen *et al.* 1996; Khatib *et al.* 1999).

Breast cancer cells preferentially metastasize to bone, as previously described (Coleman & Rubens 1987). Previous coculture studies of MCF7 breast cancer cells

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with mesenchymal stem cells (MSCs) showed induction of morphology, proliferative capacity, and aggregation pattern of the cancer cells (Fierro *et al.* 2004). In addition, interaction of MCF7 cells with MSCs down-regulated the expression of E-Cadherin and epithelial-specific antigen (Sasser *et al.* 2007). Another study emphasized the MSC's important role in the spreading and invasion capacity of tumor-derived cells (Karnoub *et al.* 2007).

The ability of tumor-derived cells to destroy and invade the ECM dictates their spreading capability. This process includes ECM and basement membrane remodeling by proteolytic enzymes including matrix metalloproteinases (MMPs), cathepsins, heparanases and uPA/plasmin, reviewed in (Duffy 1992). The urokinase plasminogen activator (uPA) system has been shown to play a critical role in early steps of carcinogenesis, as demonstrated in a uPA-deficient murine model (Gutierrez *et al.* 2000), and was also implicated in enhancing cellular proliferation by stimulating mitogenesis of epidermal tumor (Kirchheimer *et al.* 1987) and melanoma (Kirchheimer *et al.* 1989) cell lines. The uPA system is also responsible for modulation of tumor progression, ECM degradation and cell proliferation, adhesion and migration (Duffy 2004). It includes two serpin inhibitors known as plasminogen activator inhibitor PAI-1 (SerpinE1) and PAI-2 (SerpinB2). SerpinE1 and B2, for example, are directly linked to inactivation and internalization of adhesion receptors, in part through the cell surface urokinase, which plays a critical role in tumor growth, invasion, and metastasis (Andreasen *et al.* 2000).

The model system hereby presented is composed of MBA-15, a pre-osteoblastic (PO) stromal cell line (MSCs) (Benayahu *et al.* 1989) and DA3, a murine mammary adenocarcinoma cell line (Fu *et al.* 1990). We first characterized the molecular events that distinguish between the two cell lines. The basic biological context and changes in gene expression, which accompany the crosstalk between the osteoblasts and the cancer cells, were studied. We carried out coculture assays followed by a gene profiling array to compare cellular profiles of gene expression, from which functional annotations were retrieved. This study provides insights regarding gene expression profiles of PO and cancer cell lines by using an *in vitro* model system, thus shedding light on basic cellular interactions that exist in the bone microenvironment.

Results

We carried out gene profiling arrays to characterize the interactions between PO and mammary adenocarcinoma cells. The array allowed to explore the gene ontology (GO) and pathways that distinguish between the two cell lines after coculture.

Differential gene expression between pre-osteoblastic cells (MBA-15) and mammary adenocarcinoma cells (DA3)

Gene expression profiles of DA3 compared with MBA-15 cells were explored. Seventy-five differentially expressed genes [fold-change (FC) threshold cut-off >10 or less than −10 and P-value < 0.05, with false discovery rate (FDR) correction] were identified (Fig. S1, Table S1 in Supporting Information). Interestingly, the expression of all 75 genes was reduced in DA3 (blue) compared to MBA-15 cells (red) (see hierarchical cluster analysis, Fig. S1 in Supporting Information). Analysis of enriched GO terms for these genes using ToppGene (Chen *et al.* 2009) showed specific biological processes ordered by their statistical significance (Fig. 1, Table S2 in Supporting Information). The most significant category (Fig. 1) is response to wounding, which comprises 19 genes. Other categories were cell migration (14 genes), locomotion (17 genes), ECM organization (seven genes), blood vessel development (ten genes), biological adhesion (14 genes), regulation of cell proliferation (14 genes), immune system process (16 genes), response to oxygen levels (six genes), skeletal system development (eight genes) and response to steroid hormone stimulus (eight genes). The number of genes in each category is shown on top of the bars (Fig. 1).

Figure 2A,B depict Venn diagrams, demonstrating gene overlap between the various enriched functional categories (Pirooznia *et al.* 2007). Figure 2A shows that four genes (Col1a2, Col1a1, Pdgfra, and Mmp2) participate in three biological processes: response to wounding, cell migration, and skeletal system development. Figure 2B illustrates five genes (Slit2, Fn1, Vcan, Ccl2, and Serpine2) that are common to cell migration and cell adhesion. Two additional genes, Col1a1 and Mmp2, were found to also overlap with skeletal system development. Table S2 (Supporting Information) summarizes common gene lists associated with enriched functions. Genes associated with four or more functions are marked by a blue color.

We validated the gene profiling array results using qRT-PCR (TLDA) (Fig. 2C). As can be seen, genes related to wound responses, cell migration, and biological adhesion (Pdgfra, Vcan, and Col1a2) are highly expressed in the MBA-15 gene profiling array (light-gray bars) and MBA-15 qRT-PCR (dark-gray

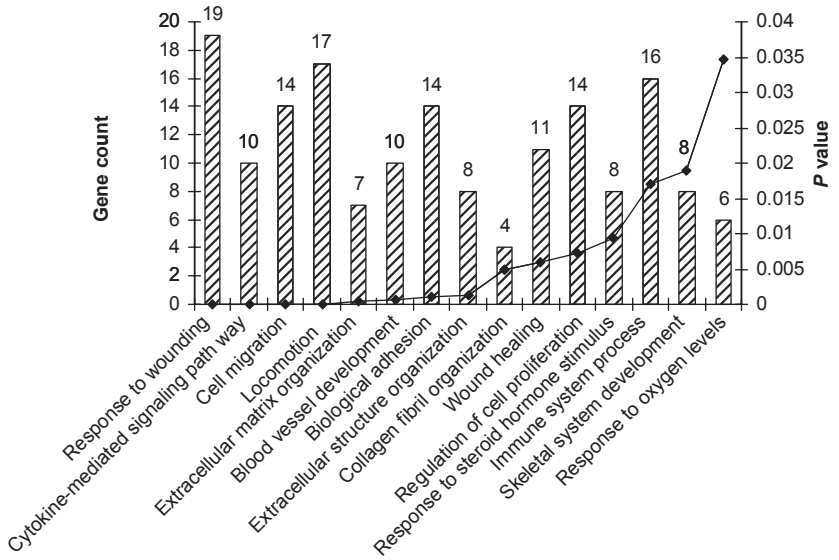

Figure 1 ToppGene-enriched biological processes of differentially expressed genes (FC > 10 or less than −10 and $P < 0.05$, FDR). Numbers above bars present the number of genes in each biological process (left *y*-axes) and its corresponding *P*-value (right *y*-axes). FC, fold change.

bars) over the DA3 (black bars) cells. The differential expression of CD326 (EpCAM-epithelial CAM) was demonstrated by immunofluorescence in both cells (Fig. S2 in Supporting Information).

Gene expression of cocultured mammary adenocarcinoma (DA3) cells compared to their corresponding mono-cultured controls. Mono-cultured DA3 cells served as controls and were compared to cocultured DA3 cells, grown together with MBA-15 cells. Expression data derived from the cocultured DA3 compared to the control cells (Fig. 3) was studied. Three hundred and eighty-two differentially expressed genes were identified (threshold cutoff: FC > 2 or FC less than −2 and *P*-value < 0.05, with FDR correction) (Fig. 3). Among these genes, 90 were up-regulated and 292 were down-regulated in cocultured cells compared to the corresponding control cells. Both the up- and down-regulated genes were individually submitted to GO enrichment analysis using ToppGene (Chen *et al.* 2009). Six specific biological processes, ordered by their statistical significance, were obtained for the up-regulated genes and deemed as most interesting (Fig. 4A) were obtained. The most significant category as summarized in the bar graph in Fig. 4A is growth, which includes 19 genes (*P*-value = 2.6×10^{-7}).

Figure 4B and Table S4 (Supporting Information) demonstrate gene overlap between the various functional categories identified by the up-regulated genes. Genes common to four or more functions are marked in the table by an asterisk. Figure 4B presents a Venn diagram that shows six genes shared by three biological processes: response to wounding, regulation of cell proliferation, and regulation of cell migration. This list includes SerpinE1 and SerpinE2, Ptgs2, Fgf7, Hbegf, and Itga2.

Down-regulated genes showed significantly enriched biological processes that are all related to lipid catabolism (summarized in the bar graph in Fig. 4C). The most significant categories discovered were lipid catabolic processes (15 genes) and lipid metabolism (36 genes).

We validated our results by using qRT-PCR (TLDA) assay, demonstrating that mRNA expression of SerpinE1, SerpinB2, Vim, and CD44 genes were significantly up-regulated in cocultured DA3 TLDA (light-gray bars), DA3 gene profiling array (dark-gray bars) as compared with the mono-cultured DA3 controls (black bars) (Fig. 4D). These four genes are related to wound healing responses, cell migration, biological adhesion, and cell proliferation.

Gene expression of cocultured pre-osteoblastic (MBA-15) cells compared to their corresponding mono-cultured controls

Differential gene expression of cocultured PO (MBA-15) cells compared to mono-cultured cells (FC threshold cutoff >2 or FC less than −2 and *P*-value < 0.05, with FDR correction) showed 463

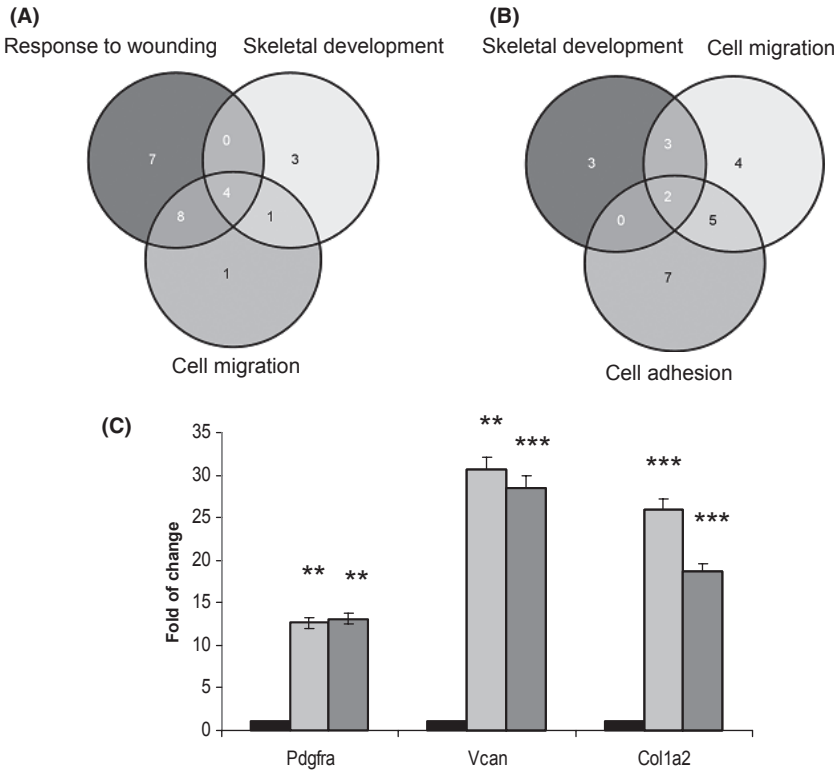

Figure 2 Venn diagrams and gene array validation. (A, B) Venn diagrams showing common genes among three of the six different most interesting biological processes shown in Fig. 1. (C) qRT-PCR validation (TLDA) of the expression levels of three representative genes from the indicated groups, in DA3 (black bars, TLDA), MBA-15 microarray (light-gray bars), MBA-15 TLDA (dark-gray bars) cells. Each histogram represents the average of two different experiments, each conducted in triplicate. $^{**}P < 0.01$, $^{***}P < 0.001$. *P*-values in Table S8 (Supporting Information). FC, fold change.

down-regulated genes, but only 40 up-regulated genes. As the number of up-regulated gene number was relatively small, ToppGene (Chen *et al.* 2009) and GATHER (Gene Annotation Tools) (Solinger *et al.* 2010) failed to assign significant enriched GO functions. We carried out the GO enrichment analysis on 142 differentially expressed genes (threshold cutoff FC > 4 or less than −4 and *P*-value < 0.05, FDR correction (Fig. 5, Table S5 in Supporting Information). Surprisingly, all 142 affected genes were down-regulated in the cocultured MBA-15 cells when compared to the control cells (Fig. 5). Analysis of ToppGene GO enrichment terms corresponding to these genes (Chen *et al.* 2009) showed 13 enriched biological processes ordered by statistical significance. We focused on four most significant biological processes (Fig. 6A), all dealing with cell adhesion related functions. The most statistically significant category was cell junction organization, which included ten genes.

Figure 6B and Table S6 (Supporting Information) demonstrate gene overlap that exists between the various enriched GO categories. Genes whose expression was shared by four or more biological processes are indicated in blue in the table. Figure 6B is a Venn diagram showing five common genes (F11r, Dsp, Jup, Lama5 and Cdh1) among three biological processes: cell junction organization, cell adhesion, and epithelium development. Four genes (Itgb4, Nf2, Cdh3, and Cadm1) are shared by the cell junction and cell adhesion processes.

Next, we validated the gene profiling array results by performing qRT-PCR (TLDA) (Fig. 6C). The mRNA expression levels of Lama5, Cadm1, Lamb3, L1cam, Elf3, and Bmp7 were significantly down-regulated in the cocultured MBA-15 TLDA (light-gray bars), MBA-15 gene profiling array (dark-gray bars) in comparison with the basal expression of mono-cultured MBA-15 cells (black bars). Lama5, Cadm1, Lamb3, and L1cam are mainly related to biological

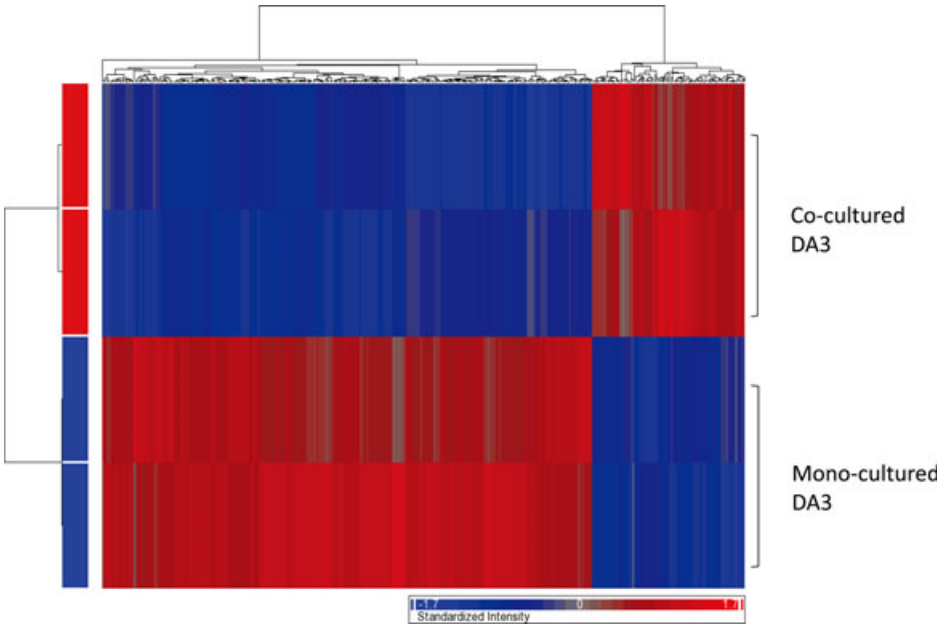

Figure 3 Differential gene expression of cocultured DA3 cells and their corresponding mono-cultured control. Cluster analysis of 384 differentially expressed genes (FC cutoff >2 or less than −2 and *P*-value < 0.05, with FDR correction). Up-regulated genes are shown in red, and down-regulated genes are shown in blue. Each experiment was carried out in duplicate. FC, fold change.

adhesion, while Elf3 and Bmp7 are related to cell proliferation and locomotion, according to the enriched GO categories.

Discussion

The relationships between cancer cells and the microenvironment play a critical role in cancer growth and metastasis. The stroma, which constitutes the microenvironment, contains locally originating or bone marrow–derived MSCs, which promote the growth and spreading of cancer cells. Paget (1989) proposed the 'seed and soil' hypothesis: the bone provides the fertile soil in which certain cancer cells prefer to grow. The mineralized bone matrix serves as a complete reservoir of growth factors (Hauschka *et al.* 1986). The metastatic cascade includes angiogenesis, degradation and remodeling of the basement membrane, detachment from the primary tumor, surviving and evading the host immune defense (Mundy 1997; Cooper *et al.* 2003). Tumor-derived cells must become motile and detach from the primary tumor, then break through the ECM barrier. Cell migration is achieved through coordination between adhesion and detachment of cells via CAMs. This occurs through induced remodeling of the ECM by tumor cells (Holly *et al.* 2000; Hornebeck *et al.* 2002).

The molecular variations between the two cell lines used in this study, MBA-15 and DA3, were established. In our experimental model, MSCs were represented by the MBA-15 PO stromal cell line. The differential phenotype of the two cell lines was demonstrated by the expression of CD326 (EpCAM) antigen that is expressed by DA3 cells, in contrast to the MBA-15 cells. EpCAM, an epithelial-specific CAM (Litvinov *et al.* 1994), was shown to be overexpressed on breast cancer cell lines (Osta *et al.* 2004). Then, a gene profiling array was carried out to compare the gene expression patterns between MBA-15 and the murine mammary adenocarcinoma cell line DA3. Differentially expressed genes showed many down-regulated genes in the cancer cells compared to the stromal cells. Those genes were implicated in pathways such as wounding responses, cell migration and locomotion, ECM organization, blood vessel development, biological adhesion, regulation of cell proliferation, immune system process, response to oxygen levels, skeletal development, and response to steroid hormone stimulus. These pathways are known to be responsible for the continuous maintenance of the basic crosstalk between PO cells and tumor cells in other systems (Mundy 1997; Boyce *et al.* 1999; Bissell & Radisky 2001; Cooper *et al.* 2003). Among the genes expressed differentially by the two cell lines

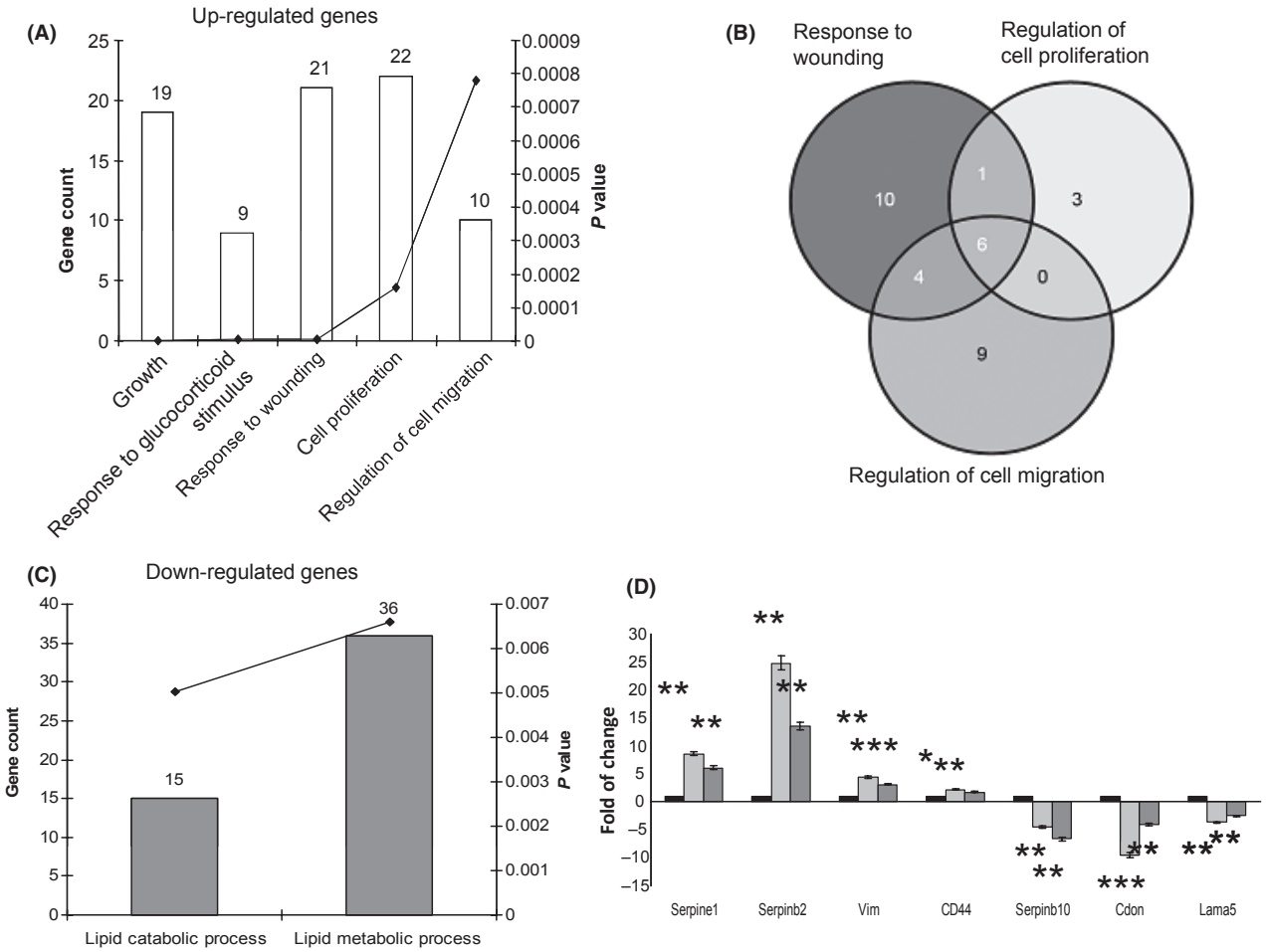

Figure 4 Gene expression of cocultured DA3 cells versus control cells (FC > 2 or less than −2 and $P < 0.05$, with FDR correction). ToppGene-enriched biological processes of (A) up-regulated (white bars) and (C) down-regulated (gray bars) genes of DA3 cells. The number of genes in each biological process is shown on top of bars (*y*-axis, left) and its corresponding *P*-value ($P < 0.05$ with FDR correction, *y*-axis, right) that is depicted by the black line. (B) Venn diagram showing common genes among three of the six different biological processes shown in (A). (D) qRT-PCR validation (TLDA) of the expression levels of a few representative genes from the indicated groups, in DA3 (black bars, TLDA), cocultured DA3 TLDA (light-gray bars), DA3 microarray (dark-gray bars) cells. Each histogram represents the average of two different experiments, each conducted in triplicate. $^{**}P < 0.01$, $^{***}P < 0.001$. *P*-values in Table S9 (Supporting Information). FC, fold change.

were Vcan (Versican), Emb (Embigin) and Ccl2 (Monocyte chemotactic protein 1, MCP1). Both Vcan and Emb regulate interactions of the cell with the ECM. Vcan is a component of the ECM that regulates adhesion, migration, proliferation, apoptosis, angiogenesis, all processes implicated in cancer metastasis biology (Kim *et al.* 2009). Emb, a CAM, regulates cell adhesion/ECM interactions, growth, apoptosis, and differentiation in response to hormonal signaling and ECM matrix (Guenette *et al.* 1997). Ccl2 (MCP1) interacts with tumor cells to create an environment that contains abundant chemo-attractants and growth factors (Lin & Pollard 2004). Ccl2 plays a critical role in tumor progression through its involvement in angiogenesis. It was previously shown that Ccl2 can act directly on endothelial cells to promote angiogenesis (Salcedo *et al.* 2000). Molloy *et al.* (2009) reported the potential important role of Ccl2 in recruitment of circulating breast cancer cells to the bone tissue. Hence, our findings correlate with the MSCs' potency to regulate tumor progression (Bergfeld & DeClerck 2010). Together with other cells (myofibroblasts, endothelial cells, pericytes, and inflammatory cells), PO cells contribute to the

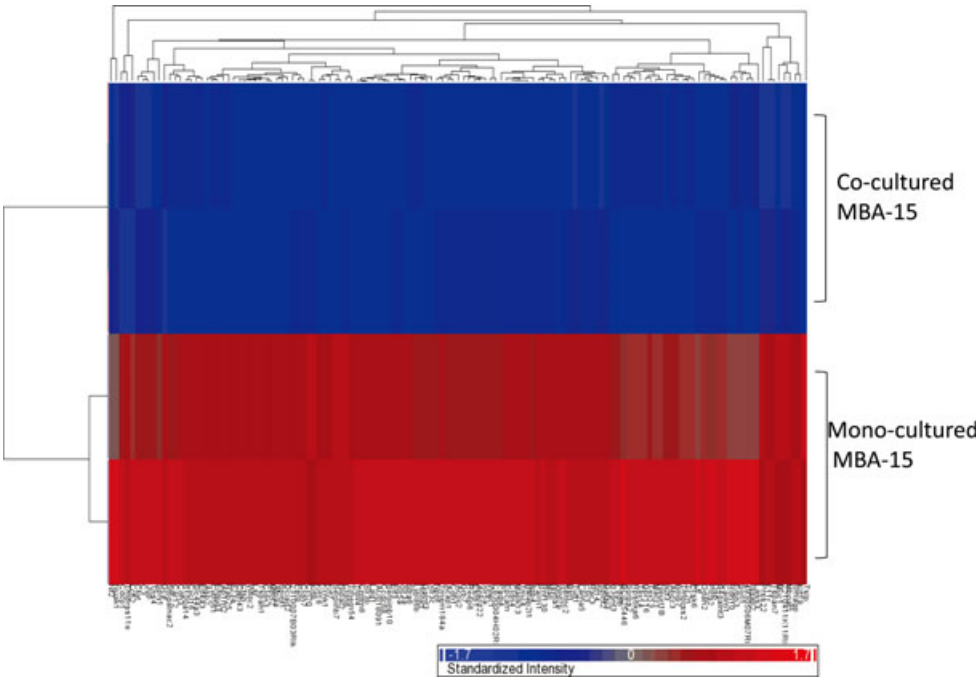

Figure 5 Differential gene expression of cocultured MBA-15 and their corresponding mono-cultured control. Cluster analysis of 142 differentially expressed genes (FC > 4 or less than −4 and *P*-value < 0.05, with FDR correction). Up-regulated genes are shown in red, and down-regulated genes are shown in blue. Each experiment was carried out in duplicate. All genes presented were down-regulated in cocultured MBA-15 cells compared to the mono-cultured ones. FC, fold change.

creation of a microenvironment that resemble the environment of a chronic wound (Dvorak 1986).

Although the differences between PO, stromal-derived and breast cancer cells were previously established, we carried out a high-throughput experiment to specifically identify the molecular characteristics of these cells. The genes reported above (Guenette *et al.* 1997; Salcedo *et al.* 2000; Kim *et al.* 2009; Molloy *et al.* 2009) were analyzed according to their cellular pathways and gene functions unique to each cell line.

The *in vitro* coculture model was utilized to mimic the basic cellular events that may exist in the bone marrow after organ-specific metastasis. Analysis of up-regulated genes after coculturing of DA3 cells compared with control cells suggests pathways that are relevant to tumor progression and metastasis, growth, proliferation, regulation of cell migration, response to glucocorticoid stimulus and wounding. These results are supported by previous coculture studies. Cooper *et al.* (2003) determined the contribution of stromal-derived factors to prostate carcinoma, which frequently metastasizes to bone. Fierro *et al.* (2004) showed that coculture of MCF-7 breast cancer cells with MSCs induces changes in morphology, proliferative capacity and aggregation pattern of the breast cancer cells. The bone tropism mechanism of breast cancer cell was extensively reviewed (Dvorak 1986; Mundy 2002).

The uPA system, which was implicated in enhancement of tumor proliferation in human epidermal tumor cell line, ovarian carcinomas, and endothelial cells (Holmberg *et al.* 1978; Kirchheimer *et al.* 1987) includes two serpin inhibitors known as plasminogen activator inhibitor (PAI)-1 (SerpinE1) and PAI-2 (SerpinB2). We show that the SerpinE1 and E2 genes, which regulate cell migration, cell proliferation, and response to wounding, were significantly up-regulated in response to coculturing of the DA3 cells. In addition, SerpinB2, usually involved in wounding responses, was also found to be highly expressed in response to coculture.

The PAI-1 modulates tumor growth, invasion, cell adhesion, and angiogenesis (Durand *et al.* 2004; McMahon & Kwaan 2008). PAI-1 and PAI-2 are involved in the regulation of cancer invasion and metastasis. These modulators catalyze the degradation of the ECM thus allowing malignant cells to invade locally and eventually spread to distant sites, as showed in ovarian carcinomas, ductal breast carcinoma, and fibrosarcomas (Dano *et al.* 1985; Andreasen *et al.* 1997). PAI-1 and PAI-2 also limit the

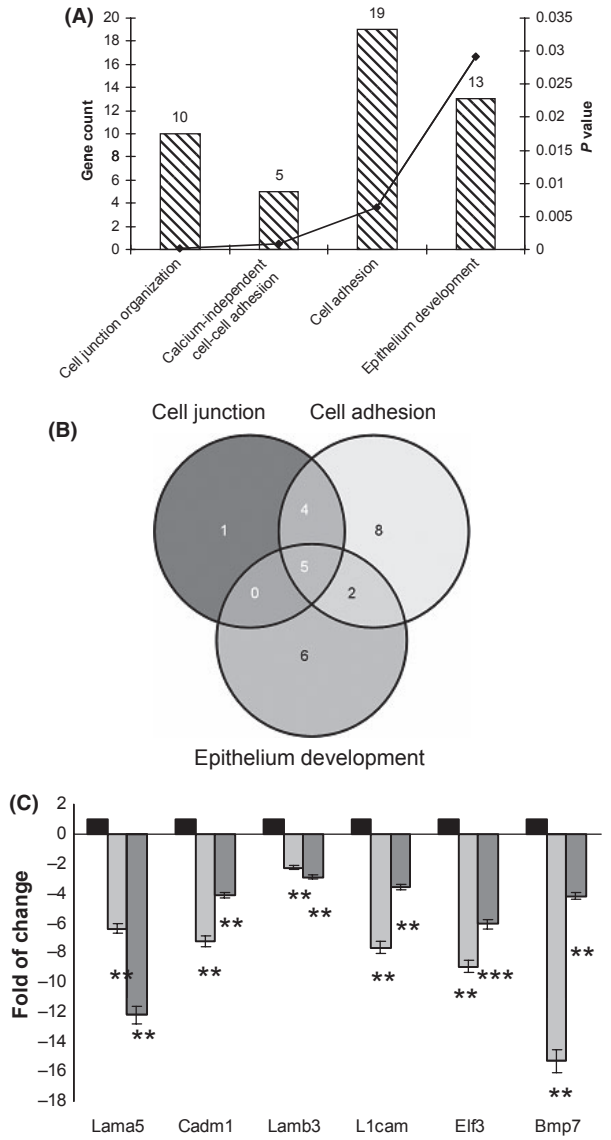

Figure 6 Gene expression of cocultured MBA-15 cells versus control cells (FC > 4 or less than −4 and $P < 0.05$, with FDR correction). (A) ToppGene-enriched biological processes of differentially expressed genes in cocultured MBA-15. Graphs show the number of genes in each biological process (shown on top of bars, *y*-axis, left) and its corresponding *P*-value ($P < 0.05$ with FDR correction, *y*-axis, right) that is depicted by the black line. (B) Venn diagram showing common genes among three of the six different biological processes shown in (A). (C) qRT-PCR validation (TLDA) of the expression levels of a few representative genes from the indicated groups, in MBA-15 (black bars, TLDA), cocultured MBA-15 TLDA (light-gray bars), MBA-15 microarray (dark-gray bars) cells. Each histogram represents the average of two different experiments, each conducted in triplicate. $^{**}P < 0.01$, $^{***}P < 0.001$. *P*-values in Table S10 (Supporting Information). FC, fold change.

proteolysis of the ECM, which appears to be necessary for the invasion process. The effect of PAI-1 on cell adhesion and migration is complex and depends on a combination of different factors, such as composition of the ECM and the specific adhesion receptors expressed by the cell [reviewed in (Duffy 2004)]. Through their activity, PAIs are organizers of cell-ECM contacts and cover a range of activities required to promote and disrupt cell attachment sites (Andreasen *et al.* 2000). To our knowledge, up-regulated expression of SerpinE1, E2 and B2 genes in response to coculturing may reflect only a minor part of the complex molecular changes that accompany the interactions between the tumor cells and the PO stromal cell line. Our results support previous studies that have demonstrated that cocultured MCF-7 cells, for example, acquire altered morphology and proliferative capacity in response to coculturing with MSCs (Fierro *et al.* 2004; Molloy *et al.* 2009).

Expression of sphingosine-1-phosphate phosphatase 1 (Sgpp1) was down-regulated in cocultured DA3 cells compared to control cells. Sgpp1 is an enzyme that mediates lipid metabolism by catalyzing the degradation of S1P (Mandala *et al.* 2000). Sgpp1 enzyme activity has a role in modulating cell fate through varying intracellular S1P levels (Long *et al.* 2005), and its mRNA levels were previously reported to be increased by glucocorticoid treatment (Bianchini *et al.* 2006). This study also presents up-regulated genes related to glucocorticoid stimulus, along with down-regulated genes related to lipid catabolism (such as Sgpp1). Lepine *et al.* (2004) report the sphingosine contribution to glucocorticoid-induced apoptosis in thymocytes. We speculate that reduced Sgpp1 gene expression upon coculturing of DA3 cells promotes cell growth, proliferation, and tumorigenicity (Olivera *et al.* 1999; Maceyka *et al.* 2002), thereby enhancing their tumorigenic properties.

By comparing the changes in gene expression, which occurred in cocultured MBA-15 with those from cells grown alone, we identified genes whose expression was down-regulated. Genes related to cell junction organization, cell adhesion, and epithelial development pathways were enriched in cocultured MBA-15 cells.

Desmosomes are intercellular junctions that function in cell–cell adhesion and attachment of intermediate filaments to the cell surface (Garrod *et al.* 2002). Desmosomes are composed of the adhesive core with desmosomal cadherins, including Dsg, which mediate intercellular adhesion, and the desmosomal plaques, Jup, which is a cytoplasmic membrane–associated protein (Garrod *et al.* 2002). Desmosomal proteins, expressed

by epithelial cells, were shown to be expressed also by odontoblasts and neighboring dental pulp fibroblasts (Sasaki *et al.* 1982; Iguchi *et al.* 1984) that are mesenchymal derived cells. Their expression in human periodontal ligament fibroblasts was also reported (Yamaoka *et al.* 1999). Desmogleins are major components of the transmembrane adhesion complex, whereas desmoplakins are the most prominent components of the cytoplasmic plaque (Green & Jones 1996). Regulating junction assembly and disassembly may be particularly important in processes such as wound healing and tumor invasion, where adhesion is compromised and motility is enhanced. In this study, we showed significantly reduced expression of claudins, desmoglein (Dsg), and junction plakoglobin (Jup) genes in cocultured MBA-15 cells compared to control cells. These genes maintain cell junction organization and cell adhesion [reviewed in (Oliveira & Morgado-Diaz 2007)] suggesting a role in regulation of tissue integrity (Green & Jones 1996), which includes the basic and the complex cell–matrix interactions. Changes in the crosstalk between the cells and their microenvironment are translated into the breakdown of tissue integrity, a prominent step in the development and the progression of cancers from the premalignancy stage to invasion (Bissell & Radisky 2001). We speculate that the crosstalk between tumor cells affects the cell–cell adherence junctions, which are part of an intact tissue architecture that under normal conditions exerts tumor suppressing functions (Bissell *et al.* 2005).

Overall, our findings of the differentially expressed genes in response to coculturing might support the well-known 'seed and soil' hypothesis (Paget 1989). The results presented here confirm the distinct influence of the marrow-derived cells on the tumorigenicity of cancer cells. These findings contribute to prior studies that demonstrated that interaction with marrow-derived cells results in changes in the cancerous cells' proliferation and adherence, thus enhancing their metastatic properties (Fierro *et al.* 2004; Karnoub *et al.* 2007; Sasser *et al.* 2007). In contrast, the PO cells lose cell junction organization, a phenomenon that may reflect the remodeling of tissue architecture. This study strengthens and expands previous findings and establishes the critical role of the microenvironment in maintaining metastatic potency.

Experimental procedures

Cell culture

Two cell lines were used in this study: MBA-15, a PO stromal cell line (Benayahu *et al.* 1989) and DA3, a mammary adenocarcinomal cell line (Fu *et al.* 1990). Both cell lines were maintained in Dulbecco's modified essential medium (DMEM) (Gibco, USA) with the addition of 10% heat-inactivated fetal calf serum, supplemented with 1% glutamine and 1% penicillin/streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C.

Coculture and cell separation

MBA-15 cells were seeded at density of 2×10^4 cells/cm^2 and allowed to adhere overnight. The following day, DA3 mammary adenocarcinoma cells were seeded on top of the MBA-15 monolayer at a density of 1.3×10^4 cells/cm^2. In parallel, both cell types were cultured separately as controls. Cells were maintained in growth medium for 24 or 48 h and then magnetically separated using the EasySep® positive selection kit (STEMCELL Technologies, USA) according to the manufacturer's instructions. Briefly, cocultured cell populations were trypsinized and dispersed into a single suspension. The separation was based on the expression of the epithelial tissue specific antigen CD326 (EpCAM; Litvinov *et al.* 1994). EasySep® positive selection cocktail, CD326-mouse specific antibody (clone number G8.8; BioLegend, USA), and magnetic nanoparticles were added during serial incubations on ice. The magnetic nanoparticles selectively bound to viable epithelial cells expressing the CD326 antigen, which were positively selected by placing the tube in a magnet (STEMCELL Technologies). Retrieved cells were centrifuged, and RNA was extracted from cultured cells using EZ RNA kit (Biological Industries, Beth HaEmek, Israel). Mono-cultured MBA-15 or DA3 cells, simultaneously seeded and maintained with identical confluence of the coculture, sorted using the EasySep® positive selection kit, were referred to as control cells.

Gene profiling array experiment and data analysis

Affymetrix GeneChip® Mouse Gene 1.0 ST arrays were used for gene expression analysis according to the instruction manual, as described in the Affymetrix website (28 853 genes across 770 317 distinct probes; Affymetrix, Santa Clara, CA, USA; 103; http://www.affymetrix.com). A total of eight arrays were carried out in biological duplicates. Gene profiling array was carried out on CEL files using Partek® Genomics Suite TM, version 6.5 Copyright © 2010 (http://www.partek.com). Data were normalized and summarized with the robust multi-average method (Irizarry *et al.* 2003), followed by analysis of variance (ANOVA). Genes of interest that were differentially expressed after the different treatments or cell lines were identified by a FC cutoff >2 or less than −2 (or higher, as specified) and $P < 0.05$ (FDR correction). Hierarchical cluster analysis was obtained using Partek® Genomics Suite TM. For analyses of biological and functional groups, the ToppGene gene ontology tool (Chen *et al.* 2009) was used. GO data were verified by four additional tools: (i) WebGestalt (Zhang *et al.* 2005), (ii) GATHER (Solinger *et al.* 2010), (iii) David (Dennis

Table 1 Validated genes and function

Gene symbol	Gene name	Accession number	Gene function	Probe assay (Applied Biosystems, Foster City, CA, USA)
Cdon	Cell adhesion molecule-related/down-regulated by oncogenes	NM_021339.2	Biological adhesion, cell proliferation	Mm00491190_m1
Serpine1	Serine (or cysteine) peptidase inhibitor, clade E, member 1	NM_008871.2	Biological adhesion, cell migration, blood vessel development, response to wounding, chemotaxis, cell proliferation	Mm00435860_m1
Serpinb2	Serine (or cysteine) peptidase inhibitor, clade B, member 2	NM_001174170.1	Response to wounding	Mm00440905_m1
Serpinb10	Serine (or cysteine) peptidase inhibitor, clade B (ovalbumin), member 10	NM_198028.3	Coagulation, cellular differentia tion, tumor suppression, apoptosis, and cell migration	Mm01291800_m1
Col1a2	Collagen, type I, alpha 2	NM_007743.2	Cell migration, blood vessel development, response to wounding, chemotaxis, extracellular region part	Mm00483888_m1
Cd44	CD44 antigen	NM_009851.2	Biological adhesion, cell migration, blood vessel development, response to wounding, inflammatory response	Mm01277163_m1
Pdgfra	Platelet derived growth factor receptor, alpha polypeptide	NM_001083316.1	Cell migration, response to wounding, cell proliferation, cellular metabolism	Mm00440701_m1
Bmp7	Bone morphogenetic protein 7	NM_007557.2	Locomotion, blood vessel development, chemotaxis, cell proliferation, extracellular region part	Mm00432102_m1
Elf3	E74-like factor 3	NM_007921.3	Response to wounding, inflammatory response, cellular metabolism	Mm00468224_m1
Vcan	Versican	NM_001081249.1	Biological adhesion, cell migration, response to wounding, extracellular region part	Mm01283063_m1
L1cam	L1 cell adhesion molecule	NM_008478.3	Biological adhesion, cell migration, response to wounding, chemotaxis	Mm00493049_m1
Cadm1	Cell adhesion molecule 1	NM_207675.2|	Cell proliferation	Mm00457551_m1
Lama5	Laminin, alpha 5	NM_001081171.2	Biological adhesion, cell migration, blood vessel development, cell proliferation, extracellular region part	Mm01222029_m1
Lamb3	Laminin, beta 3	NM_008484.2	Biological adhesion, extracellular region part	Mm00493108_m1

Table 1 (*Continued*)

Gene symbol	Gene name	Accession number	Gene function	Probe assay (Applied Biosystems, Foster City, CA, USA)
Vim	Vimentin	NM_011701.4	Immune response, cell migration and adhesion	Mm01333430_m1

A list of selected genes that their expression was validated using a custom TaqMan low-density array platform (Applied Biosystems, CA, USA). The table describes their functional and biological relevance, their accession number, according to the GeneBank and their probe assay.

et al. 2003) and (iv) GeneCodis (Carmona-Saez *et al.* 2007). Venn diagrams were used to cross between gene lists (Pirooznia *et al.* 2007) (Table S7A–C in Supporting Information).

Real-time quantitative reverse-transcription polymerase chain reaction

The expression of selected genes was analyzed using a custom TaqMan low-density array platform (Micro Fluidic Cards; Applied Biosystems, Foster City, CA, USA) in both mono- and cocultured DA3 and MBA-15 cells. Genes for assay validation were selected according to their biological relevance. Namely, genes that participate in wounding responses, cell migration, ECM organization, blood vessel development, biological adhesion, skeletal system development, cell proliferation and cell junction organization were verified (Table 1).

The reaction consisted of 1 μg of cDNA template (whose synthesis was carried out from 1 μg of total RNA with the High Capacity cDNA Archive Kit (Applied Biosystems). The total sample volume was adjusted to 100 μL with de-ionized water and 95 μL of TaqMan® Universal PCR Master Mix (Cat no: 4304437; Applied Biosystems). One hundred microliters of the aforementioned Master Mix were loaded into each of the eight array ports (every two ports comprise one sample on the array). Thus, the samples run as duplicates were only loaded into four ports of the array. Thermal cycling conditions included a 50 °C step for 2 min, denaturation for 10 min at 94 °C, followed by 40 additional cycles, each consisting of two steps: 97 °C for 30 s and 59.7 °C for 1 min for annealing and extension.

Quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) was carried out using the ABI Prism7900 HT Sequence Detection System (Applied Biosystems). Three control genes were included in the study: TBP, PPIA, and Taf1. Micro Fluidic Cards were analyzed with RQ documents and the RQ Manager Software for automated data analysis. Obtained values of target gene expression were normalized to the concentration of TBP, PPIA, and Taf1, which showed the least variation among the reference genes in the studied cell lines. A description of TaqMan Gene Expression assays (Applied Biosystems) used for validation of gene expression is provided in Table S11 (Supporting Information).

Immunofluorescence microscopy analysis

Cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min, washed with PBS, and permeabilized with 0.5% Triton in PBS for 5 min. The slides were stained with polyclonal, rabbit anti-CD326-mouse specific antibody (clone number G8.8; BioLegend, USA) (1 : 100). Slides were stained with 1 : 500 species-specific secondary antibody, conjugated to Cy-3, designed for simultaneous multiple staining (Jackson Immunoresearch Laboratories, USA) for 1 h at room temperature. Then, slides were washed with PBS and mounted with Vectashield® mounting medium including 4′,6-Diamidino-2-phenylindole (DAPI), dimethyl sulfoxide (Vector Laboratories, CA, USA) and observed using a confocal microscope (Leica TCF SP2).

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Conflict of interest

The authors declare that they have no competing interests.

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Supporting Information/Supplementary material

The following Supporting Information can be found in the online version of the article:

Figure S1 Differential gene expression between DA3 and MBA-15 cells.

Figure S2 Immunofluorescence of CD326 (EpCAM) protein sub-cellular localization.

Table S1 A list of differentially expressed genes of DA3 compared to MBA-15 cells

Table S2 Gene ontology (GO) analysis was obtained for differentially expressed genes FC > 10 or less than −10 and $P < 0.05$ under FDR (false discovery rate) adjustment criteria between DA3 and MBA-15 cells (Table S1)

Table S3 A list of differentially expressed genes in co-cultured DA3 and their corresponding mono-cultured controls

Table S4 GO analysis was obtained for differentially expressed genes FC > 2 or less than −2 and $P < 0.05$ under FDR (false discovery rate) adjustment criteria between co-cultured DA3 compared to their corresponding mono-cultured controls (Table S3)

Table S5 Differentially expressed genes between co-cultured MBA-15 compared to their corresponding mono-cultured controls

Table S6 GO analysis was obtained for differentially expressed genes FC > 4 or less than −4 and $P < 0.05$ under FDR (false discovery rate) adjustment criteria between co-cultured MBA-15 and their corresponding mono-cultured controls (Table S5)

Table S7 Comparison of enriched biological processes, molecular functions and cellular component using gene ontology tools in: DA3 alone versus MBA-15 alone (A), co-cultured versus mono-cultured DA3 (B) co-cultured versus mono-cultured MBA-15 (C)

Table S8 Validation of gene mRNA expression: comparison between DA3 and MBA-15 cells

Table S9 Validation of gene mRNA expression: comparison between DA3 their corresponding mono-cultured controls

Table S10 mRNA expression validation of genes compared between MBA-15 and their corresponding mono-cultured controls

Table S11 List of the serial numbers of TaqMan Gene Expression assays that were used for validation of the gene expression assays (Applied Biosystems)

Table S12 Differentially expressed genes between co-cultured MBA-15 compared to their corresponding mono-cultured controls

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