Article

Conversion of mesenchymal stem cells to cancer-associated fibroblasts in a tumor microenvironment: an *In Vitro* study

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SUMMARY
Carcinomas grow in a complex microenvironment which consists of stromal cells, fibroblasts, immune cells, matrix proteins, and soluble proteins. This microenvironment is critical in providing signals for tumors to proliferate and produce cytokines, which create an area of immune suppression that signals inward migration of mesenchymal stem cells. It also aids in angiogenesis, metastasis and invasion of the tumor, which further enable tumor cells to proliferate and metastasize. Further mesenchymal stem cells are known to migrate into the tumor microenvironment induced cytokines released by the tumor in the microenvironment, further differentiating them to cancer associated fibroblasts (CAF). CAFs are in turn known to promote and aid tumor migration, enabling metastasis. In this study, conditioned media from the MCF7 breast cancer cell line induced a CAF like phenotype in bone marrow mesenchymal stem cells (BMSCs), indicating the potential role of stroma in the progression of cancer. We hypothesize that MCF7-conditioned media induces expression of vimentin and α-smooth muscle actin (αSMA) when co-cultured with (BMSCs), and that this could be involved in aiding cancer metastasis and progression.

INTRODUCTION
Tumor microenvironment includes blood vessels, immune cells, fibroblasts, signaling molecules and the extracellular matrix. The tumor microenvironment is marked by a zone of immune suppression created by tumor cells invaginating across the basement membrane into the interstitial space (1). Invasion of tumor cells into the interstitium enables them to further metastasize and spread in the body. However, this process depends heavily on the stroma of the surrounding area which is comprised of fibroblasts, immune cells, and mesenchymal stem cells. This draws parallels to the "soil and seed theory", where the cancer cells being the "seeds" and the specific organ microenvironments being the "soil" (2, 3). Tumor cells secrete inflammatory factors, including cytokines, which attract bone marrow mesenchymal stem cells (BMSCs) (4). Tumor cells induce significant changes in the BMSCs and convert them to cancer-associated fibroblasts (CAFs) which further aid in tumor progression (5-7). However, the process of conversion to CAFs is not clear yet.

CAFs are present in the peritumoral area and act in an orchestrated manner, resulting in remodeling of adhesion molecules and tissue. This is reminiscent of the wound healing process, but with some exceptions, as cancer is viewed as a wound that never heals. Cancer cells are known to "hitch a ride" on CAFs to spread to other parts of the body, through lymphatics and blood vessels (8). CAFs exist in close proximity to the cancer epithelium and constitute the bulk of a tumor's stroma. They can acquire expression markers associated with metastasis, including vimentin and α-smooth muscle actin (αSMA). Vimentin, a critical prerequisite for metastasis in a number of cancers, is a structural protein that is used to maintain cell integrity and is encoded by the VIM gene (11). In recent studies, vimentin has been shown to regulate the activity between cytoskeletal proteins and cell adhesion molecules by participating in stromal cell adhesion, migration, invasion, and signaling (9). Expression of αSMA allows stromal cells to gain contractile stress fibers for better mobility and stronger capabilities (10), which aid in tumor metastasis.

We hypothesize that cancer cells secrete soluble factors that can induce mesenchymal stem cells to transform into CAFs, which are marked by the expression of αSMA and vimentin. We show that BMSCs cultured in media conditioned by MCF7 cells (a breast cancer cell line) induced expression of vimentin and αSMA. This indicates the presence of soluble factors in MCF7-conditioned medium (MCF7-CM) that convert BMSCs to CAFs. These CAFs in the tumor microenvironment can aid in cancer metastasis and progression.

RESULTS
Increased expression of αSMA and vimentin are some of the characteristic markers of CAF formation. BMSC differentiation was examined during culture in MCF7-CM through semi-quantitative analysis of αSMA and vimentin mRNA expression.
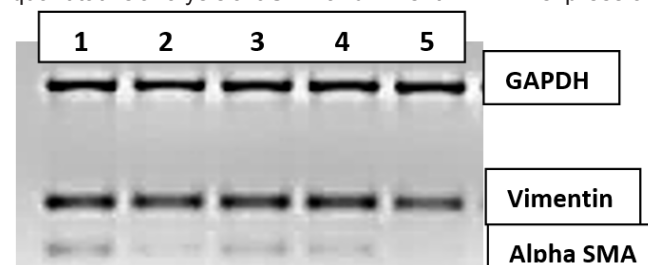

Figure 1. A representative gel electrophoresis picture showing bands for GAPDH (control), vimentin and αSMA. Groups are labeled as 1: BMSC+MCF7-CM, 2: MCF7+BMSC-CM, 3: BMSC+MCF7, 4: MCF7 CONTROL, 5: BMSC control.
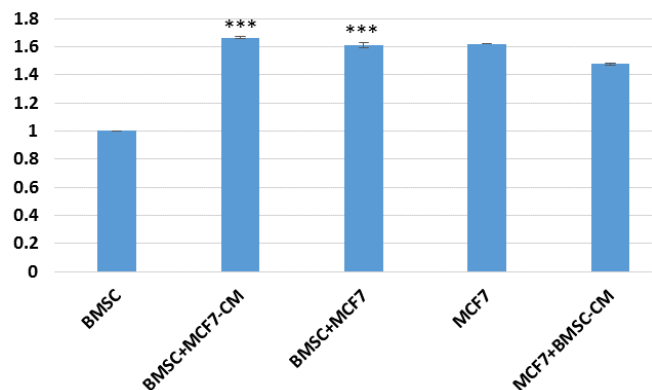
Figure 2. Fold change in mRNA levels of αSMA in the different groups tested. BMSC control, BMSC+MCF7-CM (BMSCs cultured in MCF7-CM), BMSC+MCF7 (MSCs co-cultured with MCF7), MCF7 control, MCF7+BMSC-CM (MCF7 cultured in BMSCs conditioned medium). The data was normalized to BMSC control levels. Statistical signifance was denoted as *** with p-value <0.001. Error bars represent standard error of the mean (n=3).

Figure 3. Fold change in mRNA levels of vimentin in the different groups tested. BMSC control, BMSC+MCF7-CM (BMSCs cultured in MCF7-CM), BMSC+MCF7 (MSCs co-cultured with MCF7), MCF7 control, MCF7+BMSC-CM (MCF7 cultured in BMSCs conditioned medium). Statistical signifance was denoted as *** with p-value <0.001. Error bars represent standard error of the mean (n=3).

Untreated BMSC and MCF7 were considered control groups (BMSC and MCF7, respectively) and were compared with BMSCs co-cultured with MCF7 (BMSC+MCF7), BMSCs cultured in MCF7-CM (BMSC+MCF7-CM), and MCF7 cells cultured in BMSC-conditioned media (MCF7+BMSC-CM). The BMSC control expressed the lowest levels of αSMA and vimentin (**Figures 1-3**). BMSC+MCF7-CM showed the highest expression levels ($p<0.001$) of αSMA and vimentin, indicating that the MCF7-CM induced BMSCs to acquire a CAF-like phenotype. Vimentin levels in both BMSC+MCF7 and BMSC+MCF7-CM were statistically significant ($p<0.001$) whrn compared with BMSCs control group (**Figure 3**). The αSMA expression level was significantly higher ($p<0.001$) in BMSC+MCF7-CM and BMSC+MCF7 when compared with the MCF7 and BMSC controls, whereas there was no significant difference observed between MCF7+BMSC-CM and the controls (**Figure 2**).

DISCUSSION

Tumor progression is a series of orchestrated events that take place around the interstitial space, which is heavily populated by endothelial cells, immune cells, and fibroblasts. A definite step in tumor progression is the invasion of cancer cells through the basement membrane (stroma) into the interstitial tissue. Fibroblasts form a significant proportion of the tumor microenvironment, and play a crucial role in aiding tumor progression. Tumor activity, due to cell-cell interaction or due to soluble factors released by the cancer cells, leads to changes in the extracellular adhesion molecules, which lead to molecular and architectural remodeling of the stroma. The result is the transformation of fibroblasts into CAFs, which are basic components of periglandular sheaths and play a pivotal role in wound healing and chronic inflammation (1).

These CAFs aggregate in the peritumoral area and surround the carcinoma cells which themselves surround the normal cells. In addi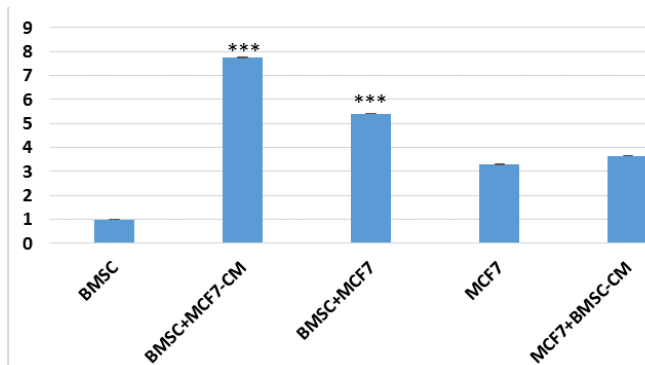tion, progenitor cells like BMSCs are recruited into the area and get converted to CAFs, which comprise the major cell type in a desmoplastic response in a tumor area. This conversion also contributes to angiogenesis, which is a major step towards tumor metastasis (12,13). Another significant phenotypic change in the tumor which indicates definite tumor progression is the endothelial to mesenchymal transition (EMT), induced by TGFβ present in the microenvironment (14).

A remarkable sequence of events mediated by tumor cells results in the EMT leading to extracellular matrix remodeling, which results in a permissive microenvironment which aids in tumor migration and immunosuppression. Transdifferentiation of cancer cells into myoepithelial cells leads to the transformation of the surrounding stroma to a CAF phenotype (15,16). Although myofibroblasts are very crucial in wound healing, they contribute to the chronic inflammation and desmoplasia that leads to chronic fibrosis seen in cancers (17).

Results from this study showed that the predicted mechanism of action is mediated by tumor-derived soluble factors that induce transition of BMSCs to CAFs, which overexpress vimentin and αSMA (18,19). The experiment clearly shows that MCF7 cells conditioned medium are crucial for BMSCs transition to CAFs (5,10) as compared to cell-cell interaction as seen in co-culture (BMSC+MCF7). This could also induce CAFs to produce inflammatory, angiogenic factors including TGFβ and VEGF which could be responsible for an immuno-suppressed tumor microenvironment and also for inducing neoangiogenesis, which is vital for tumor angiogenesis (2,10).

We would like to follow up on our findings by exploring other markers for CAFs, as well measure the levels of TGFβ and VEGF in these groups. This will help in understanding the tumor microeivronment and interaction of stem cells with tumor via different cytokines.

METHODS
Cell lines

The breast cancer cell line MCF7 was obtained from NCCS, Pune and BMSCs were obtained from Lonza. The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen) supplemented with 10% Fetal Bovine Serum (FBS) (Invitrogen). Passage number ranging from 5-7 was used for MCF7 and 2-3 for BMSCs. All the cultures were maintained at 37°C in a 5% CO_2 humidified incubator.

BMSCs were grown on a 3D collagen scaffold that provides optimal factors required for BMSCs and offers the "niche" for its growth. The collagen scaffold also helps in mimicking the 3D environment as in vivo.

Preparation of Conditioned Medium (CM)

MCF7 cells were cultured in DMEM with 10% FBS. At 70-80% confluency, media was replaced with serum-free media, and collected after 18 hours of incubation. The collected medium was centrifuged at 1500 rpm for 10 minutes. Supernatant was aliquoted and stored at -80°C. Conditioned media from BMSCs was prepared and stored in a similar manner. Conditioned media obtained from MCF7 cells and BMSCs were named as MCF7-CM and BMSC-CM, respectively.

Cell treatment with CM

To observe the effect of CM on cells, different groups of cells were cultured in a 3D scaffold (in-house technology, under patent application number: 201841037897) for 48 hours and were harvested for RNA isolation:

1. BMSC control
2. BMSC+ 50%MCF7-CM
3. MCF7+BMSC
4. MCF7 control
5. MCF7+ 50%BMSC-CM

Semi-quantitative Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from the cells of each group using a Qiagen RNA Isolation kit. RNA was quantified and purity was checked at 260 nm and 280 nm using a spectrophotometer. cDNA was prepared using a Genei RT PCR kit and stored at -80°C.

PCR was carried out with 200 ng cDNA for 50 μL PCR reaction. The forward and reverse primers used were as follows: αSMA, 5'-ACTGAGCGTGGCTATTCCTCCGTT-3' and 5'-GCAGTGGCCATCTCATTTTCA-3'; vimentin, 5'-CCAGGCAAAGCAGGAGTC-3' and 5'-CGAAGGTGACGAGCCATT-3'; GAPDH (internal control), 5'-GGTCGGAGTCAACGGATTTGGTCG-3' and 5'-CCTCCGACGCCTGCTTCACCAC-3'. PCR was set up using Jumpstart mix (Sigma), 20 pmol each of forward and reverse primer (Europhins) and the volume was attained using molecular grade water. PCR was carried out for 5 minutes at 95°C (initial denaturation), followed by 30 cycles of 95°C for 1 minute, 61.15°C (αSMA), 57.1°C (Vimentin), 66°C (GAPDH) for 30 seconds, 72°C for 1 minute, and a final extension at 72°C for 10 minutes. PCR products along with 100 bp ladder (Sigma) were run on a 3% agarose gel and were observed under a UV-transilluminator. The target gene expression was analyzed using ImageJ software and was normalized to that of GAPDH. Three independent experiments were performed.

Statistical analysis

Quantitative data is shown as mean ± standard error of the mean (SEM). Analysis of variance (ANOVA) followed by Tukey's test was performed and statistical significance was obtained using GraphPad Prism software. $p < 0.05$ was considered as significant.

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