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Publication Date	2011-02-23
Publication Information	Potter, Shirley M., Dwyer, Roisin M., Hartmann, Marion C., Khan, Sonja, Boyle, Marie P., Curran, Catherine E., & Kerin, Michael J. (2012). Influence of stromal–epithelial interactions on breast cancer in vitro and in vivo. Breast Cancer Research and Treatment, 131(2), 401-411. doi: 10.1007/s10549-011-1410-9
Publisher	Springer
Link to publisher's version	https://doi.org/10.1007/s10549-011-1410-9
Item record	http://hdl.handle.net/10379/15383
DOI	http://dx.doi.org/10.1007/s10549-011-1410-9

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Abstract

Stromal cell secreted chemokines including CCL2 have been implicated in the primary tumor microenvironment, as mediators of tumor cell migration, proliferation and angiogenesis. Expression of CCL2 and its principal receptor CCR2 was analysed by RQ-PCR in primary tumor cells and breast cancer cell lines. Breast cancer cell lines (MDA-MB-231, T47D) were co-cultured directly on a monolayer of primary breast tumor and normal stromal cells, retrieved using EpCAM+ magnetic beads, and changes in expression of *CCL2, CCR2*, *MMP11*, *ELK1*, *VIL2* and *Ki67* detected by RQ-PCR. Epithelial cell migration and proliferation in response to stromal cell-secreted factors was also analysed. In vivo, tumor xenografts were formed by co-injecting T47D cells with primary tumor stromal cells. Following establishment, tumors were harvested and digested, epithelial cells retrieved and analysed by RQ-PCR. Whole tumor tissue was also analysed by immunohistochemistry for CD31 and the *VIL2* encoded protein Ezrin. Tumor stromal cells expressed significantly higher levels of *CCL2* than normal cells, with no *CCR2* expression detected. Primary epithelial cells and breast cancer cell lines expressed elevated *CCL2*, with relative expression of *CCR2* found to be higher than the ligand. Interaction of breast cancer epithelial cells with primary tumor, but not normal stromal cells, stimulated increased expression of *CCL2* (8-fold), *ELK1* (6-fold), *VIL2* (6-fold) and *MMP11* (17-fold). Factors secreted by stromal cells, including CCL2, stimulated a significant increase in epithelial cell migration, with no effect on cell proliferation in vitro observed. In vivo, the presence of stromal cells resulted in tumors of increased volume, mediated at least in part through neoangiogenesis demonstrated by immunohistochemistry (CD31). Admixed tumor xenografts exhibited increased expression of *Ki67*, *MMP11*, *VIL2* and *ELK1*. Elevated Ezrin protein was also detected, with increased cytoplasmic

localisation. The results presented highlight mechanisms through which breast cancer epithelial cells can harness stromal cell biology to support tumor progression.

Introduction

The primary breast tumor microenvironment plays a pivotal role in cancer initiation and progression [1]. Stromal cells are the predominant cell type in this microenvironment and evidence of their active participation in tumor progression is growing rapidly [2]. Tumor stromal cells are fundamentally different from the stroma of corresponding normal breast tissue [3], and have gene expression signatures that correlate with tumor grade and poor prognosis [4,5,6]. These properties appear to be retained following separation from malignant epithelial cells [7], suggesting tumor stroma is comprised of an independent fibroblastic subpopulation which supports malignant behaviour [6,8]. In invasive breast cancer, stromal cells are found in much higher proportion than *in situ* carcinomas, and predominantly at the invasive front [9]. Allinen and colleagues showed that breast tumor stromal cells undergo extensive gene expression changes in progression from normal breast tissue to ductal carcinoma *in situ* (DCIS) to invasive ductal carcinoma [10].

In view of these central roles in the biology of breast cancer, understanding the mechanisms by which stromal cells mediate such effects is essential. Within the tumor microenvironment, stromal cells are the most active secretory cells [2,11], and various paracrine mediators of their growth-promoting signals have been proposed, including cytokines, growth factors, and proteases [7,12]. Studies implicate altered chemokine expression levels as an indicator of progression to tumorigenicity and metastatic capacity [13]. Orimo et al reported that tumor stromal cell derived CXCL12 promotes tumor growth and angiogenesis via its cognate receptor (CXCR4) expressed by carcinoma cells [7].

Indeed previous work by this group showed that whole breast tumor explants secreted high levels of CCL2 (MCP-1, Monocyte Chemotactic Protein-1) and that stromal cells

were responsible for the bulk of its secretion [14]. CCL2 is a 76-amino acid protein with a primary role in the immune context, regulating recruitment of monocytes/macrophages and other inflammatory cells to damaged or infected sites [15,16]. CCL2 is minimally expressed by normal breast epithelial ducts [16]. In contrast, extensive CCL2 protein expression has been noted in breast tumor tissue [17,18]. CCL2 functions through its main receptor CCR2, of which there are two isoforms, CCR2A and CCR2B [19,20]. Recent research has implicated CCL2 as an active participant in the tumor microenvironment, influencing factors such as tumor-associated macrophages, growth, angiogenesis and metastasis [17,21,22]. Expression of CCL2 protein in primary breast tumors was shown to have a significant prognostic value for relapse free survival, and correlated with high tumor grade [18,23]. Although the mechanisms by which stromal cells promote tumorigenesis are not yet fully understood, their potential as novel therapeutic targets in breast cancer is apparent [24,25]. However, in order for stromal-epithelial interactions, or stromal cells themselves, to emerge as appropriate targets for novel breast cancer therapies, further characterisation of the molecular crosstalk between these two cell populations is required.

The results presented show that isolation of breast cancer epithelial cells following interaction with primary tumor stromal cells in vitro and in vivo, stimulates increased expression of genes associated with invasion, angiogenesis and tumor progression. While stromal cells secreted high levels of CCL2, they were devoid of the CCR2 receptor expressed by epithelial cells, suggesting paracrine action of the chemokine, potentially mediating cell migration. Another novel finding of this study was that in vivo interaction with primary tumor stromal cells induced increased expression of *VIL2*/Ezrin, a protein that plays a key regulatory role in breast cancer metastasis. The

results presented provide a valuable insight into intracellular crosstalk between stromal and epithelial cells in the breast tumor microenvironment, highlighting how epithelial cells can harness stromal cell biology to facilitate their invasion and progression.

Methods

Primary Culture

Following ethical committee approval and written informed consent, fresh specimens of human breast cancer were harvested at surgery and primary cell cultures prepared, as described previously [14]. Normal controls were obtained from reduction mammaplasties. The digested cell suspension was separated into organoid, epithelial and stromal fractions by differential centrifugation and cultured in selective media, as described [26]. Primary stromal cells were characterised by flow cytometry using the Guava® EasyCyte™ 8HT and analysed using Incyte software. Cell type specific antibodies were used including Thy1/CD90, CD105, CD73, α-SMA, CD31, MUC1/CD221, CD34, and Cytokeratin (BD Pharmingen™). Appropriate isotype control antibodies were used and the results reflect the percent of positive cells compared with isotype controls. The level of expression of Fibroblast Activation Protein (FAP) in tumor compared to normal stromal cells was also determined by Real Time Quantitative PCR (RQ-PCR) as described below.

Primary tumor epithelial cells (n=6) were isolated from organoid and epithelial cell fractions. Epithelial Cell Adhesion Molecule (EpCAM) positive cell–enriched populations were retrieved from these single cell suspensions by positive selection using EasySep magnetic beads (StemCell Technologies Inc.), according to manufacturers' protocol.

Culture of Cell Lines

The following breast cancer cell lines were included in this study: T47-D, ZR-75-1, MCF-7, BT-474, (Oestrogen and Progesterone receptor positive, ER+, PR+), MDA-MB-231, SK-BR-3 (ER-, PR-) and MCF-10-2A (non-tumorigenic). These were purchased from the ATCC and cultured in Leibovitz-15 (MDA-MB-231), RPMI-1640 medium (T47-D, ZR-75-1, BT-474,), McCoys 5A (SK-BR-3) and Eagles Minimum Essential Medium (MCF-7) media, each supplemented with 10% FBS, and 100 IU/ml Penicillin G/100mg/ml Streptomycin sulfate. MCF-10-2A cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) -F12 medium supplemented with 5% Horse Serum, 20ng/ml Epidermal Growth Factor, 100 ng/ml Cholera Toxin, 0.01 mg/mL Insulin and 500ng/ml Hydrocortisone.

Collection of Conditioned Medium and CCL2 Quantification

Conditioned media (CM), containing factors secreted by cells, was obtained from cultures. All cell populations were seeded in 6-well plates at a density of 2×10^5 cells per well in 2mL DMEM containing 2% FBS (required for chemokine stability). DMEM was used to prepare media for all cell types to ensure that differences observed were not as a result of culture conditions. CM from co-culture experiments was harvested after 72 hours, centrifuged (1 min, 1000RPM, 4°C), and stored at -20°C until required. CM from individual stromal cell populations was harvested after 24 hours in culture and used neat/undiluted, as a chemoattractant in migration assays or as a growth medium in proliferation assays. CM from stromal cells was analysed for CCL2 content using Quantikine® Enzyme Linked Immunosorbent Assays (R&D Systems), following manufacturers' protocol.

In Vitro Cell Migration Assay

Transswell® Permeable Supports (Corning Inc, Sigma Aldrich) were used to track migration of MDA-MB-231 cells in response to factors secreted by stromal cells, as described [14]. Briefly, MDA-MB-231 cells were inoculated into the insert, and their migration in response to stromal cell CM, serum free basal medium (negative control) basal medium with 2% FBS (baseline control), or basal medium with 10% FBS (positive control) in the well below was quantified. MDA-MB-231 migration in response to stromal cells in the presence of a CCL2 monoclonal antibody (40ng/mL) was also quantified. Migrated cells were counted in 5 fields of view per membrane using an Olympus BX60 microscope and image analySIS® software. Each experiment was repeated in triplicate, with results expressed as Mean ± SEM.

In Vitro Cell Proliferation Assay

Cell proliferation was assessed using the ViaLight™ Plus Kit (Cambrex), and based on bioluminescent measurement of ATP. Breast cancer epithelial cell lines (MDA-MB-231 and T47D) were seeded into 96-well plates (6 x 10³ cells per well) and allowed to adhere overnight. Medium was removed and substrates added. Test substrates included tumor or normal stromal cell CM, and increasing concentrations of hCCL2 (50-300pg/mL) standards. Basal and complete medium were used as negative and positive controls respectively. Plates were incubated for 48 hours and ATP levels measured on a LuminoSkan Ascent Luminometer (Thermo). Data represent the Mean reading of 8 wells and are expressed as Mean ± SEM.

Real Time Quantitative PCR

Cells were homogenised in 1 mL of QIAzol Lysis reagent and total RNA isolated using the RNeasy® Mini Kit (QIAGEN Ltd.) as per manufacturer's instructions. cDNA was generated using SuperScript III reverse transcriptase enzyme (Invitrogen) and amplified by real-time quantitative PCR (RQ-PCR) using the ABI Prism 7000 (Applied Biosystems). Taqman® Universal Master Mix and Gene Expression Assays (Applied Biosystems) designed for the target genes (*FAP, CCL2, CCR2A, CCR2B, MMP11, Ki67, VIL 2, ELK 1*), and control genes *Mitochondrial Ribosomal Protein L19* (*MRPL19*) and *Peptidylprolyl Isomerase A* (*PPIA*) were used.

Due to the low yields from primary epithelial cell cultures the TaqMan® PreAmp Cells-to-Ct™ Kit (Applied Biosystems) was used for gene expression analysis on these cells. Briefly, this protocol involves an intermediate amplification step between reverse transcription and RQ-PCR in which the cDNA is enriched for target genes. The comparative C_T method for relative quantification was used, allowing determination of the quantity of the target gene in each sample population normalised to endogenous control genes (*MRPL19* and *PPIA*) and compared to a calibrator, and was expressed in a linear form using the formula $2^{-\Delta\Delta CT}$ [27]. Gene expression levels in primary tumor epithelial and stromal cells were determined relative to the levels in epithelial and stromal cells from normal breast tissue harvested at reduction mammaplasty. In the case of epithelial cell lines, levels were expressed relative to those of non-tumorigenic MCF-10-2A cells. In co-culture experiments, expression levels in the epithelial cell population post co-culture were expressed relative to expression levels in these cells cultured alone.

Co-culture of Tumor Stromal and Epithelial Cells

Primary tumor stromal cells (n=6 individual donors A-F, Luminal A n=4, Luminal B n=2), or normal stromal cells (n=4 individual donors) suspended in stromal medium were plated into $T75cm^2$ flasks (1 x 10^6 cells per flask) and allowed to adhere overnight. Breast cancer epithelial cell lines (MDA-MB-231 or T47D) were seeded onto the monolayers of stromal cells at the same density. The individual cell populations were also cultured alone in parallel. Co-cultures were all established in stromal medium, so any changes observed could not be attributed to differences in culture conditions. Following direct co-culture adherent cells were washed twice with PBS and trypsinized into a single cell suspension and the epithelial fraction retrieved using EasySep EpCAM+ magnetic beads (Stem Cell Technologies Inc.). RNA was extracted from retrieved epithelial cells and changes in gene expression resulting from their interaction with tumor and normal stromal cells identified by RQ-PCR, as described.

Growth of Breast Cancer Xenografts in Mice

Animal studies were carried out in accordance with experimental guidelines set out by the institutional ethics committee. Female athymic nude mice (Harlan Laboratories UK Ltd.) were implanted with 17-β-estradiol 60-day Slow Release pellets, (Innovative Research of America) to support growth of estrogen receptor positive T47D cells. Mice were divided into three groups and given a subcutaneous injection of T47D cells alone (5.6 x 10^5 cells), or T47D cells admixed with tumor stromal cells (1 x 10^6) derived from two individual donors (n=6 in each group). Tumors were measured weekly using callipers and volume estimated (volume (mm^3) = length x width x height x 0.52). Following 10 weeks of tumor growth, animals were sacrificed by CO_2 inhalation and tumors harvested. Tissue for immunostaining was immediately

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immersed in 4% paraformaldehyde for 24hrs, transferred to 30% sucrose for 24hrs, snap frozen in an isopentane/liquid nitrogen bath and stored at -80ºC until required for cryosectioning. Tumor tissue harvested for retrieval of epithelial cells was immediately immersed in basal culture media, minced using crossed blade scalpels and digested overnight using collagenase as described [14]. Epithelial cells were then retrieved from the mixed population using EpCAM$^+$ magnetic beads. Cells were pelleted and stored at -80ºC until required for RNA isolation.

Immunohistochemistry

Frozen tissue samples from xenografts were cryosectioned (5µm sections) and allowed to air dry at RT followed by rehydration in PBS-0.05% Tween-20. Following blocking of endogenous peroxidases, antigen retrieval was performed using citrate buffer. Sections were then analysed using the Ventana Discovery™ machine with antibodies specific to CD31 (AbCAM), and Ezrin (AbCAM). Once staining was complete sections were washed in warm soapy water, dehydrated in serial alcohol immersions, mounted using DPX mounting medium and examined using a Leica DFC 300 FX light microscope, with Leica Software, V 2.3.4.

Statistical Analysis

Data were analysed using the software package SPSS 15.0 and are presented as Mean ± SEM of triplicate experiments. Results with a $p < 0.05$ were considered statistically significant. All tests were two-tailed. Levene's test confirmed equal variance of observations in each group and permitted parametric data to be compared using a student's unpaired t-test. Normality was confirmed using the Kolmogorov-Smirnov test.

Results

Epithelial and stromal cell isolation and characterisation

Primary breast stromal cells displayed a typical fibroblastic morphology and when characterized by flow cytometry (Figure 1) were shown to be positive for CD90 (>95% positive, Figure 1A), CD105 ((>95% positive, Figure 1B), CD73 ((>95% positive, Figure 1C), and alpha-smooth muscle actin (α-SMA), and negative for CD31 (<2% positive, Figure 1D), CD34 (<5% positive), MUC1/CD221 (<5% positive), and cytokeratin (<2% positive, Figure 1E). Tumor stromal cells were found to have higher expression of α-SMA (range 60%-87% positive), a marker of activated fibrobalsts (myofibroblasts), than normal stromal cells (range 2%-68% positive). In agreement with previous reports [28,29], RQ-PCR analysis also revealed significantly higher expression of Fibroblast Activation Protein (FAP) in tumor compared to normal stromal cells (Figure 1F, p<0.001). Epithelial cells were selected using magnetic beads, based on EpCAM positivity and were confirmed to be cytokeratin positive (>95% positive). Levels of expression of *CCL2*, and its receptor *CCR2*, were determined in each cell population relative to the geometric mean expression of endogenous control genes, and values expressed relative to normal counterparts ($2^{-\Delta\Delta CT}$, Fig. 2). In the case of stromal cells, expression of *CCL2* was significantly higher in each population of tumor stromal cells (n=6, A-F) compared to mean *CCL2* expression from n=4 normal stromal cells isolated from reduction mammaplasties (mean 1.05 log fold increase p<0.05, Fig. 2A). In contrast both tumor and normal stromal cells failed to express either isoform of the CCL2 receptor, *CCR2A* or *CCR2B*. Expression of CCL2 and CCR2 was also analysed in primary breast cancer epithelial cells (n=6, Figure 2B). Relative expression of *CCR2* was higher than the

ligand (*CCR2*; mean 1.45 log RQ, *CCL2*; mean 0.78 log RQ) (Fig. 2B). *CCL2/CCR2*

expression analysis was also performed on 5 breast cancer epithelial cell lines and

mean values expressed relative to non-tumorigenic MCF-10-2A cells (Fig. 2C).

Effect of direct co-culture on CCL2 secretion and expression

In-vitro secretion of CCL2 was quantified when the cells were cultured individually

and in direct co-culture (Fig. 3A). The baseline of the graph represents the sum of

what each cell population secreted when cultured individually, with each bar

representing the amount by which the co-culture population differed from this.

Following 72-hours co-culture of breast cancer cell lines on a monolayer of stromal

cells, CCL2 levels were significantly higher than that seen from the individual

populations (mean increase for T47D + tumor stromal cells: 4901 ± 1953 pg/ml,

MDA-MB-231 + tumor stromal cells: 5035 ± 1294 pg/ml, p < 0.05, Fig. 3A). This

effect was significantly higher than that seen when these cells were co-cultured with

normal stromal cells (p< 0.05). Following in vitro co-culture with primary

tumor/normal stromal cells, epithelial tumor cells were retrieved using EpCAM$^+$

magnetic beads, and expression of *CCL2* analysed. Levels of gene expression are

expressed relative to levels detected in breast cancer cells cultured alone ($2^{-\Delta\Delta CT}$).

CCL2 gene expression levels reflected protein secretion trends, with expression

increased in both breast cancer populations following co-culture with stromal cells

(Fig. 3B). This upregulation was significantly higher when co-cultured with tumor

(mean fold increase: T47D 9.07 ± 2.85, MDA-MB-231 8.41 ± 4.36) compared to

normal stromal cells (T47D 4.03 ± 2.37 MDA-MB-231 2.47 ± 0.77, p < 0.05). In

contrast to *CCL2* expression, *CCR2* expression levels were decreased following co-

culture compared to the cells cultured alone.

Cell migration and proliferation

MDA-MB-231 showed significantly greater chemotaxis in response to tumor compared to normal stromal cells (Fig. 4A). This effect was subsequently blocked by the addition of a monoclonal antibody to *CCL2* (range 27 – 64% blockade). Breast cancer cells also displayed a dose dependent increase in chemotaxis towards commercial standards of *CCL2*, with similar results observed in SK-Br-3 and MCF-7 cells (results not shown).

There was no significant change in proliferation of breast cancer epithelial cells in response to factors secreted by tumor or normal stromal cells (Fig. 4B). Recombinant standards of CCL2 were also found to have no effect on cell proliferation (results not shown).

Expression of invasion/proliferation associated genes

In both breast cancer cell lines, expression levels of the invasion associated gene *MMP11*, was significantly increased following co-culture with tumor compared to normal stromal cells (Fig 5A and B, $p < 0.05$). While expression levels of the proliferative marker, *Ki67*, increased following co-culture with normal stromal cells, it decreased when the co-cultures involved tumor stromal cells. Expression levels of the invasion associated gene *VIL2*, and the oncogene, *ELK1*, were significantly upregulated in T47D cells following co-culture in a tumor specific fashion (Fig 5A, $p < 0.05$).

In vivo co-culture

T47D cells were injected alone or coinjected with tumor stromal cells subcutaneously into nude mice. These tumor stromal cells were derived from two separate human invasive ductal breast carcinomas, Tum A Stro (Luminal A, Grade 3, T1, N0) and Tum B Stro (Luminal A, Grade 2, T1, N0). T47D cells co-mixed with tumor stromal cells exhibited a faster rate of growth and generated tumors of greater volume at the endpoint of 10 weeks than T47D injected alone, (Mean tumor volume ± SEM: T47D 33 ± 5 mm^3, T47D + Tum A Stro 118 ± 15 mm^3, T47D + Tum B Stro 63 ± 9 mm^3, Fig. 6A). Xenografts that developed in the presence of tumor stromal cells also displayed neovascularisation, confirmed by positive staining for the endothelial marker, CD31 (Fig. 6B b [10X], c [20X], d [40X]), while tumors formed from T47D cells alone were negative for CD31 (Fig. 6B a [40X]). Mixed stromal-epithelial xenografts displayed greater intensity of ezrin staining throughout the tumors and increased cytoplasmic localisation of the protein (Fig. 6C b, arrows) compared to epithelial xenografts which predominantly displayed membranous staining (Fig. 6C a, arrows).

Upon tumor harvesting at necropsy, mixed tumor xenografts were digested and the T47D cells retrieved with epithelial specific beads. Subsequent analysis allowed identification of gene expression changes in these isolated epithelial cells resulting from their in vivo interaction with tumor stromal cells (Fig 6D). The epithelial cells displayed increased expression of the invasion and migration associated genes *MMP11*, *VIL2*, and *ELK1* following in vivo co-culture with tumor stromal cells (range 0.2 – 3.6 log fold increase, Fig. 6D). Interestingly these cells also displayed increased expression of the proliferative marker *Ki67*, which was downregulated in the in vitro co-culture set-up.

Discussion

The current study highlights potential mechanisms through which malignant epithelial cells can harness stromal cell biology to facilitate their invasion into the tumor microenvironment.

There is accumulating evidence pointing to a pivotal role for chemokines in controlling migration, growth and differentiation of tumor cells [30]. The current study shows that isolated primary tumor epithelial cells displayed elevated expression of *CCL2*, as well as its principal receptor, *CCR2*. In terms of breast cancer, the potential tumorigenic role of CCL2 and CCR2 is poorly defined to date. In the current study, primary tumor stromal cells had significantly higher expression of the CCL2 gene than normal stromal cells. CCL2 exerts its effects through a principal receptor, CCR2 [31], of which two isoforms (A and B) have been identified. *CCR2* receptor expression has previously been shown in two cell lines, MDA-MB-231 and T47D [32]. The current study reports that along with these and other breast cancer cell lines, breast cancer epithelial cells isolated from fresh breast tumors express *CCR2A*, while both isoforms were undetectable in tumor stromal cells, suggesting a paracrine role for the chemokine. CCL2 was detected in the CM of all tumor stromal cells examined and in significantly higher quantities than normal stromal cells. Directional cell migration is an integral part of cancer cell invasion during metastasis, involving changes in cell cytoskeleton and adhesion [33]. In the current study factors secreted by tumor stromal cells induced migration of breast cancer epithelial cells, shown to be mediated at least in part by CCL2. Furthermore direct contact between tumor stromal and epithelial cells induced a synergistic increase in CCL2 secretion and gene expression. The disparity between protein secretion and gene expression levels may

be accounted for by post-translational modifications, which would not be detected at the gene expression level.

It is worth noting that in vitro and in vivo interaction with tumor stromal cells induced increased expression of *VIL2* in epithelial cells. Ezrin, the coded product of the *VIL2* gene, is a membrane-cytoskeleton crosslinker known to regulate cell adhesion and motility, as well as overall metastatic potential [34]. Moreover, mixed tumor xenografts not only achieved increased tumor volume, but also displayed increased cytoplasmic localisation of ezrin protein, compared to tumors formed from T47D cells alone. The switch from apical to cytoplasmic ezrin localisation has previously been shown to correlate with high tumor grade, high *Ki67* expression (also shown in this study) and metastasis [35].

The presence of stromal cells in vivo resulted in positive staining for CD31, indicating active neoangiogenesis within tumors xenografts [36], a known effect of CCL2 [17,21]. Emphasis must be placed on the fact that in this study baseline gene expression levels were analysed in primary breast cancer epithelial cells isolated from fresh tumors; however to support triplicate repeats of all experiments performed, breast cancer cell lines were employed in functional experiments.

The results presented also show that interaction with tumor stromal cells induced *MMP11* upregulation in breast cancer epithelial cells, with a similar degree of upregulation in vitro and in vivo. MMP11 has been shown to promote tumor progression [37], and is upregulated in invasive relative to *in situ* ductal carcinoma [4]. Furthermore tumor stromal cells also induced increased expression of *ELK1* in T47D cells in vitro and in vivo. *ELK1*, a member of the ets oncogene family, promotes tumor progression, and is critical to regulation of cell proliferation and

apoptosis [38]. While ELK1 expression was increased in vitro (6 fold increase), the level of increase was markedly higher in vivo (54 fold).

A challenge in breast cancer research is the availability of models that faithfully reflect the complexity of the disease. The majority of in vivo xenograft models are homotypic, involving the introduction of breast cancer cell lines alone into the mouse microenvironment [39]. In this and other recent studies [7,40], breast cancer epithelial cells were admixed with tumor stromal cells isolated from human breast tumors and introduced into mice, forming heterotypic 3-dimensional xenografts which more accurately simulate stromal-epithelial interactions in the tumor microenvironment. This is the first study to retrieve and analyse epithelial cells following in vivo co-culture. While the majority of targets showed similar trends in both in vitro and in vivo models, the disparity in some results can be explained by the fact that cultures grown on a non-physiological 2-dimensional substratum lack exposure to components of the extracellular matrix that are present in the 3-dimensional in vivo microenvironment [40]. Given the heterogeneity of breast cancer, no individual model reflects all aspects of the disease, however every attempt should be made to reflect in vivo events and models that focus on epithelial cells alone fall short on this. Understanding the dynamic and reciprocal crosstalk between stromal and epithelial cells will deepen our knowledge of the tumorigenic process, and may also facilitate exploitation of stromal-epithelial interactions as valid targets for novel therapies.

Conflict of interests

The authors declare that they have no competing interests.

Acknowledgements:

References

1. Lorusso G, Ruegg C (2008) The tumor microenvironment and its contribution to tumor evolution toward metastasis. Histochem Cell Biol 130: 1091-1103.

2. Kalluri R, Zeisberg M (2006) Fibroblasts in cancer. Nat Rev Cancer 6: 392-401.

3. Mueller MM, Fusenig NE (2004) Friends or foes - bipolar effects of the tumour stroma in cancer. Nat Rev Cancer 4: 839-849.

4. Ma XJ, Dahiya S, Richardson E, Erlander M, Sgroi DC (2009) Gene expression profiling of the tumor microenvironment during breast cancer progression. Breast Cancer Res 11: R7.

5. Finak G, Bertos N, Pepin F, Sadekova S, Souleimanova M, et al. (2008) Stromal gene expression predicts clinical outcome in breast cancer. Nat Med 14: 518-527.

6. Singer CF, Gschwantler-Kaulich D, Fink-Retter A, Haas C, Hudelist G, et al. (2008) Differential gene expression profile in breast cancer-derived stromal fibroblasts. Breast Cancer Res Treat 110: 273-281.

7. Orimo A, Gupta PB, Sgroi DC, Arenzana-Seisdedos F, Delaunay T, et al. (2005) Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. Cell 121: 335-348.

8. Kim JB, Stein R, O'Hare MJ (2005) Tumour-stromal interactions in breast cancer: the role of stroma in tumourigenesis. Tumour Biol 26: 173-185.

9. Elenbaas B, Weinberg RA (2001) Heterotypic signaling between epithelial tumor cells and fibroblasts in carcinoma formation. Exp Cell Res 264: 169-184.

10. Allinen M, Beroukhim R, Cai L, Brennan C, Lahti-Domenici J, et al. (2004) Molecular characterization of the tumor microenvironment in breast cancer. Cancer Cell 6: 17-32.

11. Bhowmick NA, Neilson EG, Moses HL (2004) Stromal fibroblasts in cancer initiation and progression. Nature 432: 332-337.

12. Tlsty TD, Coussens LM (2006) Tumor stroma and regulation of cancer development. Annu Rev Pathol 1: 119-150.

13. Muller A, Homey B, Soto H, Ge N, Catron D, et al. (2001) Involvement of chemokine receptors in breast cancer metastasis. Nature 410: 50-56.

14. Dwyer RM, Potter-Beirne SM, Harrington KA, Lowery AJ, Hennessy E, et al. (2007) Monocyte chemotactic protein-1 secreted by primary breast tumors stimulates migration of mesenchymal stem cells. Clin Cancer Res 13: 5020-5027.

15. Zlotnik A, Yoshie O, Nomiyama H (2006) The chemokine and chemokine receptor superfamilies and their molecular evolution. Genome Biol 7: 243.

16. Christopherson K, 2nd, Hromas R (2001) Chemokine regulation of normal and pathologic immune responses. Stem Cells 19: 388-396.

17. Saji H, Koike M, Yamori T, Saji S, Seiki M, et al. (2001) Significant correlation of monocyte chemoattractant protein-1 expression with neovascularization and progression of breast carcinoma. Cancer 92: 1085-1091.

18. Ueno T, Toi M, Saji H, Muta M, Bando H, et al. (2000) Significance of macrophage chemoattractant protein-1 in macrophage recruitment, angiogenesis, and survival in human breast cancer. Clin Cancer Res 6: 3282-3289.

19. Charo IF, Myers SJ, Herman A, Franci C, Connolly AJ, et al. (1994) Molecular cloning and functional expression of two monocyte chemoattractant protein 1 receptors reveals alternative splicing of the carboxyl-terminal tails. Proc Natl Acad Sci U S A 91: 2752-2756.

20. Wong LM, Myers SJ, Tsou CL, Gosling J, Arai H, et al. (1997) Organization and differential expression of the human monocyte chemoattractant protein 1 receptor gene. Evidence for the role of the carboxyl-terminal tail in receptor trafficking. J Biol Chem 272: 1038-1045.

21. Salcedo R, Ponce ML, Young HA, Wasserman K, Ward JM, et al. (2000) Human endothelial cells express CCR2 and respond to MCP-1: direct role of MCP-1 in angiogenesis and tumor progression. Blood 96: 34-40.

22. Neumark E, Sagi-Assif O, Shalmon B, Ben-Baruch A, Witz IP (2003) Progression of mouse mammary tumors: MCP-1-TNFalpha cross-regulatory pathway and clonal expression of promalignancy and antimalignancy factors. Int J Cancer 106: 879-886.

23. Chavey C, Bibeau F, Gourgou-Bourgade S, Burlinchon S, Boissiere F, et al. (2007) Oestrogen receptor negative breast cancers exhibit high cytokine content. Breast Cancer Res 9: R15.

24. Albini A, Sporn MB (2007) The tumour microenvironment as a target for chemoprevention. Nat Rev Cancer 7: 139-147.

25. Joyce JA (2005) Therapeutic targeting of the tumor microenvironment. Cancer Cell 7: 513-520.

26. Spiers V GA, Walton DS, Kerin MJ, Fox JN, Carelton PJ, Desai SB, Atkin SL (1998) Short-term primary culture of epithelial cells derived from human breast tumours. Br J Cancer 78: 1421-1429.

27. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25: 402-408.

28. Santos AM, Jung J, Aziz N, Kissil JL, Pure E (2009) Targeting fibroblast activation protein inhibits tumor stromagenesis and growth in mice. J Clin Invest 119: 3613-3625.

29. Scanlan MJ, Raj BK, Calvo B, Garin-Chesa P, Sanz-Moncasi MP, et al. (1994) Molecular cloning of fibroblast activation protein alpha, a member of the serine protease family selectively expressed in stromal fibroblasts of epithelial cancers. Proc Natl Acad Sci U S A 91: 5657-5661.

30. Balkwill F (2004) Cancer and the chemokine network. Nat Rev Cancer 4: 540-550.

31. Charo IF (1999) CCR2: from cloning to the creation of knockout mice. Chem Immunol 72: 30-41.

32. Nam JS, Kang MJ, Suchar AM, Shimamura T, Kohn EA, et al. (2006) Chemokine (C-C motif) ligand 2 mediates the prometastatic effect of dysadherin in human breast cancer cells. Cancer Res 66: 7176-7184.

33. Jiang P, Enomoto A, Takahashi M (2009) Cell biology of the movement of breast cancer cells: Intracellular signalling and the actin cytoskeleton. Cancer Lett.

34. Li Q, Wu M, Wang H, Xu G, Zhu T, et al. (2008) Ezrin silencing by small hairpin RNA reverses metastatic behaviors of human breast cancer cells. Cancer Lett 261: 55-63.

35. Sarrio D, Rodriguez-Pinilla SM, Dotor A, Calero F, Hardisson D, et al. (2006) Abnormal ezrin localization is associated with clinicopathological features in invasive breast carcinomas. Breast Cancer Res Treat 98: 71-79.

36. Bingle L, Lewis CE, Corke KP, Reed MW, Brown NJ (2006) Macrophages promote angiogenesis in human breast tumour spheroids in vivo. Br J Cancer 94: 101-107.

37. Motrescu ER, Blaise S, Etique N, Messaddeq N, Chenard MP, et al. (2008) Matrix metalloproteinase-11/stromelysin-3 exhibits collagenolytic function against collagen VI under normal and malignant conditions. Oncogene 27: 6347-6355.

38. Hsieh YH, Wu TT, Tsai JH, Huang CY, Hsieh YS, et al. (2006) PKCalpha expression regulated by Elk-1 and MZF-1 in human HCC cells. Biochem Biophys Res Commun 339: 217-225.

39. Vargo-Gogola T, Rosen JM (2007) Modelling breast cancer: one size does not fit all. Nat Rev Cancer 7: 659-672.

40. Olsen CJ, Moreira J, Lukanidin EM, Ambartsumian NS (2010) Human mammary fibroblasts stimulate invasion of breast cancer cells in a three-dimensional culture and increase stroma development in mouse xenografts. BMC Cancer 10: 444.
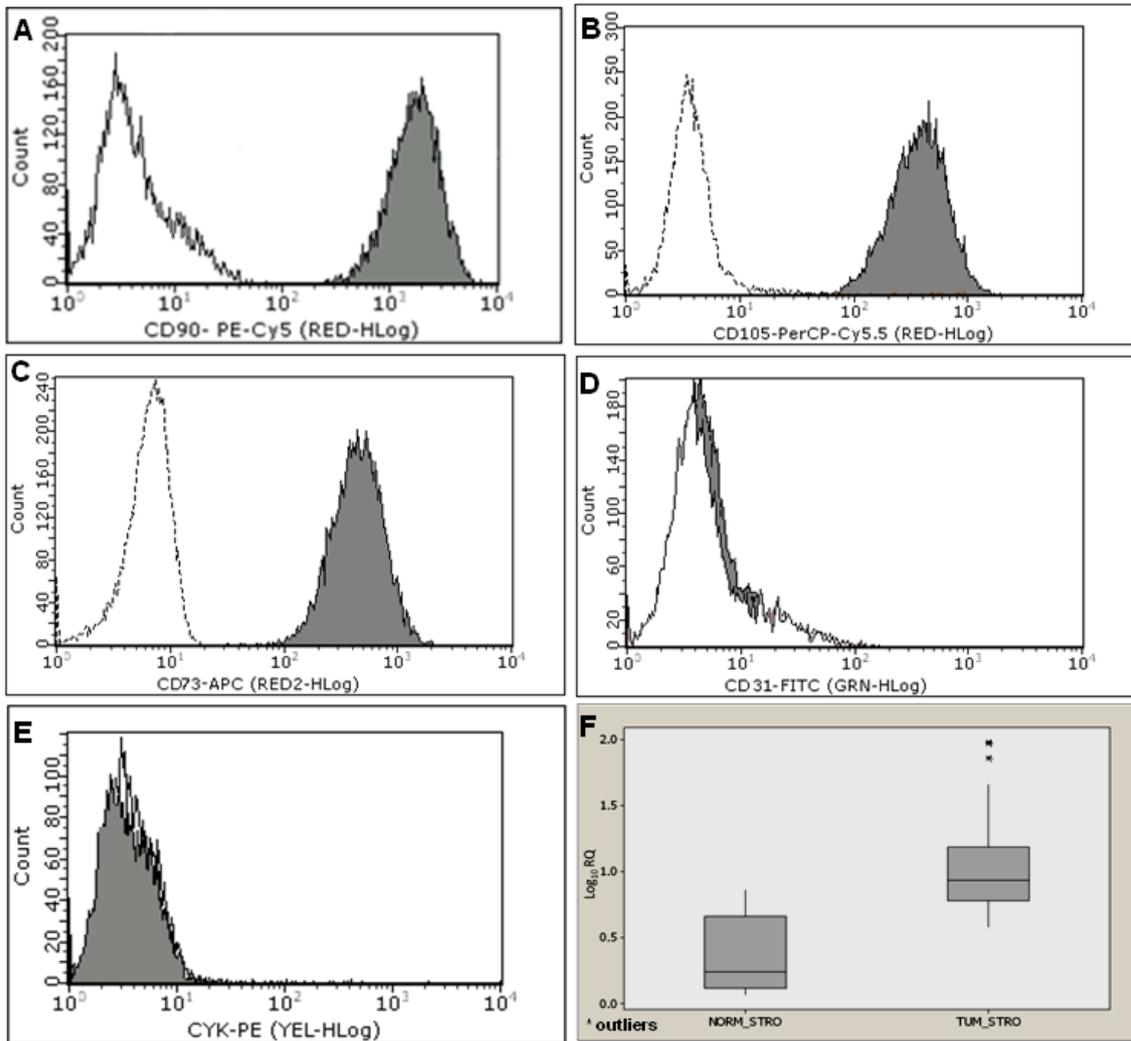
Figure 1: Primary stromal cell characterization

Example of mammary stromal cell characterization by flow cytometry (A-E). Isotype controls are shown in white, with analytical samples shown in grey. Stromal cells were shown to be positive for CD90 (A), CD105 (B), CD73 (Figure 1C), and negative for CD31 (Figure 1D) and cytokeratin (Figure 1E). RQ-PCR analysis also revealed significantly higher expression of Fibroblast Activation Protein (FAP) in tumor (n=22) compared to normal (n=8) stromal cells (Figure 1F, $p<0.001$).
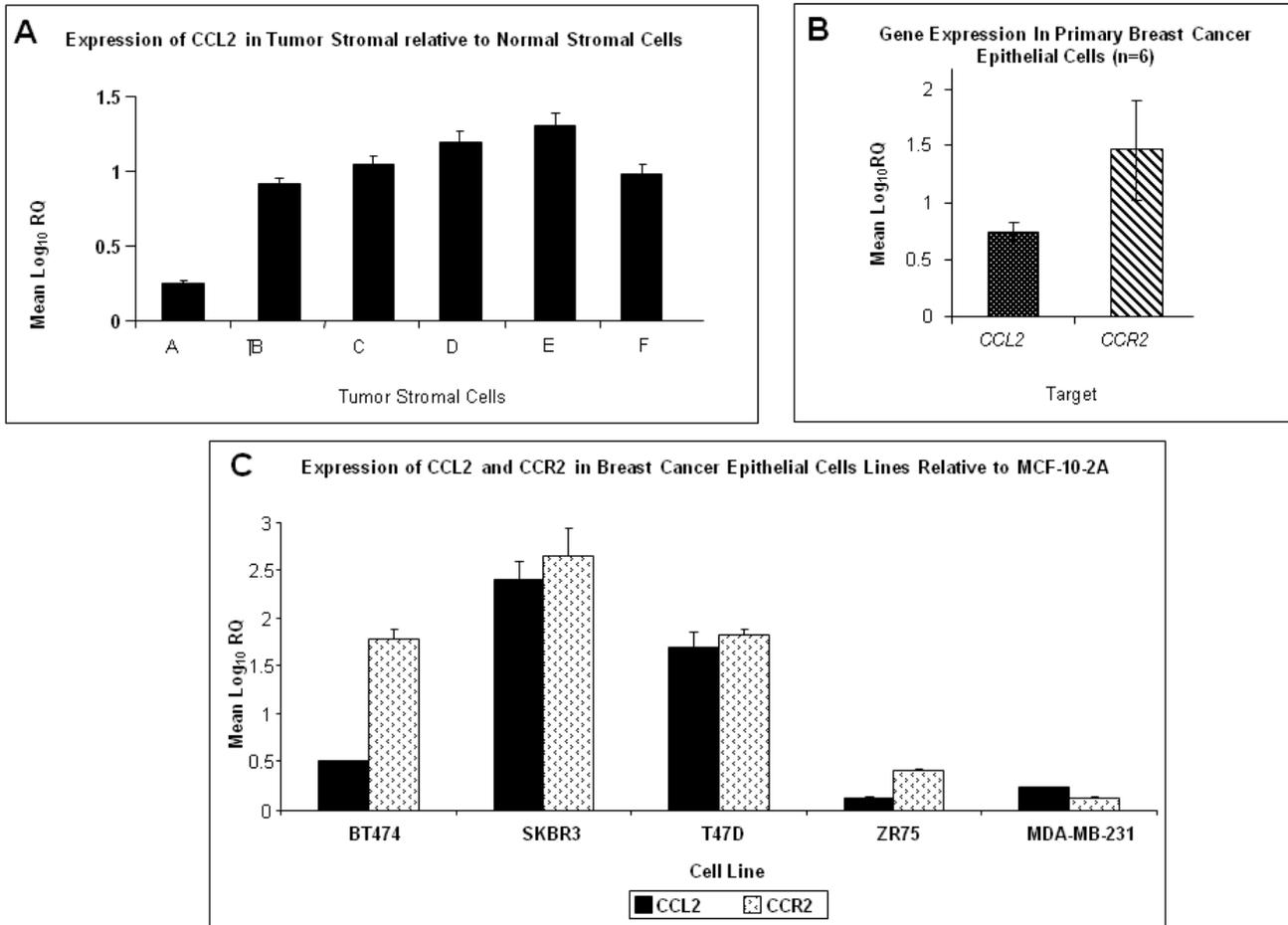
Figure 2: CCL2 and CCR2 expression in Breast Cancer Cells

A Expression of CCL2 in Tumor Stromal (n=6 individual donors) compared to Normal Stromal Cells (n=4 individual donors), represented by the baseline. CCR2 expression was not detected in any stromal cell population examined.

B CCL2 and CCR2 gene expression in primary breast cancer epithelial cells isolated from fresh breast tumors (n=6 individual donors). Results are expressed as $\log_{10}$ RQ values, relative to normal counterparts (n=2) ($2^{-\Delta\Delta CT}$).

C Breast cancer epithelial cell lines express *CCL2*, and its principal receptor *CCR2*. Results are expressed as $\log_{10}$ RQ values, and relative to non-tumorigenic MCF-10-2A cells.
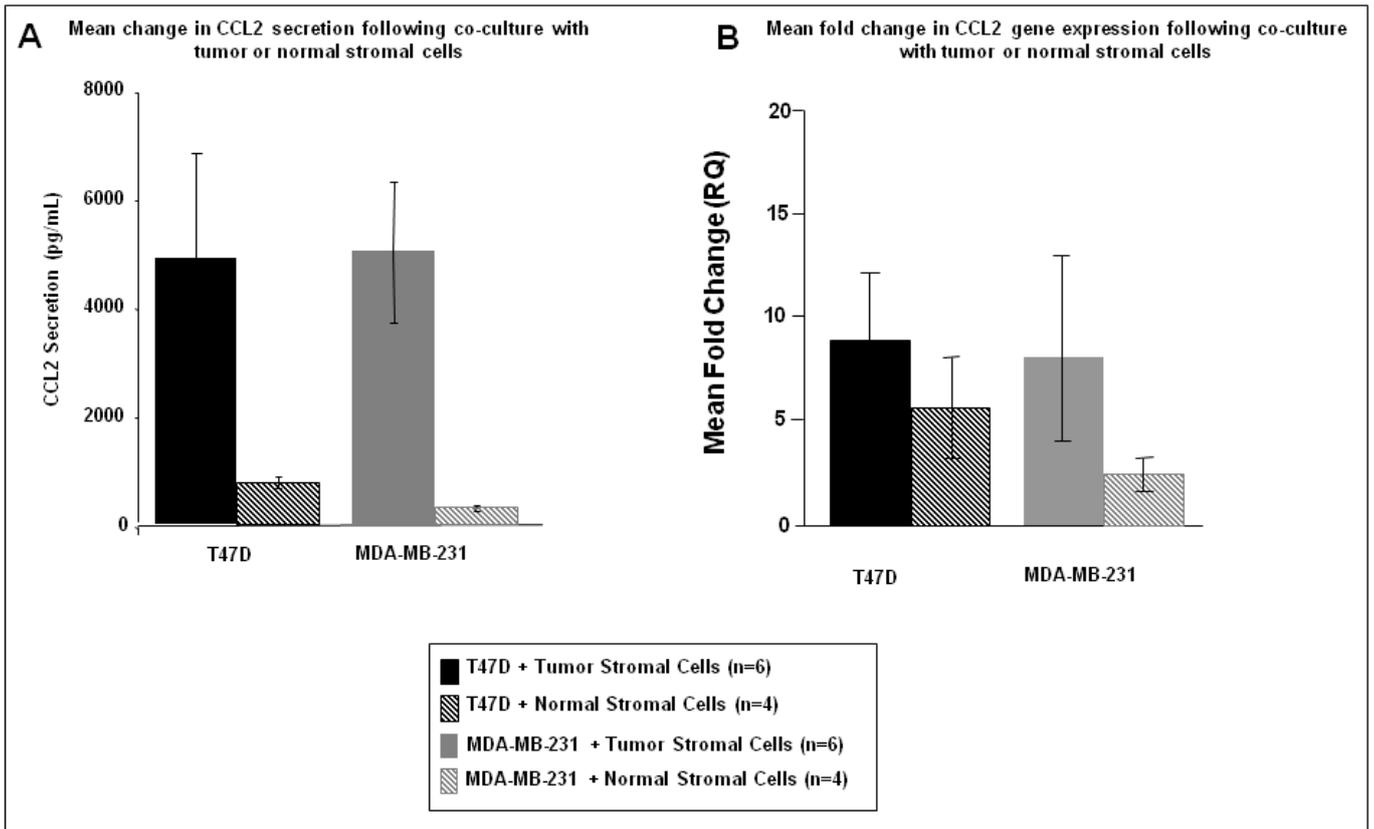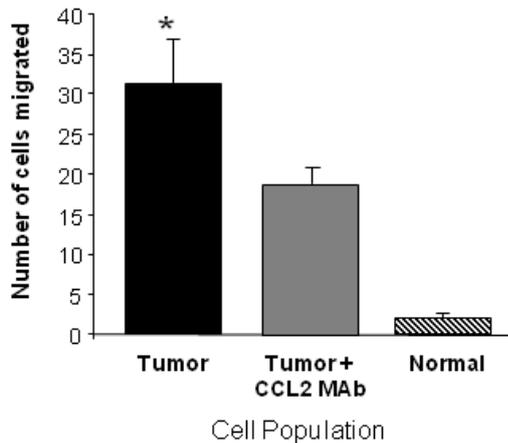
Figure 3: Interaction of tumor stromal and epithelial cells results in increased CCL2 protein secretion and gene expression.

A CCL2 secretion: the baseline of the graph represents the sum of what each cell population secreted when cultured individually, with each bar representing the amount by which the co-culture population differed from this. Data presented represent Mean ± SEM of triplicate experiments, using 6 individual tumor stromal populations and 4 individual normal stromal populations.

B Following in-vitro co-culture of primary stromal cells with breast cancer cell lines, epithelial cells were retrieved using EpCAM+ magnetic beads, and expression of *CCL2* analysed. Data presented represent Mean fold change values ± SEM of triplicate experiments, and are expressed relative to levels detected in the breast cancer cells cultured alone ($2^{-\Delta\Delta CT}$).

A Mean migration of MDA-MB-231 breast cancer epithelial cells in response to tumor (n=8) and normal (n=4) stromal cells

B Proliferation of breast cancer epithelial cells in response to tumor (n=8) and normal (n=4) stromal cells
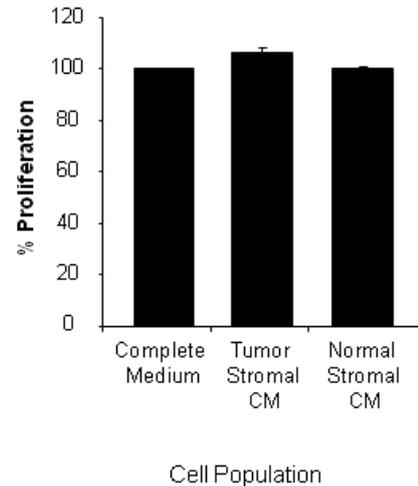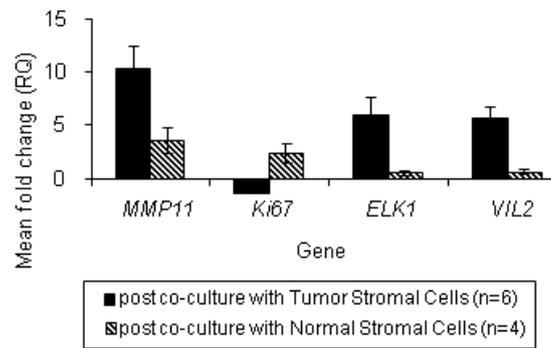
Figure 4: Interaction of tumor stromal and epithelial cells in vitro: effect on migration and proliferation.

A Factors secreted by primary tumor stromal cells induce increased migration of breast cancer epithelial cells. This effect was subsequently blocked by the addition of a monoclonal antibody to CCL2.

B A Vialight™ assay was used to assess relative proliferation of breast cancer cells (MDA-MB-231 and T47D), in response to stromal cell CM (Tumor Stromal Cells and Normal Stromal Cells). Proliferation induced by complete medium was taken as 100%. Results are displayed as Mean % proliferation of the 2 cell lines ± SEM, from triplicate experiments.

(A) Mean fold change in T47D cells following co-culture with tumor (n=6) and normal (n=4) stromal cells (Baseline represents T47D cells cultured alone)

post co-culture with Tumor Stromal Cells (n=6)
post co-culture with Normal Stromal Cells (n=4)

(B) Mean fold change in MDA-MB-231 cells following co-culture with tumor (n=6) and normal (n=4) stromal cells (Baseline represents MDA-MB-231 cells cultured alone)
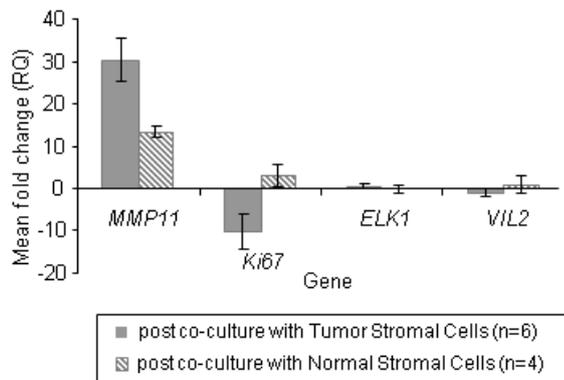
post co-culture with Tumor Stromal Cells (n=6)
post co-culture with Normal Stromal Cells (n=4)

Figure 5: Interaction of tumor stromal and epithelial cells in vitro: effect on gene expression. Interaction with tumor stromal cells induces increased expression of invasion associated genes and decreased expression of proliferative markers in breast cancer epithelial cells T47D **(A)** and MDA-MB-231 **(B)**. Data presented represent Mean fold change values ± SEM of triplicate experiments, and are expressed relative to levels detected in the breast cancer cells cultured alone ($2^{-\Delta\Delta CT}$).
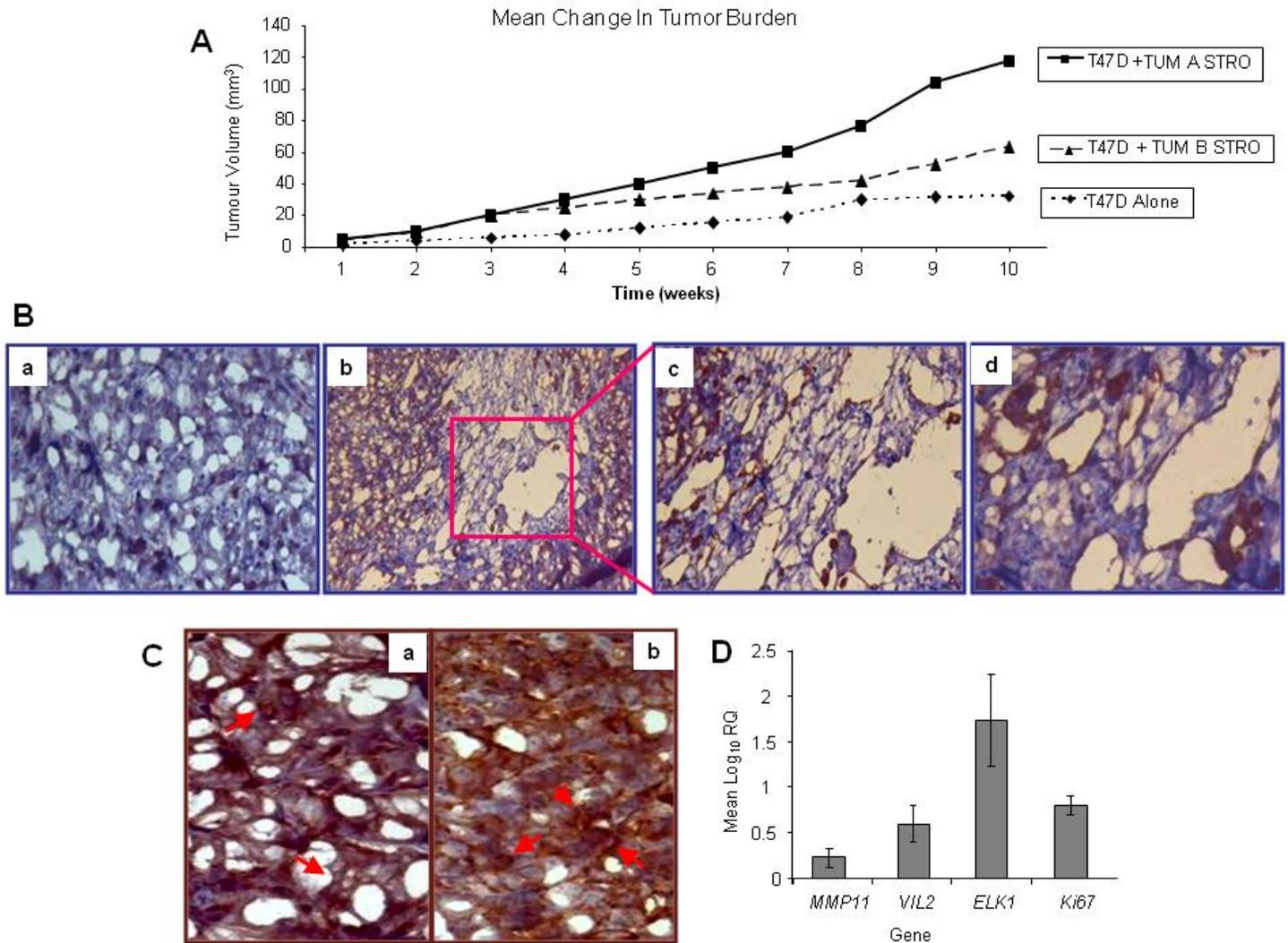
Figure 6: Interaction of tumor stromal and epithelial cells in vivo

T47D cells were injected alone or coinjected with tumor stromal cells subcutaneously into nude mice.

A Mixed stromal-epithelial xenografts exhibited increased tumor volume. Data represent Mean ± SEM for n=6 in each group.

B Xenografts that developed in the presence of tumor stromal cells (b [10X], c [20X], d [40X]) stained positive for CD31, indicating neovascularization, while those composed of epithelial cells alone did not (a, [40X]).

C Mixed stromal-epithelial xenografts displayed greater intensity Ezrin staining and increased cytoplasmic localisation of the protein (b), compared to those composed of epithelial cells alone where more membrane targeted staining was visible (a).

D Upon harvesting, tumors were digested and T47D cells were retrieved from mixed xenografts with epithelial specific beads, RNA extracted and RQ-PCR performed. Results are expressed relative to expression levels in epithelial cells isolated from tumors formed from T47D cells alone. Each bar represents Mean $\log_{10}$ RQ ± SEM for both groups (Tum A Stro and Tum B Stro, n=6 in each group).

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