RESEARCH ARTICLE

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The Effect of Mammary Extracellular Matrix in Controlling Oral and Mammary Cancer Cells

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Abstract

Extracellular matrix (ECM) plays an important role in the normal physiology of tissues and progression to disease. Earlier studies and our external microarray data analysis indicated that mammary matrix from involuting tissue showed upregulation of genes involved in ECM remodeling. The present study examines the fate of mammary and oral cancer cells grown in the ECM from lactating mammary gland. Our findings show that non-tumorigenic cells, MCF10A and DOK cells did not proliferate but the tumorigenic and metastatic cells, SCC25 and MDA-MB-231, underwent apoptosis when grown on mammary ECM isolated from lactating mice. In addition, the cytokinesis marker, CEP55, was repressed in the oral and breast cancer cells. In contrast, these cells proliferated normally on mammary ECM isolated from mice undergoing involution. External microarray data analysis of mammary tissue further revealed over expression (~16 fold) of QSOX1 gene, which promotes cellular quiescence, in lactating mammary gland. A recent study has indicated that QSOX1 overexpression in breast cancer cells led to reduced proliferation and tumorigenic properties. This extracellular protein in mammary ECM may be responsible for reduced cellular proliferation. The present study has shown that ECM from lactating mammary gland can regulate signals to oral and breast cancer cells to halt cell division. This preliminary observation provided insights into the potential role of ECM factors present in lactating mammary gland as therapeutic targets to control cancer cell division. This preliminary study is an attempt to understand not only the requirement of ECM remodeling factors essential for the growth and survival of cancer cells but also the factors present in the lactation matrix that simultaneously halts cell division and selectively inhibits the growth of cancer cells.

Keywords: Cancer- extracellular matrix- lactation- involution

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Introduction

The tumour microenvironment plays an important role in the regulation of cancer cell behavior (Bhowmick et al., 2004; Bissell and Labarge, 2005). Recent research has also revealed that non-cellular components of the microenvironment, the extracellular matrix (ECM), also play an important role in tumour progression (Erler et al., 2009; Levental et al., 2009). ECM promotes integrity, proliferation and maintenance of tissue morphology and also influences the fundamental characteristics of the cell (Hynes, 2009; Lu et al., 2011). Several mechanisms regulate the dynamics of ECM including synthesis, degradation and remodelling required during developmental processes (Page-McCaw et al., 2007) and deregulation of these mechanisms can lead to disorganized ECM with cellular abnormalities resulting in fibrotic and cancer conditions (Cox and Erler, 2011). ECM is also responsible for maintaining the polarity and architecture of epithelial tissues including mammary gland and the loss of polarity due to aberrant ECM dynamics is often associated with the cancer progression (Ghajar and Bissell, 2008; Akhtar et al., 2009).

ECM not only provides strong support to the mammary gland architecture but also acts as a communicating link between the extracellular environment and the epithelial cells (Akhtar et al., 2009). The ECM protein profile is different in various stages of mammary gland development including pregnancy, lactation and involution (Warburton et al., 1982; Schedin et al., 2004). During involution, the majority of the epithelial cells undergo apoptosis followed by ECM remodelling and an irreversible change in the mammary gland to a pre-pregnant stage (Watson, 2006). This ECM remodelling process is due to significant expression of matrix metalloproteinases (MMPs) (Green and Lund, 2005) and the expression profile of MMPs during involution was found to be similar to that of developing mammary gland tumours (Almholt et al., 2007). The activation of inflammatory responsive elements during involution is highly similar to that of the tumour microenvironment and in the wound healing process (van Kempen et al., 2003; Schedin et al., 2007).

Previous studies have indicated that mammary gland proliferation is low during lactation and mammary

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epithelial cells undergo a quiescence stage (Knight and Peaker, 1982; Capuco et al., 2003). A recent study from our lab has indicated that ECM isolated from different phases of lactation provides regulatory signals in-vitro to mammary epithelial cells (MECs) so they behave in a similar way to that observed at the phase of lactation from which the ECM was isolated (Wanyonyi et al., 2013). From the studies stated above, there is clear evidence that lactating glands show less proliferation and as the ECM can govern tumour progression, it would be interesting to observe if factors in lactating ECM inhibits metastatic and tumourigenic potential on breast and oral cancer cells, irrespective of their tumourigenic potential. Hence, the present preliminary study addressed whether extracellular matrix isolated from mammary gland during lactation included factors for inhibiting cell division of breast and oral cancer cells.

Materials and Methods

Animals

BALB/c mice were used and were obtained from Monash Animal Services, Melbourne, Australia. The experiments were approved by Deakin University Animal Ethics Committee. Mammary glands were dissected from the lactating mice (day 15). In addition, lactating mice (day 15) were separated from pups and mammary glands were dissected from mice at day 4 of involution. All tissues were immediately frozen and stored at -80^{o}C till further use.

Cell lines

MCF10A was a generous gift from Dr. Patrick Humbert, PeterMac Cancer Research Institute, Melbourne. Cells were grown as previously described (Bruzzone et al., 2014). Briefly cells were maintained in Dulbecco`s modified Eagle`s medium (DMEM) and Ham's F12 medium containing 10% fetal bovine serum (Life Technologies), 20 ng/ml epidermal growth factor, 2 µg/ml insulin, 0.1 µM hydrocortisone, 100 IU/ml penicillin and 100 µg/ml streptomycin. MDA-MB-231 and SCC25 were obtained from American Type Culture Collection (ATCC). MDA-MB-231 cells were cultured in DMEM media supplemented with 10% fetal bovine serum, 100 IU/ml penicillin and 100 µg/ml streptomycin. SCC25 cells were grown in 1:1 mixture of DMEM and Ham‟s F12 media supplemented with 10% fetal bovine serum, 100 µg/ml streptomycin, 100 IU/ml penicillin and 400 ng/ml hydrocortisone. DOK cells were purchased from the European Collection of Authenticated Cell Cultures (ECACC) and the cells were grown in DMEM media supplemented with 2 mM glutamine, 5 µg/ml hydrocortisone, 10 % fetal bovine serum, 100 IU/penicillin and 100 µg/ml streptomycin. All cells were cultured at 37^{o}C with 5% CO_2 conditions.

External microarray dataset and data analysis

Data analysis was performed using publicly available microarray data from the studies as represented in the Table 1 (SSD 1). Lactating mammary gland (day 9 and day 15) and involuting mammary gland (day 4) data files were obtained from three individual studies as represented in the Table 1. The data considered for the comparative analysis from three individual studies used the same array platform (Affymetrix MG U74 Aver 2). The CEL files (n=11) were normalized using gene spring software (Agilent) by robust multi-array average method (RMA). Normalized intensity values of late lactation data (day 9 and day 10; n=6) was compared to the late involution (day 4; n=5) and the fold change values were obtained after employing Benjamin-Hochberg multiple testing correction. Genes that were differentially regulated were considered with significant P-Value <0.01 and with fold change value > 2.

Extracellular matrix preparation from mammary gland tissue

Extracellular matrix (ECM) was isolated from mammary gland using a homogenization method (O'Brien et al., 2010). Briefly, frozen mammary gland tissue was pulverized into fine powder using a mortar and pestle containing liquid nitrogen. Homogenization of powdered tissue (1 g) was performed in high salt buffer (2 ml) composed of 200 µg/ml phenyl methyl sulfonyl fluoride (PMSF), 2 mM n-ethylmaleimide (NEM) and protease inhibitory cocktail (Sigma). ECM was dialysed sequentially to eliminate urea and protease inhibitors. Ham's F12 and M199 (1:1 ratio) media without serum was used for the final dialysis of ECM and ECM was removed from the dialysis membrane, aliquoted and stored at -20^{o}C until use.

Cell culture on ECM

ECM (40 µL) from lactating and involuting mammary gland were coated on to each well of a 96- well plate which was incubated at 37^{o}C for 1h. Cells (4 x 10^3) were seeded onto the extracellular matrix for further experimental analysis (Figure 1).

Results

Mammary ECM during lactation inhibits cell proliferation

Cells (MCF10A, DOK, SCC25 and MDA-MB-231) grown on the ECM isolated from lactation mammary gland (lactation matrix) and involution mammary gland (involution matrix) were further analysed for proliferation. Cells cultured on plastic for each cell line were also considered as a reference. Analysis of cell proliferation and viability revealed a significant reduction in cell viability at day 1 and 2 of culture in both the cancer cell lines, MDA-MB-231 and SCC25, when grown on lactation matrix (Figure 2). MDA-MB-231 did not show a significant reduction in viable cells on day3 on lactation matrix in comparison to day2 but SCC25 cells showed significant reduction in viable cells at both time points (Figure 2). Cells grown on involution matrix showed no growth inhibition and the growth pattern of the cells was almost equivalent to that of cells grown on plastic (Figure 2). The non-tumorigenic cells, DOK and MCF10A, showed no growth in the lactation matrix and there was no significant cell death observed (Figure 2). Although DOK cells showed no change in the cell growth on lactation matrix during the first 2 days of culture, there was a significant increase in the growth on

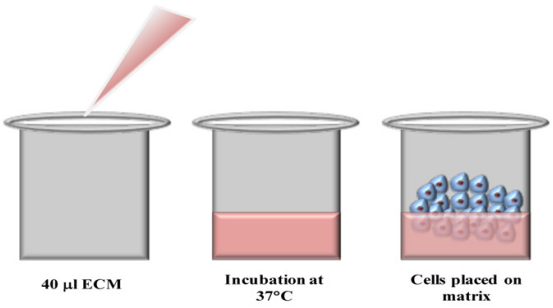

Figure 1. Schematic Representation of Culture of Cells on ECM Extracted from Lactating and Involuting Mammary Gland. Each well of a 96 well plate was coated with 40 µl of ECM and incubated for 1h at 37°C. Cells were seeded on the ECM.

day 3. The cells grown on lactation matrix, involution matrix and plastic for 48 h were subjected to live/dead cell staining using calcein AM (green) and propidium iodide (red). No effective cell death was found in the cells grown on involution matrix and plastic (Figure 3A to D). Non-tumorigenic cells, DOK and MCF10A, did not show significant cell death when grown on lactation matrix (Figure 3A and C). In contrast, there was a considerable effect on the cell viability of tumorigenic MDA-MB-231 and SCC25 cells, grown on lactation matrix (Figure 3B and D).

Morphology of the cells cultured on mammary ECM

The morphology of SCC25 and MDA-MB-231 cells grown on the lactation matrix were round and the cells grown on the ECM isolated from the involution matrix had a flat morphology due to cell attachment to the plastic as revealed by the actin staining (Figure 4). Actin staining also revealed formation of sphere like structures by MDA-MB-231 cells (Figure 4) on the lactation matrix.

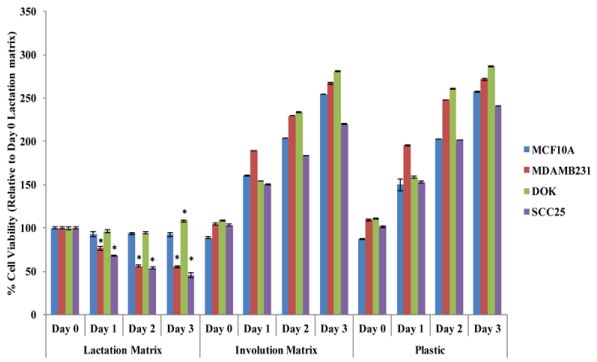

Figure 2. Effect on Proliferation of Cells Grown on Lactation Matrix, Involution Matrix and Plastic. MCF10A, MDA-MB-231, DOK, SCC25 Cells Grown on Lactation Matrix, Involution Matrix and Plastic were Measured for Cell Viability Using MTS Reagent on day 0, day 1, day 2 and day 3 at 490 nm Wavelength. The asterisk (*) indicates significant P-Value < 0.05 reduction in the cell viability of tumorigenic cells, MDA-MB-231 and SCC25 cells (Students-t-test). In this experiment, biological and technical triplicates were considered for the statistical analysis

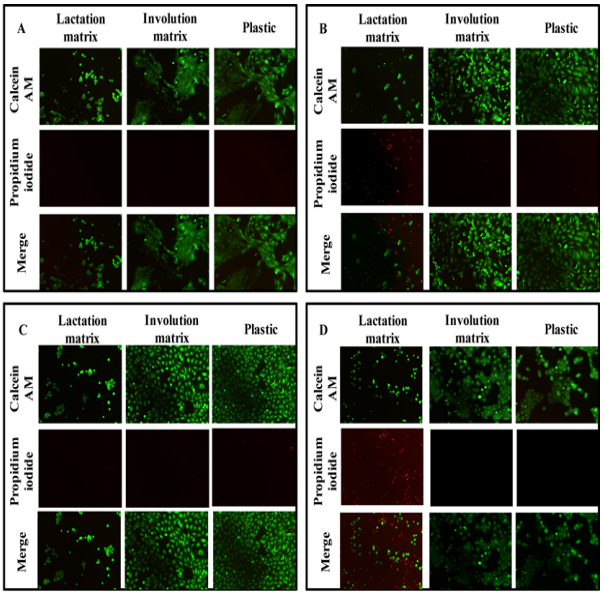

Figure 3. Live/Dead Cell Staining of Cells Grown on Lactation Matrix, Involution Matrix and Plastic Surface for 48h. MCF10A (A), MDA-MB-231 (B), DOK (C) and SCC25 (D) cells were grown for 48 h on lactation matrix, involution matrix and plastic surface. Further cells were stained with calcein AM and propidium iodide stains. Calcein AM stains live cells (green) and propidium iodide stains dead cells (red).

Lactation ECM induces apoptosis in cancer cells

A significant reduction in cell viability was observed in cells grown on lactation matrix from day 1 as shown in the Figure 2. Therefore, the SCC25 and MDA-MB-231 cells grown on lactation and involution matrix were subjected to annexin V staining for the detection of apoptosis. SCC25 and MDA-MB-231 cells grown on involution matrix were stained only with Hoechst dye indicating presence of live cells (Figure 5). Both the cancer cell lines grown

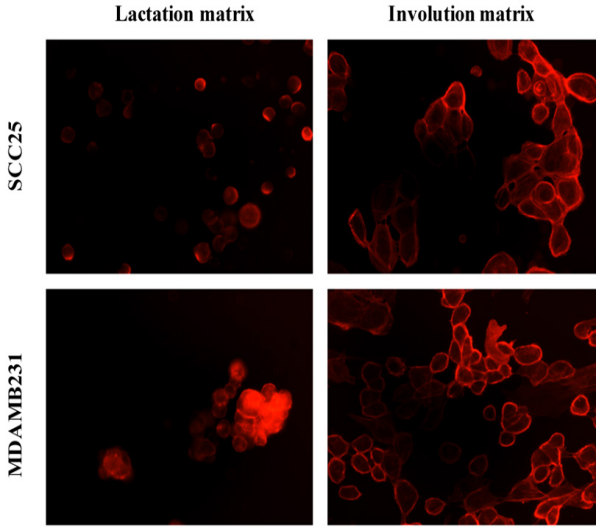

Figure 4. Actin Distribution in the Two MDA-MB-231 and SCC25 Cell Lines by Fluorescence Microscopy. Morphology of the tumorigenic cells, MDA-MB-231 and SCC25 cells, were observed by actin staining using rhodamine-phalloidin reagent (Red) that detected distributed actin filaments in the cell body after growing cells on lactation and involution matrix for 48 h at 37°C.

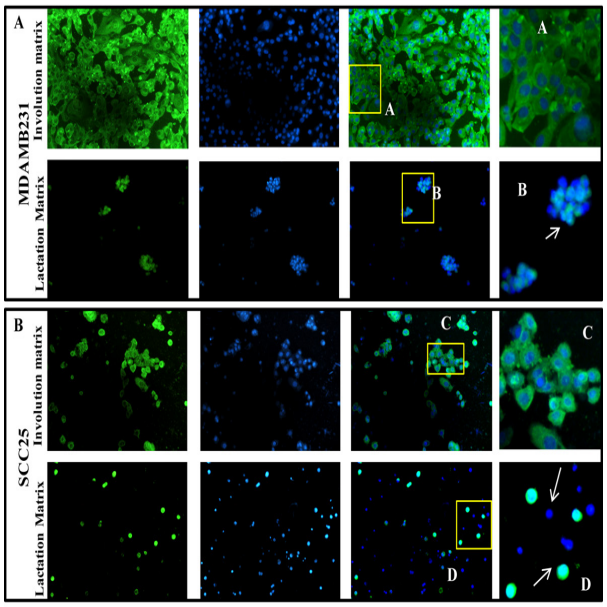

Figure 5. Annexin V Staining of Breast and Oral Cancer Cells Grown on Lactation (LM) and Involution Matrix (IM). SCC25 (oral) and MDA-MB-231 (breast) cancer cells were grown on lactation matrix for 24 h and annexin V staining was performed. Early apoptotic cells were stained with annexin V (green) and nuclei with Hoechst dye (blue). Late apoptotic cells were stained with annexin V (green) and nuclei with propidium iodide (red). Dead cells show no annexin V staining but only nuclei staining with propidium iodide (red). Live cells only show nuclei staining with Hoechst dye (blue).

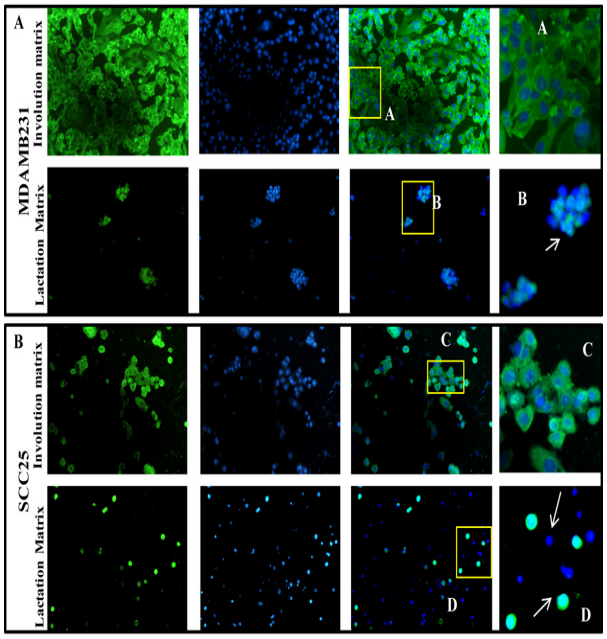

Figure 6. Immunofluorescence Analysis of CEP55 Expression in Cells Grown on Lactation and Involution Matrix. MDA-MB-231 (A) and SCC25 (B) cells were grown on lactation and involution matrix for 48 h and the cells were further analysed for CEP55 expression. Cells were treated with rabbit anti-CEP55 primary antibody and further anti-rabbit alexafluor 488 seccondary antibody was used for detection of CEP55 expression (green). Cells were counterstained with Hoechst dye (blue). Arrow marks indicate cells without CEP55 expression.

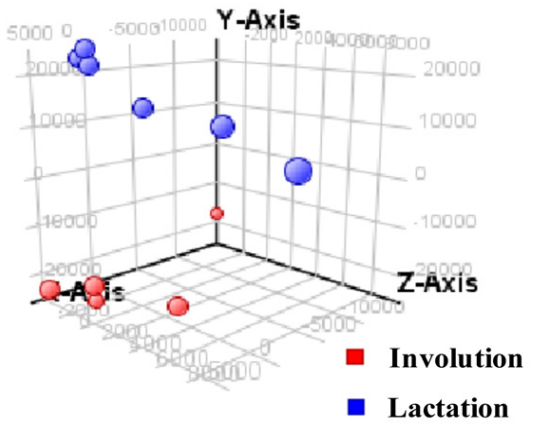

Figure 7. Principal Component Analysis of Involution and Lactation Microarray Datasets. Principal component analysis (PCA) was performed using Gene Spring software on the sample data sets belonging to lactation (blue) and involution data (red) using gene spring software.

on lactation matrix were apoptotic with the presence of early and late apoptotic cells (Figure 5).

Repression of CEP55 expression in the cells grown on lactation ECM

Mammary epithelial cells during lactation showed low proliferation and cellular quiescence was observed (Capuco et al., 2003) which may result from ECM regulation. CEP55 protein is often expressed during cytokinesis (van der Horst et al., 2009). SCC25 cells and MDA-MB-231 cells grown on lactation and involution matrix for 48 h were analysed for CEP55 expression using immunofluorescence. Results indicated all cells of both tumorigenic cell lines grown on involution matrix expressed CEP55 but only a few cells grown on lactation matrix showed CEP55 expression (Figure 6A and B).

Microarray data analysis

External datasets were analysed for the comparison of gene expression in late lactation and late involution stages of mammary gland in mice. Principal component analysis through Gene spring analysis showed a clear distinction between the two sample types (lactation and involution) considered for comparison (Figure 7).

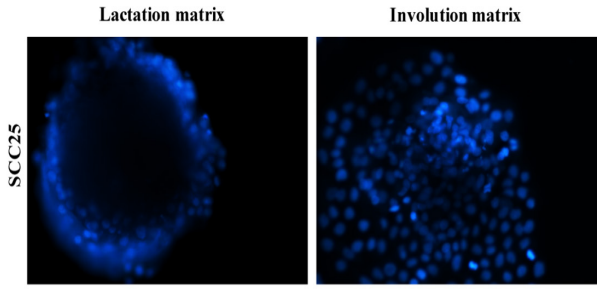

Figure 8. Growth of SCC25 in-vitro Tumour Spheres in Lactation and Involution Matrices. SCC25 cells were enriched for formation of spheres on agarose. Spheres were then placed on lactation and involution matrices and were cultured for 72 h. Spheres were stained with Hoechst dye (blue).

Further analysis using Gene Spring software revealed significant differentially regulated genes (1,000) with significant P-Value less than 0.01 and fold change greater than 2 (Supplementary file 2 (SSD 2)). In addition, the gene profile was analysed for the expression of genes involved in ECM remodelling and genes promoting cellular quiescence using the Database for Annotation, Visualization and Integrated Discovery (DAVID 6.7) program (Dennis et al., 2003), details of which are incorporated in the supplementary file 3 (SSD 3). The data analysis that was performed includes whole tissue of late lactating and late involuting mammary gland and hence the present study focuses on the secretory proteins that might have been secreted in the ECM. Most of the genes related to ECM remodelling were up regulated in involuting mammary gland (Table 2 in SSD 1). Further analysis of the genes indicated important inflammatory response-promoting genes that were up regulated in involuting mammary gland compared to the lactating mammary gland (Table 3 in SSD 1). Analysis of the genes among the significant differentially regulated genes promoting cellular quiescence revealed over expression of the QSOX1 gene. This gene is related to cells undergoing cellular quiescence (Coppock et al., 2000) and was up regulated in lactating mammary gland (Table 4 in SSD 1). The gene for beta actin (ACTB) expression was found to be down regulated and epidermal growth factor (EGF) was up regulated in lactating mammary gland in comparison to the involuting mammary gland (Table 4 in SSD 1).

Growth of in-vitro tumour spheres on the lactation and involution matrices

Spheres were produced from SCC25 cells as described in the supplementary file SSD 1. Spheres grown on the ECM from lactating mammary gland did not show attachment to the plastic whereas the spheres grown on the involution matrix attached to the plastic (Figure 8).

Discussion

Extracellular matrix (ECM) is the major part of the epithelial cell microenvironment and plays a major role in controlling the cellular behavior, adhesion, proliferation, differentiation, migration and apoptosis (Hynes, 2009). Changes in ECM composition lead to modification of ECM dynamics thereby resulting in the altered tissue architecture (Lopez et al., 2008; Engler et al., 2009). Mammary gland is emerging as a powerful model to investigate the mechanoinduction of the signals induced by ECM that can lead to the changes in cellular behavior further affecting the tissue. Previous studies indicated that cell proliferation in the mammary gland is low during lactation and most of the mammary epithelial cells are quiescent (Knight and Peaker, 1982; Capuco et al., 2003). An earlier study indicated that mammary epithelial cells (MECs) grown on the mammary ECM of a specific phase of lactation behave and respond like cells belonging to the phase of ECM irrespective of the phase of lactation from which the MECs were isolated (Wanyonyi et al., 2013). This is consistent with the conclusion that ECM plays a major role in promoting cellular quiescence during lactation. This preliminary study has explored the effects of lactation matrix on the growth of non-tumorigenic and tumorigenic oral and breast cancer cells.

Lactation matrix promotes cellular quiescence in all cell types and induces apoptosis in tumour cells

In the current study, tumorigenic cells (SCC25 and MDA-MB-231) and non-tumorigenic cells (MCF10A and DOK) demonstrated considerable proliferation when cultured on involution matrix, most likely due to the cells being able to penetrate the matrix and reach the plastic underneath. In contrast, there was no growth observed when the cells were cultured on lactation matrix which did not allow the cells to penetrate, consistent with previous findings that lactation matrix promotes cellular quiescence (Capuco et al., 2003). It was observed that some of the tumorigenic cells showed cell death when cultured on lactation matrix. The presence of factors in the mammary ECM during lactation may promote cell death only in tumorigenic cells and/or lack of the factors essential for the tumorigenic cell survival may induce apoptosis in these cells (Elmore, 2007). Actin staining revealed cells cultured on lactation matrix showed a rounded shape and were unable to penetrate the matrix and attach to the plastic surface whereas the cells cultured on the involution matrix were able to penetrate and attach to the plastic. In addition, enriched SCC25 tumour spheres did not attach to the plastic when cultured on lactation matrix but did attach to plastic following culture on involution matrix. Analysis of microarray data revealed over expression of epidermal growth factor (EGF) gene in lactating mammary gland compared to involuting mammary gland. An earlier study revealed EGF induced detachment of cells from the extracellular matrix (ECM) and induced dephosphorylation of focal adhesion kinase (FAK) reducing the cell attachment and motility (Lu et al., 2001). The mammary matrix from lactating gland has retained EGF that has been sequestered to ECM to reduce the cell attachment. Tissue inhibitor of metalloproteinases (TIMPs) inhibit the activity of MMPs (Bourboulia and Stetler-Stevenson, 2010) and the current microarray analysis did not show any significant over expression of TIMPs in lactating mammary gland suggesting that there might be other factors in the mammary ECM that block the cells to penetrate and attach to the plastic surface.

Reduction of cytokinesis marker expression, CEP55, in tumour cells grown on lactation matrix

Centrosomal protein 55kDa, CEP55, is a cell division marker (van der Horst et al., 2009) and is often overexpressed in several types of cancers including oral and breast cancers (Inoda et al., 2009; Waseem et al., 2010). When grown on involution matrix, SCC25 and MDA-MB-231 cells expressed CEP55 protein but on lactation matrix only few cells expressed CEP55 which is consistent with the fact that cancer cells require survival signals for their proliferation (Elmore, 2007). Earlier study showed that breast cancer cells mixed with mammary involuting ECM further increases tumorigenicity (McDaniel et al., 2006) and it would also be interesting to perform these tumorigenic experiments by mixing the

tumour cells with mammary ECM from lactating tissue. This will clearly suggest whether the mammary ECM during lactation has any capacity to inhibit the growth of the cancer cells in- vivo.

Genes promoting ECM remodeling and inflammatory genes in involution

In the current microarray data analysis, over expression of genes related to chemokines and several inflammatory response mediators and over expression of genes related to ECM remodeling (matrix metalloproteinases), cysteine proteinases, ECM components, cell surface receptors were observed in involuting mammary gland. The present analysis correlates with previous studies indicating involution matrix undergoes ECM remodeling and induces an inflammatory response which is similar to the cancer microenvironment (van Kempen et al., 2003; Green and Lund, 2005; Almholt et al., 2007; Schedin et al., 2007). Lactation matrix did not allow the cells to penetrate and it has further promoted cell death in the tumorigenic cells. However, an important question remaining to be addressed is what limits the cells from penetrating the matrix. In contrast, involution matrix might lack these factors and hence the cells can penetrate and attach to the plastic surface. A comparison of these 2 matrices will potentially provide this information.

Cell quiescence promoting gene, QSOX1, may play a prominent role in controlling the cell proliferation

Cellular quiescence is one of the prominent features of the mammary epithelial cells during lactation (Capuco et al., 2003). Earlier studies indicated that laminin-1 promoted cellular quiescence (Spencer et al., 2011) but it was also observed in other studies that there was no change in the expression of the laminin during several stages of mammary gland development (Woodward et al., 2001; Schedin et al., 2004). Differential expression of the laminin-1 gene was not observed in lactating mammary gland in comparison to the involuting mammary gland which is consistent with the earlier studies (Woodward et al., 2001; Schedin et al., 2004). Further the current microarray analysis showed beta actin (ACTB) levels in lactation mammary gland were reduced in comparison with the involution mammary gland. An earlier study indicated that during cellular quiescence, promoted by laminin 1 molecule, depletion of nuclear beta actin levels were also observed (Spencer et al., 2011). Studies have shown nuclear actin is essential for transcriptional activity and chromatin arrangement during cell division (Lenart et al., 2005). Microarray data analysis also revealed significant up regulation of QSOX1 gene in lactation mammary gland (in comparison to involution mammary gland). QSOX1, an extracellular protein, plays an important role in the promotion of cellular quiescence and was inversely correlated with the aggressiveness of the breast tumours (Pernodet et al., 2012). Recent studies suggested that QSOX1 is essential for the incorporation of laminin into the extracellular matrix and blocking QSOX1 leads to reduced cellular adhesion and migration (Ilani et al., 2013). From these observations it is clear that over expression of QSOX1 might play a role in recruiting laminin into the ECM of lactating mammary gland for promoting cellular quiescence. To our knowledge, the complete profile of ECM composition at different stages of mammary gland development (pre-pregnancy, pregnancy, lactation and involution) has not been determined. However, in the current study the question remains as to why the cells were unable to divide on matrix from lactating mammary gland. The plausible explanation might be attributed to the local factors in the mammary gland (Sinowatz et al., 2000). Further studies to identify the ECM proteins might be beneficial to better understand role of these proteins and their function.

Collectively, the current study has provided basic insights into the importance of lactation matrix to reduce proliferation of oral and breast cancer cells. However, a comprehensive identification of the ECM components and their potential signaling mechanisms may provide strategies for developing novel therapeutic interventions for cancer treatment. Unravelling the factors in the lactating mammary matrix that induces cell death only in the tumorigenic cells might provide new strategies in tissue engineering for cancer patients that have undergone surgical tissue excision.

Conflict of interest

No conflict of interest.

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