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► To cite this version:

F. T. Martin, R. M. Dwyer, J. Kelly, S. Khan, J. M. Murphy, et al.. Potential role of mesenchymal stem cells (MSCs) in the breast tumour microenvironment: stimulation of epithelial to mesenchymal transition (EMT). *Breast Cancer Research and Treatment*, 2010, 124 (2), pp.317-326. 10.1007/s10549-010-0734-1 . hal-00530824

HAL Id: hal-00530824

<https://hal.science/hal-00530824>

Submitted on 30 Oct 2010

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Title: Potential Role of Mesenchymal Stem Cells (MSCs) in the Breast Tumor Microenvironment: Stimulation of Epithelial to Mesenchymal Transition (EMT)

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Abstract

Bone marrow derived Mesenchymal Stem Cells (MSCs) are known to specifically migrate to and engraft at tumor sites. Understanding interactions between cancer cells and MSCs has become fundamental to determining whether MSC-tumour interactions should be harnessed for delivery of therapeutic agents or considered a target for intervention. Breast Cancer Cell lines (MDA-MB-231, T47D & SK-Br3) were cultured alone or on a monolayer of MSCs, and retrieved using epithelial specific magnetic beads. Alterations in expression of 90 genes associated with breast tumorigenicity were analyzed using low density array. Expression of markers of Epithelial-Mesenchymal transition and array results were validated using RQ-PCR. Co-cultured cells were analyzed for changes in protein expression, growth pattern, and morphology. Gene expression and proliferation assays were also performed on indirect co-cultures. Following direct co-culture with MSCs, breast cancer cells expressed elevated levels of oncogenes (NCOA4, FOS), proto-oncogenes (FYN, JUN), genes associated with invasion (MMP11), angiogenesis (VEGF) and anti-apoptosis (IGF1R, BCL2). However, universal downregulation of genes associated with proliferation was observed (Ki67, MYBL2), and reflected in reduced ATP production in response to MSC-secreted factors. Significant upregulation of Epithelial-Mesenchymal Transition specific markers (N-cadherin, Vimentin, Twist and Snail) was also observed following co-culture with MSCs, with a reciprocal downregulation in E-cadherin protein expression. These changes were predominantly cell contact mediated and appeared to be MSC specific. Breast cancer cell morphology and growth pattern also altered in response to MSCs. Mesenchymal Stem Cells may promote breast cancer metastasis through facilitation of Epithelial-Mesenchymal Transition.

Keywords: Mesenchymal Stem Cells (MSCs), Breast Cancer, Epithelial-Mesenchymal Transition (EMT), Invasion, Co-culture

Introduction

Breast cancer remains the most common malignancy in women, accounting for one quarter of all female cancers [1]. The preferential spread of tumour cells to bone and subsequent development of osteolytic metastatic deposits remains a devastating event in the course of the disease [2, 3]. It is now understood that tumour epithelial cells develop in a symbiotic rather than an independent manner with surrounding stroma. This stromal environment consists of a dynamic network of immune cells, fibroblasts, tumour vasculature and extracellular matrix [4]. Tumours actively recruit cells, including bone marrow derived mesenchymal stem cells (MSCs), into the tumour microenvironment and these cells may play a role in facilitating cancer progression [5]. MSCs are a subset of non-haematopoietic cells found within the bone marrow stroma that have an innate ability both to self renew and to differentiate into cells of multiple lineages, including osteoblasts, chondrocytes and adipocytes [6]. They have also been seen to influence the morphology and proliferation of cells within their vicinity through both cell to cell interactions and the secretion of chemoattractant cytokines and paracrine factors [7-10]. Studies assessing systemically delivered MSCs have confirmed that these circulating cells engraft and facilitate healing at sites of inflammation and injury including head trauma, stroke and myocardial infarction [10, 11]. Malignancy may also be considered as a nidus of chronic inflammation or “wound that never heals” [12] and reports have shown a similar pattern of MSC engraftment at these sites [11]. This tumor homing ability has prompted researchers to analyse MSCs as possible vectors for the targeted delivery of anti-cancer agents to tumor microenvironments [13]. However evidence suggests that interactions between MSCs and breast cancer cells may impact upon the phenotype of the cancer cell and promote their metastatic potential [7-9, 14-16]. Understanding these interactions has become fundamental to determining whether the homing ability of MSCs should be harnessed for delivery of therapeutic agents or whether the MSC-tumour interactions should be considered a target for intervention.

Studies that have analysed direct interactions between breast cancer cells and MSCs report distinct proliferative and morphological changes in the cancer cells [7, 8]. Growth patterns of cancer cells in co-culture change from a clustered to a single cell distribution, and these morphological alterations have been related to a significant downregulation of

cell adhesion molecules E-cadherin and Epithelial Specific Antigen (ESA) [7, 8]. Conflicting reports exist with relation to the effect of MSCs on proliferation of breast cancer cells, with some studies reporting no change [7] and others suggesting proliferative changes occurring in an estrogen dependant manner [8, 9].

More recently a pivotal study by Karnoub et al [14] reported that, when mixed with breast cancer cells prior to implantation, MSCs enhance breast cancer cell motility, invasion and metastatic potential in vivo. Knockdown of the CCL5–CCR5 loop led to an abrogated metastatic response confirming that these paracrine interactions play an important role in MSC-mediated metastatic spread [14]. These studies highlight the distinct effect that MSCs have on breast cancer cells, and thus understanding the pathways governing these effects remains imperative.

Epithelial to Mesenchymal Transition (EMT) is a process essential to organogenesis during embryonic development [17], however its reactivation during adult life has been ascribed to certain pathological processes including the facilitation of carcinogenesis [18]. EMT has been shown to promote the detachment of cancer cells from the primary tumour and facilitate their subsequent migration through the acquisition of stem like properties, including a loss of cellular polarity, adhesion and proliferation [18, 19]. Studies have demonstrated evidence of EMT in primary human breast carcinomas showing a proclivity toward the more invasive basal breast cancer phenotype [20, 21]. Despite recognition of the role EMT plays in the metastatic cascade, stimuli inducing EMT at the primary tumour site remain largely unknown.

Further understanding of MSC/tumour cell interactions is required to determine their role in breast cancer progression or therapy. This study aimed to further elucidate the effect MSCs have on breast cancer cells and to potentially identify pathways mediating these effects.

Materials and Methods

Cell Culture

Breast cancer cell lines included MDA-MB-231 cells cultured in Liebowitz-15 medium (L-15); T47D cells cultured in RPMI 1640 medium; and SK-Br-3 cells cultured in McCoys-5a medium. Normal human embryonic lung fibroblasts (WI-38 cells) were

cultured in Eagle's minimal essential medium (EMEM). All media were supplemented with 10% Fetal Bovine Serum (FBS), 100 IU/ml Penicillin /100µg/ml Streptomycin (P/S) and 1% L-glutamine.

Mesenchymal Stem Cell (MSCs) were supplied by the Regenerative Medicine Institute (REMEDI) at NUI Galway. With ethical approval and informed consent, bone marrow was aspirated from the iliac crests of healthy donors following a defined clinical protocol [22]. MSCs were isolated from the marrow aspirates by direct plating and subsequently cultured for 12-15 days to deplete the non-adherent haematopoietic cell fraction. Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with pre-selected FBS (10%) and P/S. The ability of MSCs to differentiate into chondrocytes, adipocytes and osteoblasts was confirmed prior to use. Characterization of surface receptors was performed targeting the markers CD105, CD73, CD90 (positive) and CD34, CD45 (negative). MSCs derived from three separate donors were utilised for experiments. All cells were maintained at 37°C and 5% CO₂.

Direct Co-Culture: Primary MSCs or normal fibroblasts (WI-38 cells) were seeded at a density of 2×10^4 cells/cm² and allowed to adhere overnight. Breast cancer cell lines were then seeded at a density of 1.3×10^4 cells/cm² onto the monolayers of MSCs or normal fibroblasts. All cell types were cultured individually in parallel as controls. Cells were maintained in MSC specific medium and following a 3 or 7 day incubation, media was harvested and epithelial cells retrieved as described below.

Retrieval of Epithelial Cells: Following direct co-culture epithelial cells were separated from MSCs in co-culture using an EasySep[®] positive selection kit (Stem Cell Technologies). As per manufacturer's instructions, co-culture populations were trypsinized and dispersed into a single cell suspension, and EasySep[®] positive selection cocktail and magnetic nanoparticles were added during serial incubations on ice. The magnetic nanoparticles bind selectively to viable epithelial cells which are positively selected by placing the tube in a magnet. Retrieved cells were centrifuged and stored at -80°C until required for RNA extraction.

Gene Expression: RNA was extracted from both cells cultured alone and epithelial cells retrieved following co-culture with MSCs or WI-38 cells using the RNeasy[®] Mini Kit (QIAGEN Ltd.) following manufacturer's protocol. cDNA was generated using SuperScript III reverse transcription enzyme and analyzed by both Taqman[®] Low-Density array (TLDA) and relative quantitative-PCR (RQ-PCR). The array plate was designed to simultaneously measure expression of 90 genes specifically associated with breast cancer tumorigenicity and 6 endogenous controls. Following identification of target genes of interest, co-culture experiments were repeated in triplicate and results validated by RQ-PCR using the ABI Prism 7000 sequence detector system (Applied Biosystems). PreDeveloped Taqman[®] Assay Reagents (PDARS) specific to genes associated with EMT, including N-cadherin, Vimentin, Twist and Snail, were also used to quantify changes in expression by RQ-PCR. The comparative C_T method was used to quantify expression of genes and this was normalized to the endogenous control, Peptidyl-Prolyl Isomerase A (PPIA). Results from cells retrieved from co-culture were expressed relative to the same cells cultured alone. Changes in gene expression were expressed using the $2^{-\Delta\Delta C_T}$ method [23] and the fold change in triplicate experiments was recorded and presented as Mean \pm SEM.

Collection of Conditioned Media (CM) for indirect co-culture: MSCs were seeded at a density of 2×10^4 cells/cm² in DMEM supplemented with pre-selected FBS (10%) and P/S. Media was aspirated at 24hr intervals and transferred to breast cancer cells lines to determine the effect of MSC secreted factors on cell proliferation and gene expression (indirect co-culture) as described below. Breast cancer cells grown in MSC medium that had not been exposed to MSCs served as a control.

Proliferation Assay: Breast cancer cell lines were seeded onto 96 well white walled plates at a density of 8×10^3 cells/well in 100 μ l media and allowed to adhere overnight. Media was aspirated and replaced with MSC-conditioned medium (CM) as described above at 24hr intervals for 72hrs. An Apoglow[®] assay was performed to assess changes in proliferation based on the level of ATP production as quantified by a luminometer. Results presented represent triplicate experiments and are expressed as Mean \pm SEM.

Indirect co-culture: Breast cancer cells were cultured in media that had been exposed to MSCs as described above. Following culture in MSC CM, cells were lysed and RNA extracted. Changes in expression of genes associated with EMT were quantified by RQ-PCR as described for the direct co-culture experiments.

Western Blot Analysis. Protein was extracted from cells cultured individually and those retrieved following co-culture. Briefly, cells were washed and resuspended in Triton-X lysis buffer [150mM NaCl, 20 mM HEPES, 2 mM EDTA, 1% Triton-X100, 2mM Sodium Orthovanadate, 10mM Sodium Fluoride, 10ul/mL Protease inhibitor cocktail (Fisher Scientific)], frozen at -20°C and then centrifuged at $500 \times g$ for 15 mins at 4°C to remove cellular debris. The protein content was determined using the Micro BCA™ Protein Assay Kit (Thermo Scientific). Protein (40 μg) was reduced in DTT (0.5 M) for 10 mins at 70°C and samples run on a 4-12% gradient pre-cast NuPAGE Bis-Tris polyacrylamide gel for 1 hr at 200V. Protein molecular weight standards (20-220 kDa) were run simultaneously on each gel. Electroblotting was performed for 1hr at 25V to transfer protein samples to a nitrocellulose membrane. Blots were blocked in 5% milk in TBS-T [20 mM Tris, 137 mM NaCl, 0.1% Tween-20] for 1 hr, and probed with antibodies targeting E-cadherin (1 $\mu\text{g}/\text{mL}$, R & D Systems), Vimentin (1:100, Abcam), or Snail (1 $\mu\text{g}/\text{mL}$, Abcam) for 1.5 hrs and washed in TBS-T. β -actin was used to confirm equal loading in wells. Horseradish peroxidase labelled goat anti-rabbit (1:3,000; Abcam) or rabbit anti-mouse antibody (1:2,000; Abcam) was then added to the membranes for 1.5 hrs. Following washing steps, SuperSignal West Pico Chemiluminescent substrate (Thermo Scientific) was applied to the membranes for 5min. Images were captured using a Syngene G-Box and GeneSnap software.

Immunohistochemistry & Fluorescent Microscopy: After 72hrs co-culture in chamber slides, cells were fixed in methanol. Immunohistochemical analysis was performed using monoclonal antibodies targeting E-cadherin (R&D Systems), MNF116 pancytokeratin (Dako, Denmark) and CD90 (Dako, Denmark). E-cadherin and CD90 were visualized

using the chromagen 3,3'-Diaminobenzidine (DAB), with Acid fast red (RED) used for detection of pancytokeratin in dual staining experiments.

To assess changes in breast cancer cell morphology in response to MSCs, cells were dual labelled and examined by fluorescence microscopy. Prior to mixing the cell populations, epithelial breast cancer cells were labelled with PKH26 (red fluorescent label, Excitation 551nm, Emmission 567nm, Sigma). Following 72hrs co-culture, cells were fixed in 4% paraformaldehyde and the cytoskeleton of the mixed populations was labelled with Alexafluor[®] 488 phalloidin (green fluorescent label, Excitation 495nm, Emmission 518nm, Invitrogen, Eugene, OR). Cells were examined using an Olympus IX81-ZDC[®] microscope and Confocal Andor Revolution spinning disc system[®].

Results

Cell Separation: Cell separation using the EasySep[®] positive selection kit (Stem Cell Technologies) was assessed. Following two washes, a positive retrieval rate of $94.4 \pm 1.1\%$ was achieved (range 92 - 97.5% retrieval). It has previously been shown in an extensive study by Woelfle et al. [24], that the immunoselection procedure does not alter breast cancer cell gene expression. To further confirm this, expression of Vimentin, E-cadherin, CXCL12 and CXCR4 in breast cancer cells selected with beads, was compared to unselected cells with a <1-fold change in gene expression detected.

Analysis of Gene Expression: Low density array analysis of 90 genes associated with breast cancer tumorigenicity was performed on all breast cancer cell lines retrieved following 72hrs co-culture with MSCs, relative to the same cells cultured alone. Any change >2.5 fold is presented (*Table 1*). Upregulation of oncogenes, proto-oncogenes and genes associated with angiogenesis, anti-apoptosis and invasion was observed. A range of genes exhibited greater than 10 fold upregulation (FOS, FYN, MET, VEGF, CD68 and MMP11) while others were upregulated over 1,000 fold (CAV-1, TGF β R2 and CXCL12). However down-regulation of genes associated with proliferation (Ki67, CCNE1 and MYBL2) was recorded across all breast cancer cells following co-culture with MSCs.

Observed changes in specific genes of interest were validated in triplicate experiments using RQ-PCR (Figure 1). Significant upregulation of the chemokine, CXCL12, was observed in SK-Br3 cells ($9,949 \pm 4,787$ fold, $p < 0.05$) following co-culture with a reciprocal downregulation in its cognate receptor CXCR4 (3 ± 1 fold). In MDA-MB-231 cells, CXCL12 expression was significantly increased ($17,066 \pm 1,109$ fold) whereas T47D cells exhibited upregulation of its receptor, CXCR4 (6 ± 2 fold, $p < 0.05$). The proliferation marker, Ki-67, was downregulated in all breast cancer cells (Range: 2 – 4 fold decrease, T47D $p < 0.05$), while the invasive marker, MMP11, was significantly upregulated.

Cell Proliferation in response to MSC secreted factors: Breast cancer cells were cultured in the presence of MSC CM for 72 hours after which ATP levels were quantified using a luminometer based Apoglow[®] Assay (Figure 2). There was a significant reduction in proliferation observed in all three breast cancer cell lines cultured in the presence of factors secreted by MSCs (SK-Br-3 $p < 0.05$; T47D and MDA-MB-231 $p < 0.001$).

Expression of markers associated with EMT: Significant upregulation in defined markers of EMT were observed in both SK-Br3 and T47D cells retrieved following 72hrs co-culture with MSCs (Figure 3a). Due to the magnitude of the increases seen, results are expressed as Log_{10} values. Upregulation of most EMT markers in the MDA-MB-231 cell line occurred to a lesser degree: Vimentin (3 fold), Snail (5 fold), N-cadherin (50 fold), while Twist expression increased $>10,000$ fold. To determine whether the effects seen were transient, T47D and Sk-Br3 cells were also retrieved following 7 days direct co-culture with MSCs. In the case of the T47D cells, a significant increase in Vimentin (244-fold) and Snail (5-fold) was still detected, while Twist and N-cadherin had returned to baseline. At Day 7 the SK-Br3 cells retained increased expression of N-cadherin (28-fold), Vimentin (153-fold) and Snail (10-fold).

To determine whether the changes in gene expression were detected at the protein level, protein was extracted from cells cultured individually and those retrieved following co-culture. Lysates were then subjected to western blot using antibodies directed against Vimentin, Snail (Figure 3b) and E-cadherin (Figure 5c). To confirm that differences seen

were not as a result of variation in protein sample, β -actin was also targeted and found to be at similar levels in all samples. Increased expression of Vimentin and Snail protein was detected in both Sk-Br3 and T47D protein lysates harvested from cells retrieved following direct co-culture with MSCs (Figure 3b).

Overall the greatest increase in all EMT markers examined was seen in Sk-Br3 cells. To determine whether this was an MSC specific effect, SK-Br3 cells were cultured directly on a confluent monolayer of normal fibroblasts (WI-38 cells). No significant change in expression of genes associated with EMT was observed following co-culture with WI-38 cells. Mesenchymal markers N-cadherin and Vimentin were downregulated 1.5 and 2.1 fold respectively, with expression of the transcription factors Twist and Snail both decreased by 1.5 and 1.4 fold respectively (results not shown).

Expression of EMT markers following indirect co-culture: To determine whether results observed were due to cell contact mediated effects, breast cancer cells were exposed to MSC conditioned medium and changes in expression of the same EMT markers were analysed (Figure 4). In T47D and SK-Br3 cells, a small increase in expression of Twist and Snail was observed (range 1 - 2 fold and 4 - 7 fold respectively). A greater upregulation was seen in N-cadherin (range 9 - 32 fold) with the most marked increase observed in vimentin expression (range 158 - 276 fold). Although the changes in expression were significant ($p < 0.05$) for Snail, Twist and Vimentin, the increase was considerably lower than that seen in the same cells following direct co-culture with MSCs (Figure 3). When the length of exposure to MSC secreted factors was increased to 7 days, levels of target expression had returned to baseline (< 2 -fold change in gene expression compared to cells cultured in standard medium). No change in expression of EMT markers was observed in MDA-MB-231 cells following indirect co-culture (results not shown).

Immunohistochemistry: Breast cancer cells and MSCs cultured individually and in co-culture were stained with cell type specific antibodies to distinguish populations and analyse changes in morphology and growth pattern. Changes in E-cadherin protein expression were also examined. E-cadherin has strong membrane targeted expression in

T47D cells (Figure 5a), while MSCs have no detectable expression. When T47D cells were cultured on a monolayer of MSCs a marked decrease was observed in the intensity of E-cadherin staining (Figure 5b). E-cadherin expression was particularly reduced at junctions where T47D cells were in direct contact with the MSCs (indicated by arrows) compared with cells located within a cluster of breast cancer cells. This change in E-cadherin protein expression was confirmed by western blot of T47D protein lysates harvested from cells cultured individually, and those retrieved following co-culture (Figure 5c).

Dual Staining: Breast cancer cells (MDA-MB-231) cultured alone stained positive for the epithelial specific cytokeratin, MNF116 (red), with nuclei counterstained with haematoxylin (blue), and grew in a typical random asymmetric pattern (Figure 5d). Stromal cells (MSCs) staining positive for CD90 grew in a symmetrical pattern with a typical parallel alignment of spindle shaped cells when cultured alone. When cultured on a monolayer of MSCs, MDA-MB-231 cells altered their growth pattern from the random cellular distribution observed to align in parallel with adjacent MSCs (Figure 5e) reflecting a change in cellular polarity.

Confocal Fluorescent Microscopy: PKH26 labelled (red) T47D cells when cultured alone were seen to grow in a typical clustered growth pattern, with the Alexafluor labelled cell cytoskeleton (green) seen to be non-branching and closely adherent to the nuclei (Figure 5f). These same cells, when co-cultured directly on a monolayer of MSCs, appeared to lose cellular adhesion leading to a more dispersed single cell distribution. Furthermore, the breast cancer cell cytoskeleton was more branching and elongated (indicated by arrows), and appeared to polarize in the direction of adjacent Mesenchymal Stem cells (Figure 5g).

Discussion

Mesenchymal Stem Cells have been reported to interact with breast cancer cells that have metastasised to bone marrow [25] as well as being actively recruited to the primary tumour stromal interface [15]. This tumour homing quality has prompted investigators to assess MSCs as possible delivery vectors for anti-cancer therapies [13]. To realise their

therapeutic potential, interactions between MSCs and breast cancer cells must be fully elucidated.

Studies have previously analysed breast cancer cells and MSCs in direct co-culture noting specific morphological and phenotypical alterations in the breast cancer cells [7-9, 14]. However isolation of the cells following co-culture and analysis of changes in gene expression has not previously been assessed. Immunomagnetic selection targeting antigens such as EpCAM is used to capture circulating tumor cells or enrich tumor cells from mixed cell samples. The immunomagnetic enrichment technique itself has previously been shown to have no significant effect on the gene expression profile of breast cancer cells [24].

Reports from this laboratory and others have shown a significant increase in migration of breast cancer cells in response to factors secreted by MSCs [25, 26], and this was reflected by increased expression of migratory genes seen here including MMP11 and CXCL12 [27]. Oncogenes and proto-oncogenes were upregulated both in a cell specific manner and, in the case of FOS and JUN, across all breast cancer cells retrieved following co-culture with MSCs. FOS and JUN are both major components of the activator protein-1 (AP-1) transcription factor complex which has been shown to positively regulate cellular motility and migration [28].

CAV-1 is now considered a marker of poor prognosis with up-regulation correlated to increased cellular dissemination and cell survival [29]. CAV-1 has been shown to mediate its anti-apoptotic properties through upregulation of IGF-1R which was also elevated in the cells following co-culture with MSCs. Interestingly a universal downregulation of genes associated with proliferation (Ki-67, MYBL2, CCNE1) was observed in all breast cancer cells retrieved from co-culture. Subsequent analysis of ATP production by breast cancer cells in the presence of MSC secreted factors (indirect co-culture), revealed a significant reduction in proliferation of all cancer cells. These results concur with those of Hombauer and Minguell [7] who noted no increase in proliferative activity when MCF-7 cells were grown alone or on a monolayer of MSCs.

The apparent promotion of oncogenes and genes associated with invasion and migration with an inhibition of proliferation fits a profile seen in EMT [18, 19]. In order to further investigate whether MSCs were exerting their effects through induction of EMT, a

number of specific genetic markers of EMT were examined in breast cancer cells following co-culture. Anti-apoptotic transcription factors, Twist and Snail, and mesenchymal protein markers, Vimentin and N-cadherin have been consistently associated with mesenchymal transition in epithelial cells [21, 30]. Vimentin upregulation is commonly observed in more invasive basal cancer subtypes and has been positively correlated with poor prognosis in breast cancer patients [31]. Interestingly, Vimentin was upregulated in both T47D and SK-Br-3 cells with no significant upregulation in the MDA-MB-231 breast cancer population. This may be due to the relatively high expression of Vimentin already present in the more invasive MDA-MB-231 cells [32]. This upregulation in Vimentin was confirmed at the protein level, and also detected following 7 days of in vitro co-culture. Significant upregulation of N-cadherin, Twist and Snail was recorded across all breast cancer cells retrieved from co-culture with MSCs although to a lesser extent in the MDA-MB-231 cells. This proportional difference in EMT changes recorded between MDA-MB-231 cells and other less invasive breast cancer subtypes coincides with findings recorded by Karnoub et al [14] who noted that MDA-MB-231 cells exist in a state of “partial EMT” and that, within their study, CCL5 secreted by MSCs did not lead to advancement of this EMT phenotype.

Further analysis of array data confirmed upregulation of a number of genes associated with EMT induction including TGF β R2 [33] and ACVR1 [34], both receptors for TGF β , which is known to stimulate mesenchymal transition in epithelial cells [35]. Research suggests that upregulated expression of TGF β R2 is an absolute requirement for TGF β mediated EMT [36]. Vascular endothelial growth factor (VEGF), typically associated with angiogenesis, was also upregulated. Non-Angiogenic functions of VEGF include anti-apoptotic and pro-migratory properties [37] as well as an important role in the initiation of EMT through upregulation of Snail expression [38]. EMT appears to be at least partly dependant on VEGF signalling as studies that have blocked VEGF noted a proportional decrease in EMT [39].

To investigate whether changes seen were specific to MSCs, breast cancer cells were directly cultured with normal fibroblasts (WI-38 cells), resulting in no significant change in EMT related gene expression. This suggests the effects observed were MSC specific. To assess whether changes in gene expression were mediated solely through cell to cell

contact, breast cancer cells were also cultured in MSC conditioned medium. No change in expression of EMT markers was seen in MDA-MB-231 cells exposed to MSC conditioned medium. Both T47D and SK-Br3 cells exhibited a relatively mild upregulation in expression of Twist, Snail and N-cadherin, with the most marked increase seen in Vimentin expression. Although significant, these changes following indirect co-culture occurred to a much lesser degree than those seen in cells directly cultured with MSCs. Also, the effects were found to be transient in the indirect co-culture model used, with gene expression returning to baseline following 7 days of indirect co-culture. This may be due to cell-contact mediated inhibition of MSC proliferation, and resultant reduction in secretion of mediating factors. Overall the data suggests that changes in gene expression observed were predominantly mediated through direct cell to cell contact.

Decreased expression of the cell adhesion protein E-cadherin and the resultant cellular dissociation is another marker consistent with the process of EMT [18]. Previous studies investigating breast cancer cells directly co-cultured with MSCs have shown a significant downregulation in E-cadherin protein expression in breast cancer cells [7, 8] an observation also noted in the current study. Dual staining to distinguish between cell populations in co-culture also highlighted alterations in morphology and growth patterns of breast cancer cells. T47D cells appeared to lose adhesiveness and separate from their normal clustered growth pattern, with cells adjacent to MSCs branching and polarizing toward the mesenchymal cells. These changes coincide with the loss of apico-basal polarity seen in cells that undergo EMT [18].

Recent literature has significantly advanced our understanding of the pivotal role EMT plays in the metastatic cascade. Initially regarded with a degree of scepticism, mesenchymal transition has been observed at the primary tumour site in a cohort of 479 human breast cancer samples and a positive correlation with basal breast cancer phenotype confirmed [20]. Despite these developments the stimulus inducing EMT at the primary tumour site remains unknown. The current study suggests that MSCs that are actively recruited to tumour stromal microenvironments may act as a stimulus to induce EMT in breast cancer cells and actively increase breast cancer metastatic potential.

Acknowledgements: This study was supported by funding from the National Breast Cancer Research Institute (NBCRI), a Royal College of Surgeons in Ireland (RCSI) Surgical Research Grant (F.T. Martin), a Health Research Board Project Grant (R.M. Dwyer), and a Science Foundation Ireland CSET award (J.M. Murphy, F.P. Barry and T. O'Brien).

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Figure legends

Table 1: Results from Low Density Array analysis of breast cancer cells retrieved following co-culture with MSCs. Results presented show genes where at least one cell line had ≥ 2.5 fold increase or decrease in expression following co-culture with MSCs. *Genes which were then validated in triplicate experiments by RQ-PCR.

Figure 1: Change in breast cancer cell gene expression following co-culture with MSCs. Results presented as Mean \pm SEM Log₁₀ Relative Quantity in triplicate experiments. The baseline represents the level of expression in breast cancer cell lines cultured individually. *denotes $p < 0.05$

Figure 2: Changes in proliferation of breast cancer cells exposed to factors secreted by MSCs for 72hrs. Cell proliferation was measured using an Apoglow® assay in T47D, SK-Br3 & MDA-MB-231 cells cultured alone and in MSC conditioned media. Results presented represent mean of triplicate experiments \pm SEM. RLU = Relative Light Units detected on luminometer. * $p < 0.05$ ** $p < 0.001$

Figure 3: (A) Changes in breast cancer cell expression of specific EMT genes following co-culture with MSCs. The baseline represents the level of expression in breast cancer cell lines cultured individually. Results presented represent mean of triplicate experiments \pm SEM. * denotes $p < 0.05$ (B) Protein analysis: Western blot of Sk-Br3 and T47D cells alone and following direct co-culture with MSCs, targeting Vimentin and Snail. β -actin was used to confirm uniform sample loading.

Figure 4: Changes in expression of EMT genes in breast cancer cells following culture in

MSC conditioned medium (Indirect co-culture). The baseline represents the level of expression in breast cancer cell lines cultured in the same medium that had not been exposed to MSCs. Results presented represent mean of triplicate experiments \pm SEM. No change in expression of EMT markers was observed in MDA-MB-231 cells following indirect co-culture (results not shown).

Figure 5 (a-g): Immunostaining of breast cancer cells and MSCs cultured individually and in direct co-culture on chamber slides for 72hrs.

E-cadherin staining: (a) T47D cells, (b) T47D + MSCs, (c) Confirmation of reduced E-cadherin by western blot of protein lysates from T47D cells cultured individually and those retrieved following direct co-culture with MSCs. β -actin was used to confirm uniform sample loading.

Dual staining with cell type specific antibodies; Red – epithelial specific pancytokeratin, Blue – haemotoxylin stained nuclei. (c) MDA-MB-231 cells, (d) MDA-MB-231 + MSCs.

Confocal fluorescent images of cell cytoskeletons stained with Alexafluor (green).

Epithelial cells were also labeled with PKH26 (red) prior to mixing: (e) T47D cells (f) T47D + MSCs. All images presented are at 200x magnification.

Fig. 1: Change in gene expression in breast cancer cells retrieved following direct co-culture with MSCs.

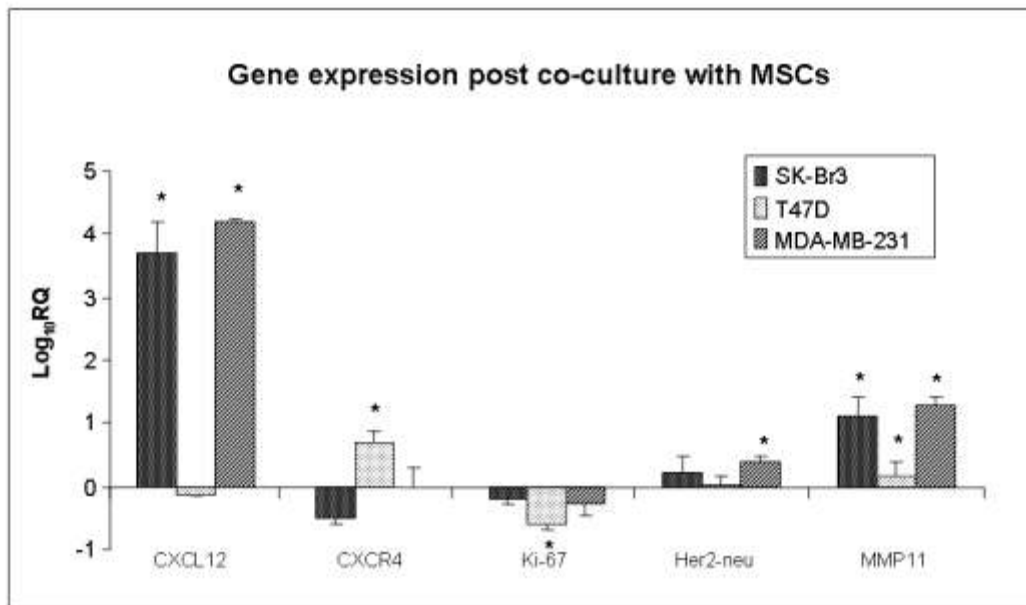


Fig. 2: Changes in proliferation of breast cancer cells exposed to factors secreted by MSCs for 72hrs

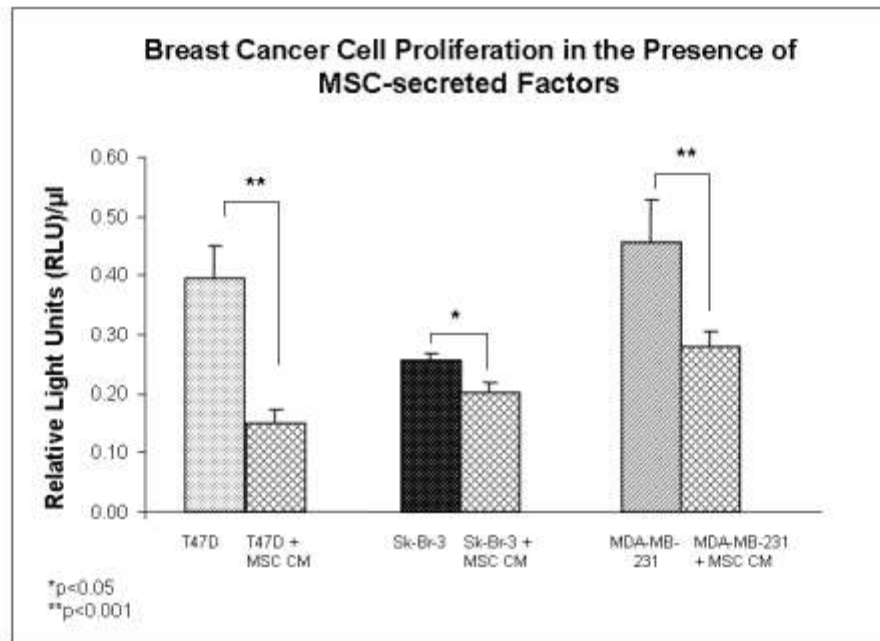


Fig. 3: (A) Changes in expression of EMT specific genes in breast cancer cells retrieved following direct co-culture with MSCs. (B) Representative samples showing increases in expression at the protein level by western blot.

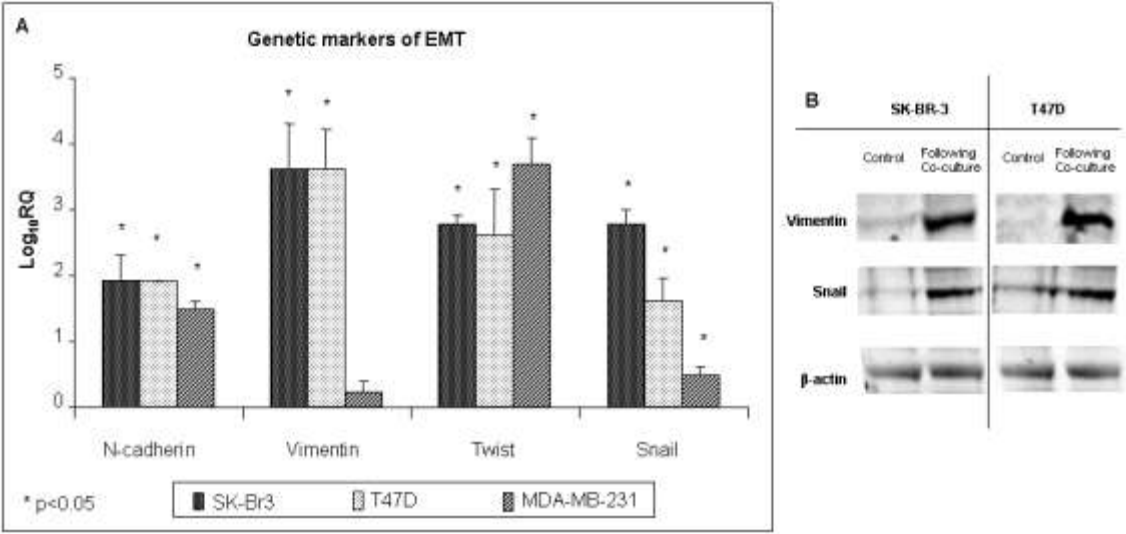


Fig. 4: Changes in expression of EMT genes in breast cancer cells following culture in MSC conditioned medium (indirect co-culture).

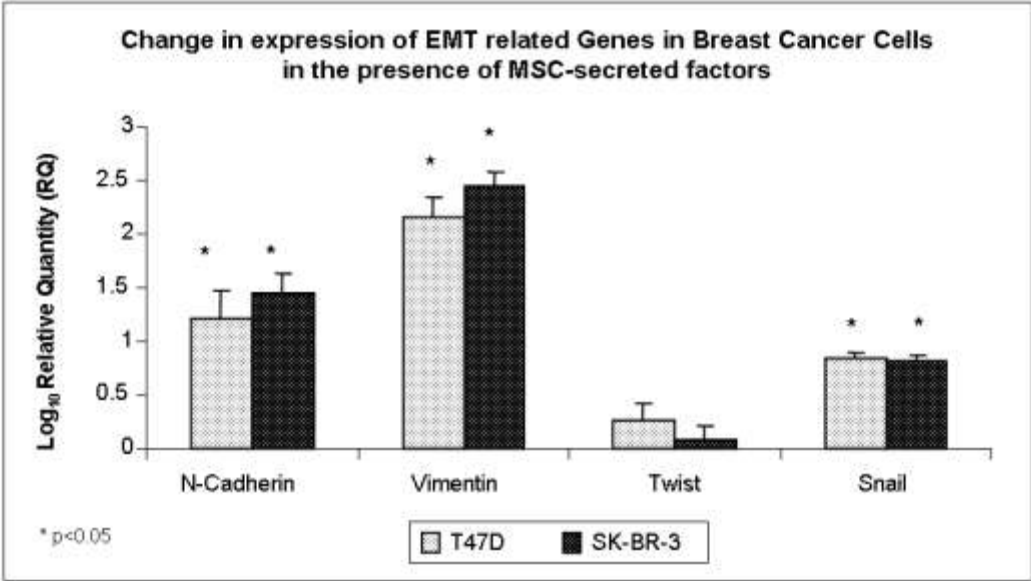
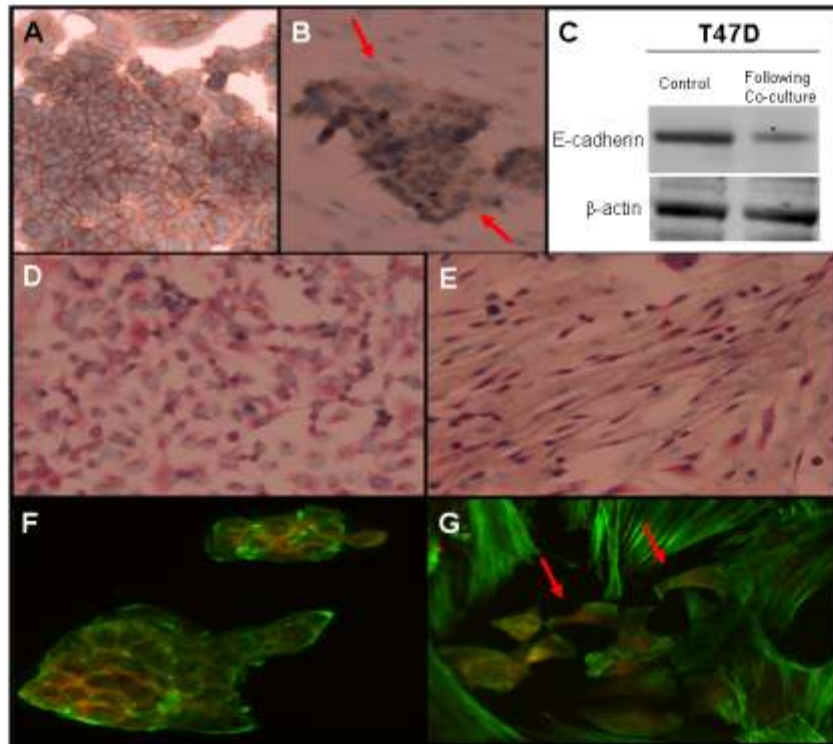


Fig. 5



Gene Function	Gene Symbol	Gene Name	Fold Change		
			Sk-Br3	T47D	MDA-MB-231
Oncogene & proto-oncogenes	NCOA4	Nuclear receptor co-activator 4	3.7	3.7	N/A
	FOS	Osteosarcoma oncogene	6.1	38.9	5.5
	MUC1	Mucin 1	6.1	1.6	N/A
	FYN	FYN oncogene	11	17.6	1.9
	JUN	JUN oncogene	3.2	3.4	2.7
	MET	MET proto-oncogene	3.3	15.4	N/A
	EPHA2	Ephrin receptor A2	2.3	5.5	1.5
Macrophage marker	CD68*	CD68 macrophage antigen/microsialin	8.1	35	3.7
Angiogenesis	VEGF	Vascular endothelial growth factor A	7.3	12	N/A
Anti-apoptosis	IGF1R	Insulin-like growth factor 1 receptor	3.5	1.2	2
	BCL2*	B-cell CLL/lymphoma 2	9.4	6.3	N/A
	CAV-1*	Caveolin 1	8.5	3994	1.2
EMT induction	TGFBR2*	Transforming growth factor-beta receptor type II	8.7	2142	1.8
	ACVR1*	Activin A receptor type 1/ TGF beta superfamily receptor 1	6.7	3.3	3.3
Proliferation	CCNE1	Cyclin E1	-2.8	-2.1	N/A

	MKi67*	Antigen identified by monoclonal antibody Ki-67	-3.3	-4.9	-2.4
	MYBL2	Myeoblastosis oncogene	-5.5	-4.4	-2
Invasion & Migration	MMP11*	Matrix metalloproteinase 11 (Stromelysin 3)	15.2	2.9	20
	CXCL12*	Stromal cell-derived factor 1	9,949	-1.2	17,066

Table 1: Low Density Array analysis of breast cancer cells retrieved following co-culture with MSCs. * selected genes validated in triplicate experiments by RQ-PCR