ELUCIDATE THE MECHANISM OF PLATELET ACTIVATING FACTOR INDUCED TRANSFORMATION IN BREAST EPITHELIAL CELLS.

ASHIQ K A
Reg. No. 20111016

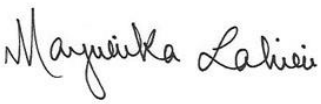
A thesis submitted in partial fulfillment of the requirements for the BS-MS dual degree programme in IISER Pune

Research Advisor:

Dr. Mayurika Lahiri, Associate Professor
Biology Division, IISER Pune

Certificate

This is to certify that this dissertation entitled ‘Elucidate the mechanism of platelet activating factor induced transformation in breast epithelial cells’ towards the partial fulfillment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research (IISER), Pune represents original research carried out by Ashiq KA at IISER Pune under the supervision of Dr. Mayurika Lahiri, Associate Professor, Biology Division, IISER Pune during the academic year 2015-2016.



Dr. Mayurika Lahiri

Associate Professor

Biology Department

IISER Pune

Declaration

I hereby declare that the matter embodied in the thesis entitled ‘Elucidate the mechanism of platelet activating factor induced transformation in breast epithelial cells’ are the results of the investigations carried out by me at the Biology Division, IISER Pune under the supervision of Dr. Mayurika Lahiri, Associate Professor, Biology Division, IISER Pune and the same has not been submitted elsewhere for any other degree.

ASHIQ K A

20111016

BS-MS Student

IISER Pune

Abstract

Microenvironment and tumour infiltrates play a crucial role in the different stages of development of cancer. Studies revealed that breast cancer cells upon stimulation with growth factors secrete phospholipid mediators such as lysophosphatidic acid, prostaglandins and platelet activating factor (PAF). PAF has been shown to play a role in platelet aggregation, inflammatory response, stimulation of neutrophil and macrophages, in allergic response and also in cancer development such as ovarian cancer, esophageal squamous cell carcinoma and breast cancer. It was also shown that cigarette smoke can inhibit platelet-activating factor acetylhydrolase in breast cancer cells and result in increased motility due to accumulation of PAF. Recent studies in lab demonstrated that PAF has the ability to enhance the migratory potential of breast cancer cells through JNK and/or PI3K pathway apart from this,it was also demonstrated that PAF treatment on MCF10A- an immortalized non tumorigenic near normal breast epithelial cell grown as 3D ‘on top’ cultures lead to morphological changes such as partially filled lumen, increased acinar volume and formation of protrusions; suggesting a potential role of PAF in transformation. The present study investigated the role of PAF induced transformation in the breast epithelial cells and its underlying mechanism. The result showed that PAF treatment on 3D ‘on top’ cultures leads to apical and basal polarity disruption and diffused cell-cell junction resembling epithelial to mesenchymal transition further confirmed using western blotting for EMT markers.Studies also confirmed over production and secretion of matrix metalloproteinases (MMP) such as MMP9 and MMP2 upon PAF treatment and also enhanced cell proliferation.

Table of Contents

List of Figures

List of Tables

Acknowledgements

My project here would not be possible without the help and support of many people.

I would start by thanking Mayurika Lahiri for providing me with an opportunity to carry out this project in her lab. Her constant support, encouragement and patience has made this project possible. Today I cannot imagine myself working in any other lab.

I would also like to thank Richa Rikhy for her valuable remarks and suggestions.

I thank Libi Anandi V for mentoring and guiding me through the project. Her guidance and help has made this project a reality. She did a lot of standardization in 3D which helped a lot in this project. She did a lot of things to inspire me about this project and push me to do lot of work. Another important person who helped me with my project is Vaishali. Both of them helped me a lot with imaging and western blotting and discussing about the project. I would also like to thank Satish who made the lab life interesting and giving suggestions about the work. I also thank my colleagues from and outside ML lab for making life at IISER so beautiful.

I would also like to give my gratitude to Vijay and IISER microscopy department without which half of the data cannot be generated. I would also like to thank all the biology department people for their support during the entire carrier. I thank all the other Lahiri lab members namely Marina Victor, Rintu Umesh, Nitheesh K, Satish, Vaishali Chakraborty, Faseela, Kezia, Vishaka, Tanmoya, Amruta and Aishwarya for their generous help and support during the work

Finally I would like to thank my parents for their encouragement and support during the five years of life at IISER Pune.

Introduction

According to World Health Organisation (WHO) Cancer Country Profile 2014-India, approximately 0.7 million deaths were reported due to cancer (Figure1). Cancer is abnormal growth due to uncontrolled proliferation of cells. Some cancer even shows the potential to metastasize that is to spread to different part of the body. Deletion, duplication and mutation lead to gain of function of oncogenes and loss of function of tumour suppressor genes. Accumulation of such changes leads to development of cancer.

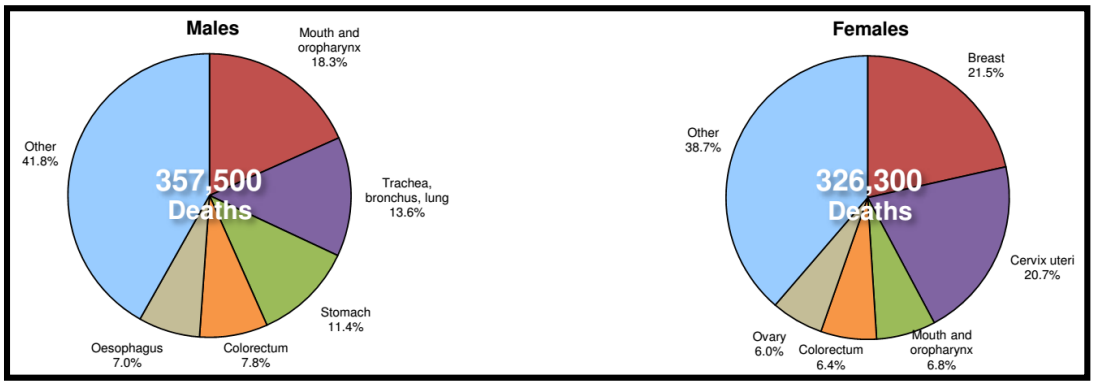


Figure 1 Pie Chart showing the cancer mortality profile of India (WHO Cancer country profile 2014-India)

Breast Cancer:

Data shown in Figure 1 suggest that cancer deaths in women is mainly because of breast cancer and cervix uteri cancer. Studies showed that genes such as BRCA1, BRCA2 and p53 down regulation can increase the risk of breast cancer in females (Evans et al., 2008; Welcsh and King, 2001). Other than genes, life expectancy, obesity, hormone replacement therapy, alcohol consumption, and radiation exposure can increase the risk of breast cancer. Prostaglandins (PG), lysophosphatidic acid (LPA) and platelet activating factor (PAF) are a few phospholipidmediators secreted by breast cancer cells which have been shown to play important roles in cancer development (Prescott et al., 2000; Zhu et al., 2006). Several studies have been conducted to identify the underlying mechanism of cancer development using different model systems. In the recent past, breast epithelial 3D “on top” culture model has become a powerful tool to investigate the molecular mechanism of morphogenesis and cancer development (Hebner et al., 2008).

Breast epithelial 3D “on top” culture Model:

Acini is the smallest functional unit of a mammary gland (Vidi et al., 2013). It is made up of mainly two types of cells- luminal cells and myo-epithelial cells. Luminal cells surround the lumen and helps in milk secretion. Hence they are also known as secretory cells (Vidi et al., 2013). The other layer of cells called myo-epithelium cells surrounds the luminal cells and helps in the constriction of lumen to secrete milk into the ducts (Debnath and Brugge, 2005; Vidi et al., 2013). These myo-epithelial cells also help in signalling between extracellular matrix and luminal cells.(Debnath and Brugge, 2005; Vidi et al., 2013). These acinar structures have various features such as hollow lumen with single layer of polarized epithelial cells, cell-cell junction and cell-matrix contacts (hemidesmosomes) (Debnath and Brugge, 2005; Hebner et al., 2008; Vidi et al., 2013). The maintenance of these features are critical for the proper functioning of the acini (Hebner et al., 2008). Any alterations or modifications to these features results in functional abnormalities (Hebner et al., 2008). Studies showed that during cancer development most of these features are disrupted (Hebner et al., 2008; Vidi et al., 2013). Even small modifications to a gene or genes can disturb these phenotypes.

MCF10A, an immortalized non tumorigenic breast epithelial origin cells grown on laminin III, collagen IV and entactin containing extra cellular matrix (Matrigel®) can recapitulate these *in vivo* acinar structures *in vitro* (Figure2) (Vidi et al., 2013). This 3D “on top” culture models are widely used to study the mechanism of morphogenesis.

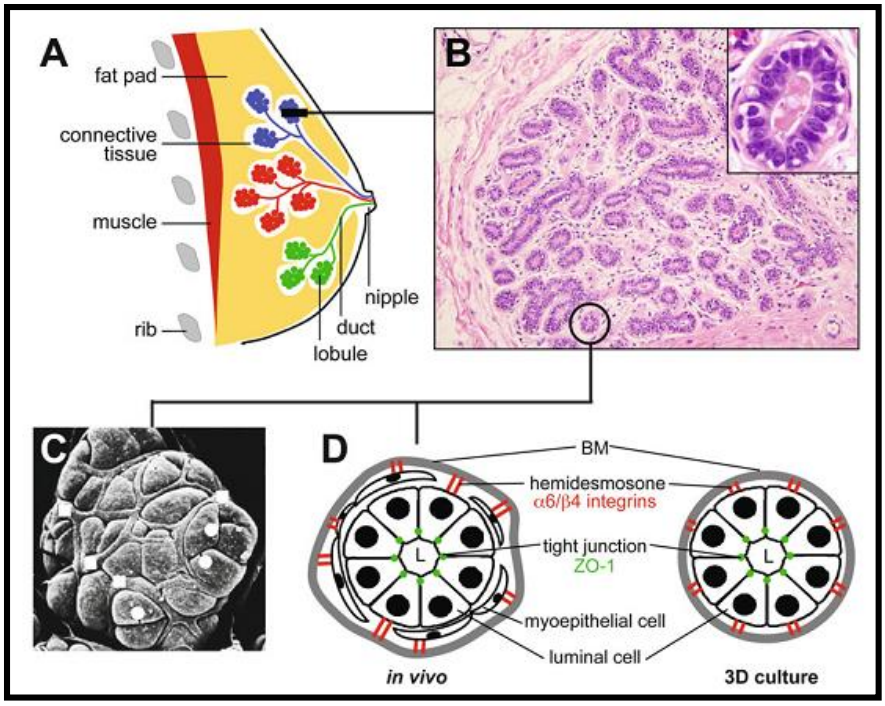


Figure 2: Anatomy of mammary gland.(A) Schematic representation of mammary gland. (B) Cross sectional area of breast lobule stained with hematoxylin and eosin. (C) Scanning electron micrograph from an acinus (D) Schematic representation cross section of 3D culture and *in vivo* acini reproduced from (Vidi et al., 2013).

When MCF10A cells are grown on Matrigel® they proliferate to form spheroids (Hebner et al., 2008; Vidi et al., 2013). By day 3, cells in the spheroid differentiate into an outer layer of polarized epithelial cells and inner cell mass (Figure 3) (Debnath and Brugge, 2005; Hebner et al., 2008; Vidi et al., 2013). These polarized cells then start to secrete their own basement membrane. The cells which lose their contact with the extracellular matrix (ECM) undergo apotosis that leads to the formation of a hollow lumen. By day 16, growth arrested acinar structures are formed (Debnath and Brugge, 2005; Vidi et al., 2013).

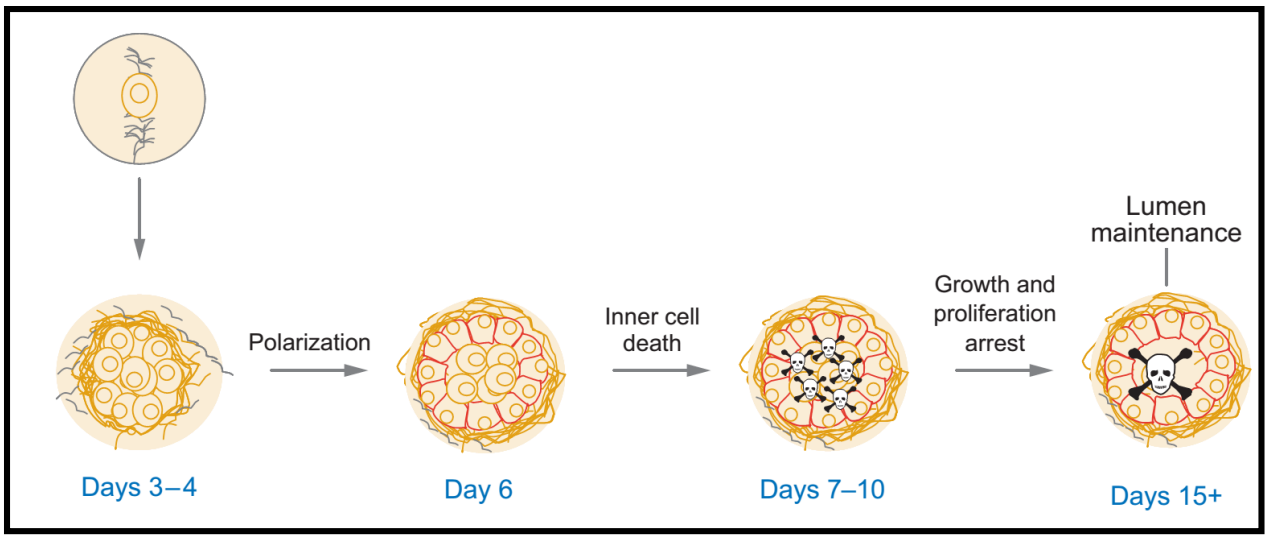


Figure 3: Schematics showing the morphogenesis of MCF10A acini in Matrigels® · Reproduced from(Hebner et al., 2008).

When malignant cells are grown on Matrigel®, they tend to form filled or partially filled lumen, multiple lumens, multi acinar structures (Figure4B-D) (Debnath and Brugge, 2005). They can also form protrusion like phenotype that may indicate epithelial to mesenchymal transition (Figure 4E) (Debnath and Brugge, 2005).

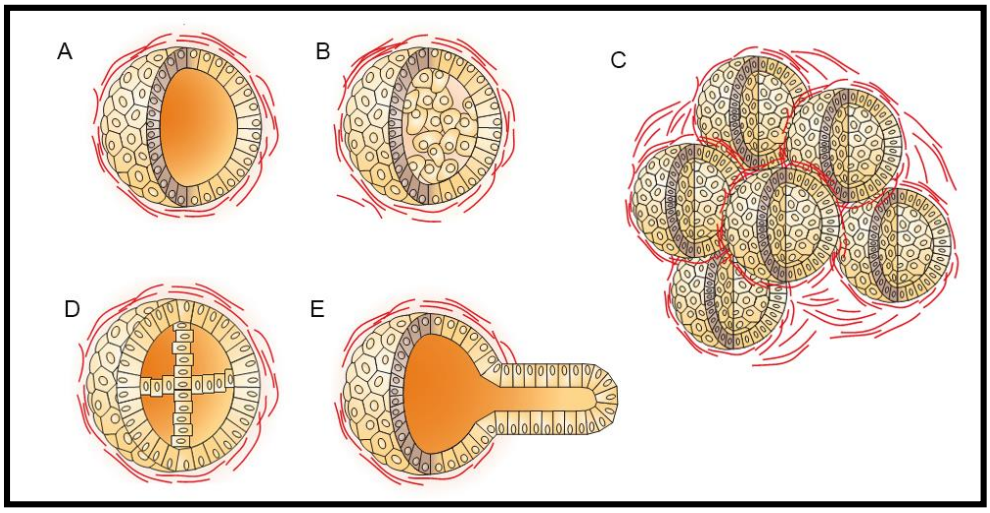


Figure 4: Phenotypes shown by non-malignant and malignant breast epithelial cells grown on 3D "on top" culture model.

Where non-malignant cells show (A) hollow lumen and malignant cells shows (B) filled lumen (C) Multi-acinar structure (D) multiple lumen and (E) tubule formation. Adapted from Debnath, J. and J.S. Brugge,Nat Rev Cancer, 2005

Role of Platelet activating Factor:

Tumour infiltrate secreted by macrophages, cancer associated fibroblasts and cancer cells plays an important role in development of cancer such as initiation and progression (Ramos-Nino, 2013; Sounni and Noel, 2013). PAF- a phospholipid mediator, which was shown to play critical roles in platelet aggregation, inflammatory response, stimulation of neutrophil and macrophages, in allergic response and cancer development (Chao and Olson, 1993). Studies also revealed that PAF plays a role in BRCA1 mutant ovarian cell transformation (Kim et al., 2012). PAF treatment on rat embryonic cells leads to anchorage independent growth and survival in low serum media suggesting its role in malignant transformation (Bennett et al., 1993). PAF acetylhydrolase hydrolase the sn-2 acetyl residue and helps in maintaining the levels of PAF in tissues.

PAF receptor is a G protein coupled cell surface receptor mediates PAF signalling (Bussolati et al., 2000). PAF receptor overexpression was correlated with the tumorigenic potential of the breast cancer cells (Anandi et al., 2015). PAF receptor overexpression in nude mice induce tumorigenesis (Robert and Hunt, 2001). Inhibiting PAF in breast cancer cells demonstrated tumour growth inhibition by inhibiting angiogenesis (Bussolati et al., 2000; Melnikova and Bar-Eli, 2007). Treatment with PAF receptor antagonist showed inhibition of growth and development in breast cancer cells and also showed inhibition of xenograft formation in nude mice (Robert and Hunt, 2001). Studies revealed that breast cancer cell lines such as MDA MB 231 and MCF7 secretes PAF upon treatment with growth factors (Bussolati et al., 2000).

PAF was shown to induce pulmonary metastasis of B16F10 through PTEN/MAPK pathways (Kim et al., 2012). PAF receptor activation was shown to activate PI3K/AKT pathway leading to up-regulation of oncogenisc proteins such as c-myc, survivin, MMP2, MMP9 and VEGF in esophageal squamous cell carcinoma (Chen et al., 2015). They also showed that activated PAF receptor signals to NF-κB to up-regulate the PAF receptor level in cell (Chen et al., 2015).

Earlier studies in the laboratory reported PAF to increase motility in MDA MB 231 cells and also continues PAF treatment on MCF10A 3D “on top” cultures can cause formation of abnormal phenotypes such as protrusions, increased cell number,

increased volume of acini and cells in lumen (Anandi et al., 2015). This suggests a potential role of PAF in inducing transformation. My study involved identifying the underlying mechanism of PAF induced transformation on MCF10A using 3D “on top: cultures.

Aim:

To study the role of PAF in transformation of breast epithelial cells and elucidate the underlying mechanism.

Objective

- To study the role of PAF in the transformation of breast epithelial cells.
    - Investigate the phenotypic changes upon PAF treatment.
        1. Apico-basal polarity markers
        2. Cell- cell junction markers
        3. Invasion and migration
        4. Epithelial to mesenchymal transition (EMT)
- To elucidate the pathway by which PAF induces transformation.

Materials and Methods

Cell Culture:

MCF10A cells obtained as a generous gift from Prof. Raymond C. Stevens (The Scripps Research Institute, California) were cultured in growth medium containing high glucose DMEM without sodium pyruvate (Lonza) with 5% horse serum (Invitrogen), 10 μg/ml insulin (Sigma-Aldrich), 0.5 μg/ml hydrocortisone (Sigma-Aldrich), 100 ng/ml cholera toxin (Sigma-Aldrich), 20 ng/ml EGF (Sigma-Aldrich) and 100 units/ml penicillin-streptomycin (Lonza) as 2D monolayer culture in 100mm tissue culture treated dishes (Corning and Eppendoff). The cultures were maintained in humidified 5% CO_2 incubators at 37ºC. During sub culturing the trypsinised cells were re-suspended in high glucose DMEM medium without sodium pyruvate containing 20% Horse serum and 100 units/ml penicillin-streptomycin.

3D on top culture:

Culture dishes were coated with Matrigel® and were allowed to polymerise at 37ºC for 15- 30 minutes. The amount of Matrigel® used for each culture dishes is tabulated in Table1. MCF10A cells suspended in DMEM containing high glucose without sodium pyruvate with 2% horse serum, 10 μg/ml insulin, 0.5 μg/ml hydrocortisone, 100 ng/ml cholera toxin and 100 units/ml penicillin-streptomycin (Assay Medium) were seeded on to these pre-coated dishes. The cell density used for each dish is mentioned in Table1. Then these cultures were maintained for 20 days at 37ºC. The cultures were replenished with fresh assay medium with 2% Matrigel® and 5 ng/ml EGF every 4th day. 200 nM PAF (ProLabs [Cayman chemicals]) was added to medium after 4 hours of seeding and every 4 days. That is PAF treatment was given on day 0, 4, 8, 12 and 16. The dose regiment was decided from the previous study conducted in PAF. For all the experiments done, we used carbamyl PAF which cannot be catabolised by PAF acetylhydrolyse to maintain continuous activation of PAF receptor.

Table 1: Details about cell density, amount of Matrigel® and assay media used in different dishes.

Dish	Volume of Matrigel®/Well	Cell density/Well	Amount of medium/Well
8 Well chamber cover glass (Lab-Tek, Thermo fisher	50 μl	6x10^3 cells	400 μl

scientific)			
12 well Plate (Corning)	125 μl	1.25x10^5 cells	1 ml
6 well Plate (Corning and Eppendoff)	250 μl	2.5x10^5 cells	2 ml

Immunofluorescence For 3D 'on top' cultures.

Medium from the 8 well chamber cover glass was removed and was fixed with freshly prepared 4% paraformaldehyde with 0.06% gluteraldehyde for 20 minutes at room temperature in dark. While staining for apically or laterally localised markers cells were incubated with PBS-EDTA (PBS containing 5 mM EDTA, 1 mM sodium orthovanadate, 1.5 mM sodium fluoride and 1X protease inhibitor cocktail) at 4^{o}C for 15 minutes to dissolve the Matrigel® layer. This helps in better penetration of antibodies. After fixation the wells were washed twice with PBS for 10 minutes at room temperature. Then the cells were incubated in ice cold 0.5% Triton-X-100 in PBS for 10 minutes at 4^{o}C to permeabilise the acini. One PBS-Glycine wash and two PBS washes of 10 minutes each were given at room temperature. The culture then was blocked with primary blocking solution containing 10% (v/v) goat serum in IF buffer (1X PBS containing 0.05% (w/v) sodium azide, 0.1% (w/v) BSA, 0.2% (v/v) Triton-X-100 and 0.05% Tween 20) for 1 hour at room temperature. Then the culture was again blocked using secondary blocking solution, which is 1% F(ab')2 fragment goat anti-mouse IgG in primary blocking solution for 1 hour. Cultures were then incubated overnight (approximately 16 hours) at 4^{o}C with primary antibodies (Table 2) prepared in primary blocking solution (1:100 dilution). The wells were then washed thrice with IF buffer for 10 minutes at room temperature to remove excess stain and was incubated with respective Alexa Flour® secondary antibody (Table2) in primary blocking solution (1:200 dilution) for 1 hour at room temperature. For counter staining actin, Phalloidin 568 or 633 (Invitrogen) was used along with the secondary at 1:100 dilution. The culture were again washed once with IF buffer and twice with PBS for 10 mins. Then cultures were counter stained with 0.5 μg/ml Hoechst 33342 (Molecular Probes) for 5- 7 mins and was washed twice with 1x PBS for 10 minutes at room temperature. Slow fade gold anti-fade reagent (Molecular probes) was used to mount the slides and was kept inside 4^{o}C overnight. Images of the cultures were captured under 63X oil immersion objective or 40X oil immersion objective of LSM 710 laser scanning confocal microscope (Carl Zeiss, GmBh).

Table 2: Details about Primary and secondary antibodies used for immunofluorescence

Primary Antibodies	Secondary Antibodies (Invitrogen)
α6 Integrin, anti-Rat (Millipore)	Alexa Fluor® 568goat anti-rat IgG (H+L)
Vimentin, anti-Mouse (Abcam)	Alexa Fluor® 488goat anti-rat IgG (H+L)
E-Cadherin, anti-Mouse (Abcam)	Alexa Fluor® 568goat anti-mouse IgG (H+L)
β-Catennin, anti-Rabbit (Abcam)	Alexa Fluor® 488goat anti-mouse IgG (H+L)
GM130, anti-Mouse (BD)	Alexa Fluor® 568goat anti-rabbit IgG (H+L)
Ki67, anti-Rabbit (Abcam)	Alexa Fluor® 488goat anti-rabbit IgG (H+L)
Laminin V, anti-Mouse (Abcam)	

Immunoblotting:

The culture was washed with PBS and incubated at 4^{o}C with PBS-EDTA for 20-30 minutes. Then the cells were centrifuged at 4^{o}C for 5 minutes at 900 rpm. 2X sample buffer (Tris with SDS, bromophenol blue, dithiotreitol, and glycerol, pH 6.8) was added to lyse the cells and the lysate was stored at -40^{o}C. These lysates were then run on a SDS-PAGE at 120V at room temperature in a SE 260 mini-vertical gel electrophoresis unit (GE Healthcare)to separate out our protein of interest. The percentage of gel is determined by the weight of the protein. The proteins were then transferred to Immobilon-P polyvinyldifluoride (PVDF) membranes (Millipore) in TE22 mighty small transfer unit (GE Healthcare) for 3 hours at 250 mA or 16 hours at 125 mA at 4^{o}C. The blots were then blocked using 5% v/v skimmed milk (SACO Foods, US) or in blockace solution (4% Block Ace (AbD Serotech) w/v and 0.2% sodium azide v/v) in TBS-T (25 mM Tris (pH 7.6), 150 mM NaCl, 2 mM KCl and 0.1% Tween 20) for 1 hour at room temperature. Then the blots were incubated in primary antibody prepared in 1:10 diluted blockace solution for 3 hours at room temperature or 16 hours at 4^{o}C. In case of GAPDH probing primary was kept for 1 hour. After giving three 10 minutes TBS-T wash on shakers the blots were incubated in HRP-conjugated secondary antibody at a dilution of 1:10,000 in TBS-T for 1 hour at room temperature. Blots were then washed thrice with TBS-T (each of 10 minutes) and were developed using Immobilon Western reagent (Millipore). Images were captured using ImageQuant LAS4000 gel documentation system (GE Healthcare).

Table 3: Details about primary antibodies and their dilution for Immunoblotting

Primary Anitibody	Dilution Used	Dilution used for gelatin zymography lysates
Vimentin, anti-Mouse (Abcam)	1:10000	1:5000
E-Cadherin, anti-Mouse (BD bioscience)	1:7500	1:5000
β-Catennin, anti-Rabbit (Abcam)	1:20,000	1:20,000
N-Cadherin, anti-Rabbit (Abcam)	1:2500	1:2500
GAPDH, anti-Rabbit (Sigma)	1:20,000	1:5000
Cytokeratin14, anti-mouse(Abcam)	1:1000	-
Cytokeratin19, anti-rabbit(Abcam)	1:1000	-
Fibronectin, anti-mouse (BD bioscience)	1:20,000	1:20,000

Wound Healing:

MCF10A cells grown on 3D on top cultures were dissociated using Trypsin (Invitrogen) containing EDTA. These cells after one passage were seeded onto two sides of wound healing inserts (Ibidi GmbH) placed on a 35mm dish at a cell density of $5x10^5$cells/ml as mentioned by the manufacturer protocol. The cells were then allowed to attach and grow for 16-18 hours in growth media. Cells were then treated with 10 μg/ml of mitomycin C (Sigma) in growth media for 2 hours. Inserts were removed and replenished with fresh media after one PBS wash. The wound area is then imaged at 4x and 10x under microscope at regular intervals of 6 hours for 24 hours. Wound area was calculated using ImageJ and using the equation ($\% \, Wound \; Closure = \frac{Initial \; wound \; area - Final \; wound \; area}{Initial \; wound \; area} * 100$)percentage of wound closure was calculated.

Gelatin Zymography:

After 20 days of culturing, the assay media from untreated MCF10A and 200 nM PAF treated MCF10A were collected and was stored at -80^{o}C. The cells in the wells are also collected in gelatin lysis buffer (25mM Tris-HCl (pH 7.5), 100mM NaCl, 1% NP-40) and was stored at -80^{o}C. The lysate is then mixed with 6X Sample Buffer (Tris with SDS, bromophenol blue, dithiotreitol, and glycerol, pH 6.8) in a ratio 2:1

and probed for GAPDH to adjust the loading. These lysates were also used to probe other markers mentioned in immunoblotting. The media was mixed with Gelatin Zymography (GZ) sample buffer in a ratio 13:87. This mixture was added to an 8% SDS PAGE containing 0.1% gelatin with a ratio obtained from the GAPDH levels. Then it was allowed to run at 120V at 4^{o}C till the dye front runs out. If the current goes below 10mA then we remove and add fresh 1X SDS running buffer. Then the gel is incubated in 1Xrenaturing buffer (2.5 % v/v of Triton X-100 in water) for 30 minutes in a shaker at room temperature after a gentle wash with distilled water. The gel was then washed with approximately 300 ml of distilled water and was incubated in developing buffer (0.5 M Tris-HCl pH-7.8, 2M NaCl, 0.05M $CaCl_2$ and 0.2% Brij35) for 30 minutes in a shaker at room temperature. Then the gel was replenished with fresh developing buffer and was kept in 37^{o}C. 24 hours later the buffer was replaced with fresh developing buffer and keep it back at 37^{o}C. After 48 hours the gel is removed and is stained using Coomassie blue staining solution (0.1% w/v Coomassie blue (Sigma-Aldrich), 50% v/v Methanol (Fisher Scientific),10% v/v Acetic acid (Fisher Scientific and 40% v/v distilled water). The gel images were captured using ImageQuant LAS4000 gel documentation system (GE Healthcare) after destaining in destaining solution (50% v/v Methanol (Fisher Scientific),10% v/v acetic acid (Fisher Scientific and 40% v/v distilled water).

Results

PAF disrupted apico-basal polarity

Maintenance of apical and basal polarity is important for the proper function of the mammary gland (Vidi et al., 2013). Most of the breast epithelial cancers show disruption of this apico-basal polarity (Hebner et al., 2008). To study PAF's effect on apico-basal polarity, 20 days MCF10A 3D cultures treated with or without 200nM PAF and were immunostained for GM130- an apical marker. The results showed GM130 mislocalization in PAF treated MCF10A (Figure 5a). Upon quantification we found that 70% of the PAF treated acini have mislocalized golgi (Figure 5b)

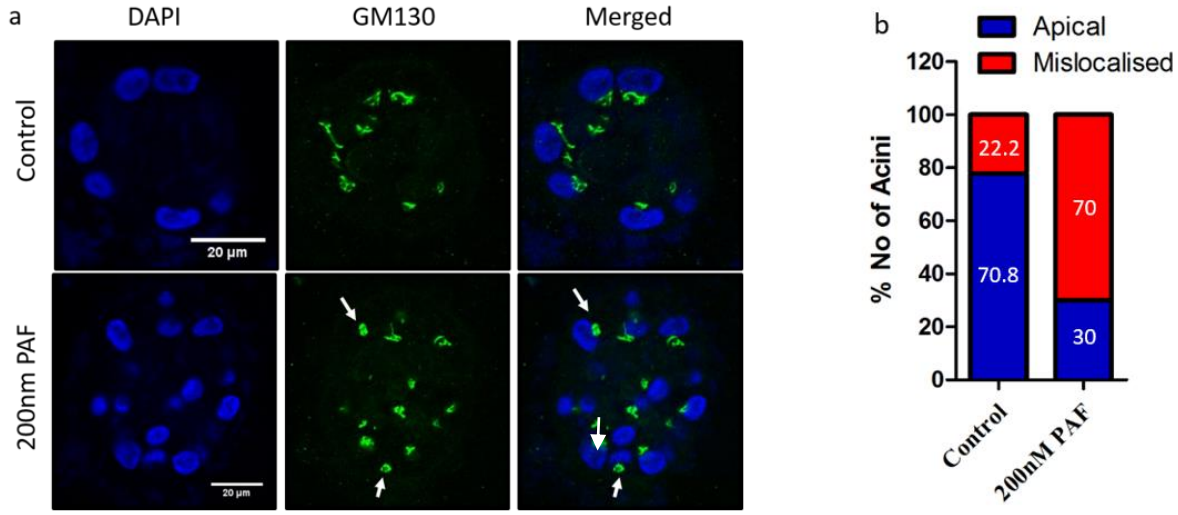


Figure 5: PAF disrupts apical polarity

MCF10A 3D "on top" cultures treated with or without PAF for 20 days were immunostained with GM130 (green) and Hoechst 33342 (blue) (n=20 N=1). (a) Image showing the golgi staining and arrows represent the golgi which are mislocalised from its apical region. (b) Graph representing percentage of acini showing apical and mislocalised GM130.

To investigate the integrity of basal polarity, 20 days MCF10A 3D cultures treated with or without 200nM PAF were immunostained for α6 Integrin- a basal polarity marker (Debnath and Brugge, 2005). The study showed 87% of acini have a loss or discontinuous α6 Integrin staining upon treatment with PAF (Figure 6a & b). These results suggest that PAF disrupted the apico-basal polarity which can lead to transformation.

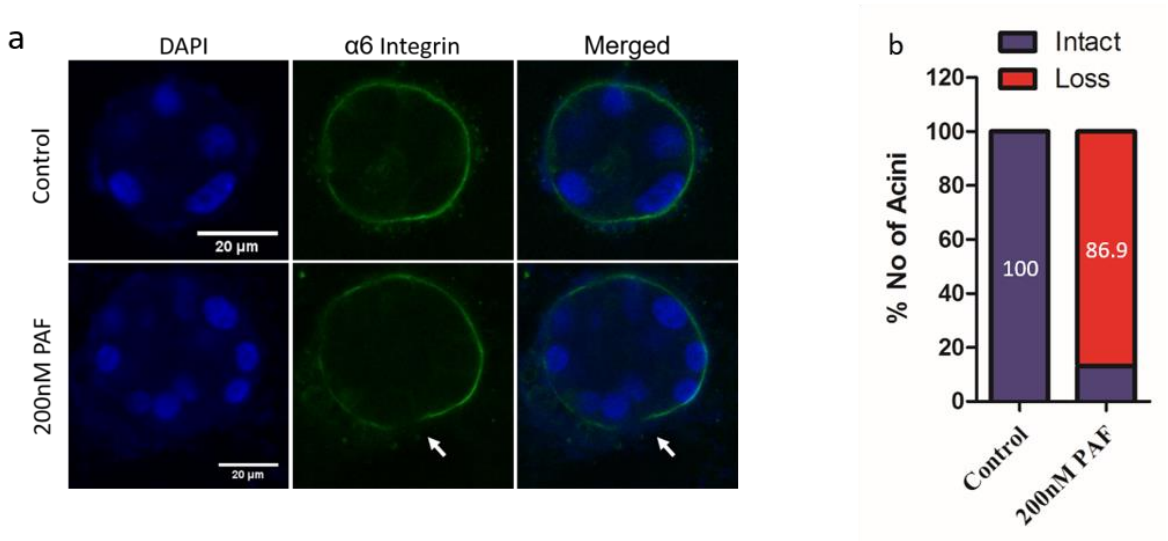


Figure 6: PAF disrupts basal polarity

MCF10A 3D "on top" cultures treated with or without PAF for 20 days were immunostained with α6 Integrin (green) and Hoechst 33342 (blue) (n=22 N=1). (a) Image showing α6 Integrin staining and arrows represent the loss of staining. (b) Graph representing percentage of acini showing Intact and loss of α6 Integrin staining.

PAF affected cell-cell junctions:

Loss of cell-cell contact is another distinct feature of EMT transition. MCF10A 3D culture grown for 20 days treated with or without PAF when immunostained for β catenin showed more diffused localisation in the treated acini compared to control (Figure 7). About 95% of the acini imaged in treatment showed such an aberrant staining pattern suggesting a loose cell- cell junction.

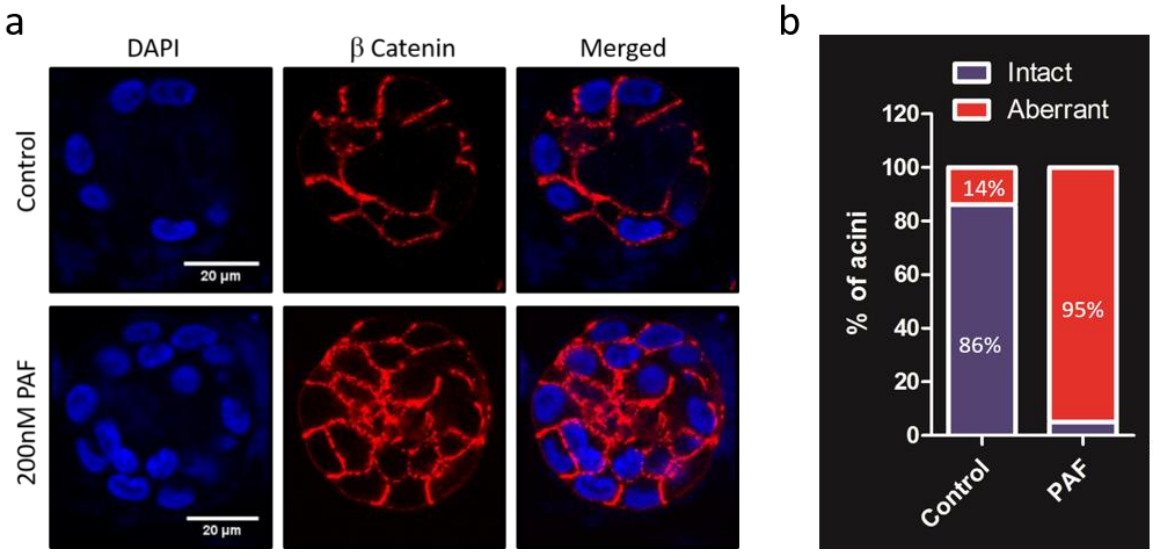


Figure 7: PAF affects cell-cell junction

MCF10A 3D "on top" cultures treated with or without PAF for 20 days were immunostained with β catenin (red) and Hoechst 33342 (blue) (n=25 N=1). (b) Graph representing percentage of acini showing aberrant β catenin staining.

PAF induces epithelial to mesenchymal transition:

During epithelial to mesenchymal transition cells undergo different morphological changes as well as up regulation of proteins such as Vimentin, N cadherin, Fibronectin, Snail and twist. Continuous PAF treatment was shown to cause protrusion like phenotype resembling EMT transition (Anandi et al., 2015). To confirm this we did immunoblotting for some of these markers on lysates collected from 20 days MCF10A 3D cultures with or without 200nM PAF treatment. The results showed increase in levels of Fibronectin and N cadherin in PAF treated acini (Figure 8a). It also showed down regulation of epithelial marker- E cadherin upon PAF treatment (Figure 8a). β catenin which is a cell-cell junction had shown changes in immunofluorescence but did not show any changes in the protein level upon treatment with PAF. This suggests that PAF affects the localisation of β catenin but not the amount of protein. Cytokeratins which are epithelial markers should show a down regulation showed up-regulation in the treated samples. Vimentin up-regulation upon PAF was also observed both by immunoblotting as well as immunostaining (Figure 8b). These data confirms that continuous treatment with PAF leads to epithelial to mesenchymal transition.

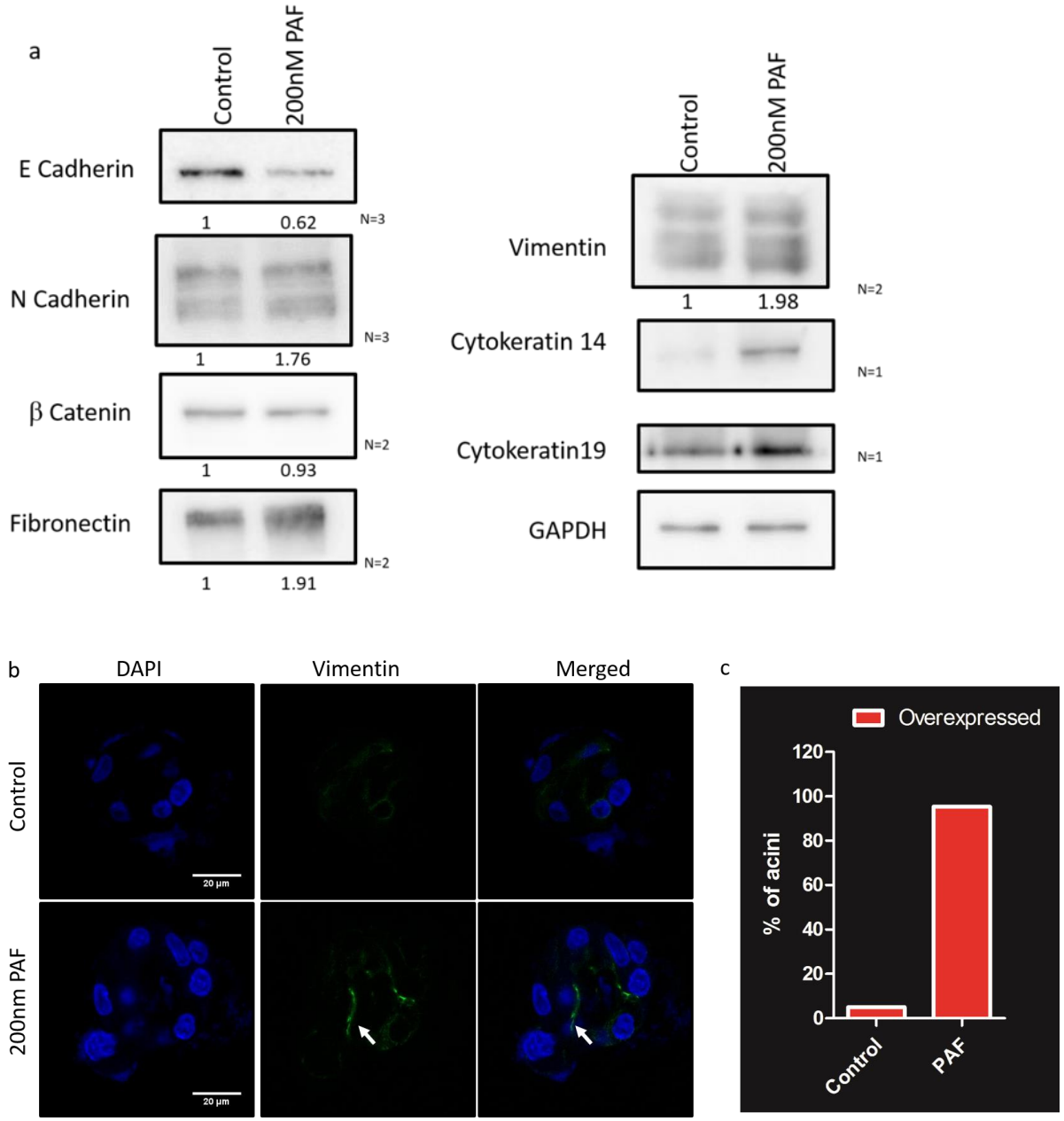


Figure 8: PAF induce Epethelial to mesenchymal transition

(a) Lysates collected from MCF10A 3D “on top” cultures treated with or without PAF for 20 days were immunoblotted against different marker proteins.(b) MCF10A 3D on top cultures treated with or without PAF for 20 days were immunostained with Vimentin (red) and Hoechst 33342 (blue) (n=42 N=2). (c) Graph representing percentage of acini showing overexpressed vimentin staining.

PAF induced secretion of MMP9 and MMP2.

Invasive cells secretes proteinase to degrade the extracellular matrix (ECM). Matrix metalloproteinase (MMP’s) are proteinases which are secreted to degrade ECM.

MMP2 and MMP9 are two MMP's which have gelatinase activity. To study the effect of PAF in secretion of MMP2 and MMP9 gelatin zymography was performed. Media collected from MCF10A 3D culture plate was loaded on SDS-PAGE gel containing gelatin as a substrate. Active MMP9 and MMP2 degrades this gelatin. The 3D lysates were also collected from the same culture plate and was probed for GAPDH as loading controls. The fold change was calculated with respect to control after normalizing the levels with GAPDH levels. The results showed more than two fold increase in the level of MMP9 secretion as well as a considerable increase in MMP2 upon PAF treatment (Figure 9). This suggests continuous treatment of PAF results in secretion of MMP's leading to degradation of extracellular matrix which may aid in the process of invasion. The invasion potential will be further confirmed using invasion assays such as collagen-matrigel assay or collagen invasion assay.

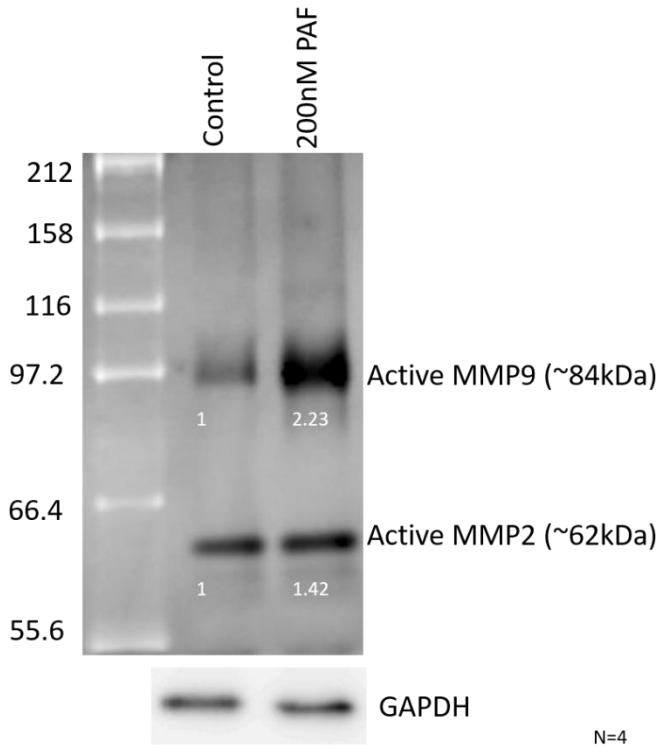


Figure 9: PAF enhance secretion of Matrix metalloproteinase

Gelatin zymography gel showing gelatin clearance by MMP9 (~84kDa) and MMP2(~62kDa). The value below the lane represent the fold change with respect to control normalised to GAPDH.

PAF-transformed cells did not show enhanced collective cell migration.
Dissociated cells from 3D cultures after 20 days were seeded on two sides of the Ibidi™ inserts to check the migratory potential of the cell upon PAF treatment. Preliminary data did not show a significant difference in the percentage wound closure (Figure10), however the experiment demands further validations.

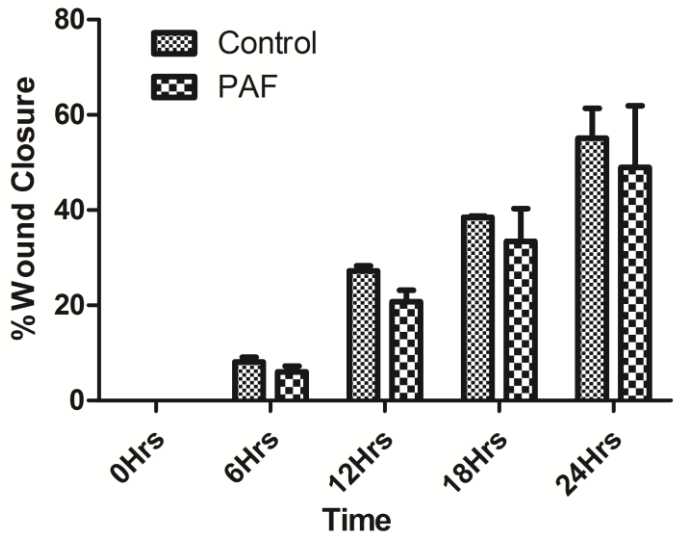


Figure 10: PAF- transformed cells did not show enhanced collective cell migration.

Wound healing data showing quantification of percentage wound closure at different time point with and without PAF (n=2 N=1). The graph represent mean closure of two technical replicate experiment and standard error mean.

PAF induced enhanced cellular proliferation even after 20 days:
MCF10A grown as 3D cultures forms growth arrested acinar structures by day 16 (Hebner et al., 2008). 20 day MCF10A 3D cultures when immunostained for Ki67- a proliferation marker, showed increase in number of acini showing more than six Ki67 positive cells upon treatment with PAF (Figure 11). Normal MCF10A culture showed some Ki67 positive cells but only 14% of acini showed more than six Ki67 positive nuclei whereas 67% of the acini imaged were having more than six Ki67 positive nuclei.

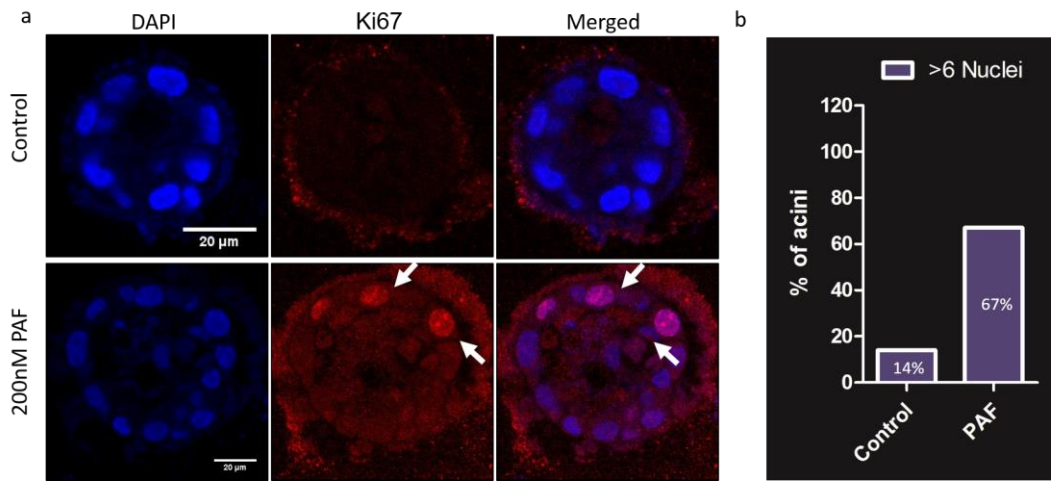


Figure 11:PAF enhance proliferation even after 20 days.

MCF10A 3D “on top” cultures treated with or without PAF for 20 days were immunostained with Ki67 (red) and Hoechst 33342 (blue) (n=40 N=2). (a) Image showing Ki67 positive acini.

Discussion:

Studies showing trace of PAF in the microenvironment lead to lot of discoveries in tumourogenisis (Ramos-Nino, 2013; Sounni and Noel, 2013) . Bussolati and group showed that cancer cells have the potential to secrete PAF upon stimulation with growth factor (Bussolati et al., 2000). The studies done on rat embryonic cell for the first time showed metastatic transformation upon treatment with PAF (Bennett et al., 1993). Later on PAF was established to induce transformation in BRCA1 mutant ovarian cells (Welcsh and King, 2001). PAF inhibition lead to inhibiting of tumour growth by inhibiting angiogenesis (Bussolati et al., 2000; Melnikova and Bar-Eli, 2007). PAF receptor over expression played a potential role in tumorigenesis of nude mice (Robert and Hunt, 2001). But none of the studies were able to investigate the role and mechanism of PAF in breast tumorigenesis. The present study investigated the role of PAF in transformation of breast epithelial cells and its underlying mechanism. The mechanism of morphogenesis of breast epithelial cell was well studied using 3D “on top” culture model (Hebner et al., 2008). Earlier studies in lab showed overexpression of PAF receptor in MCF7-a breast adinocarcinoma and MDA-MB 231- an invasive ductal carcinoma cells (Anandi et al., 2015). Along with this study, we also reported that incubation with PAF for 20 days in MCF10A 3D “on top“ cultures can lead to formation of abnormal structures such as partially filled lumen, protrusions and increased volume and number of cells per acini (Anandi et al., 2015). These characteristics shown by the treated cells resembles the characteristics of a malignant cell grown on Matrigel®. To validate whether the PAF induced changes were indeed transformation, we further investigated the phenotypic changes upon PAF treatment. To answer this, we examined the integrity of cellular polarity upon PAF treatment.

Establishment and maintenance of apico-basal polarity is critical for the normal functioning of the acini. Golgi marker, GM130 is a widely used to determine the apical polarity (Debnath and Brugge, 2005). Our preliminary results suggest that PAF treatment leads to mis-localisation of GM130 (Figure 5). We also looked into a basal polarity marker, $\alpha 6$ Integrin which showed a loss phenotype with treatment (Figure 6). It was reported that invasive cells degrades their ECM and loss contact with their basement membrane to invade to different part of the body (Debnath and Brugge, 2005; Hebner et al., 2008). This loss phenotype we observed may be due to

degradation of ECM by proteases secreted upon PAF treatment. To further study this we did a wound healing assay to observe the migratory potential of the PAF transformed cells. Interestingly there was no significant change in the migratory potential. This may be because the cells are mostly transformed to mesenchymal and are having mesenchymal migratory profile rather than a collective cell migration profile. To validate whether PAF treated cells are transformed to mesenchymal phenotype we immunoblotted for different epithelial and mesenchymal markers. The results obtained showed significant up-regulation of mecenchymal markers such as Fibronectin and N cadherine and down regulation of epithelial marker E cadherine. We also obtained considerable up-regulation in levels of Vimentin both by immunofluorescence and immunoblotting. When cells undergo EMT they tend to loss their cell-cell contact. Now to substantiate this hypothesis we did immunoblotting and immunofluorescence assay for β catenin a cell- cell junction protein. Interestingly there was a diffused staining pattern in the immunofluorescence without any change in the level of protein. This may be because the cells are now loosely attached to each other. Protrusion of the acini also suggests the EMT phenotype which was observed earlier. To validate this further, single cell migration assay and collagen invasion assay can be used.

Cells undergoing invasion or mesenchymal migration secretes matrix metalloproteinases to degrade the ECM (Chen et al., 2015). The loss of basement membrane in figure 6 also suggests degradation of the matrix up on PAF treatment. To validate this point we performed gelatin zymography assay. In this assay we can identify the level of active MMPs secreted having gelatinase activity. Our data confirmed that there is enhanced secretion of active MMP 2 and MMP 9 into the media. Studies on esophageal squamous cell carcinomas already reported these MMP up-regulation (Chen et al., 2015) but has been shown for the first time in breast cancer cells.

Future Plans:

My data from this project clearly shows PAF has the potential to induce EMT and invasion. This can be further validated using collagen invasion assay and single cell migration assay. PAF was shown to play a potential role in malignant development through PI3K/AKT pathway in esophageal squamous cell carcinoma (Chen et al., 2015) (Figure 12a). PAF has been shown to interact with JAK2 initiating JAK/STAT pathway and lead to increased migration and invasion along with proliferation (Deo et al., 2002) (Figure 12b). In figure 12 (a and b) activated PAF receptor signaling can lead to production of more PAF receptor through two different pathway. We can study this phenomenon by observing PAF receptor levels with and without PAF after 20 days in 3D using RT PCR. To identify the pathways involved PAF receptor antagonist WEB2086 treated or PAF receptor knock down studies need to conducted. Comparing these with control and PAF treated cells we can elucidate the mechanism of PAF induced transformation.

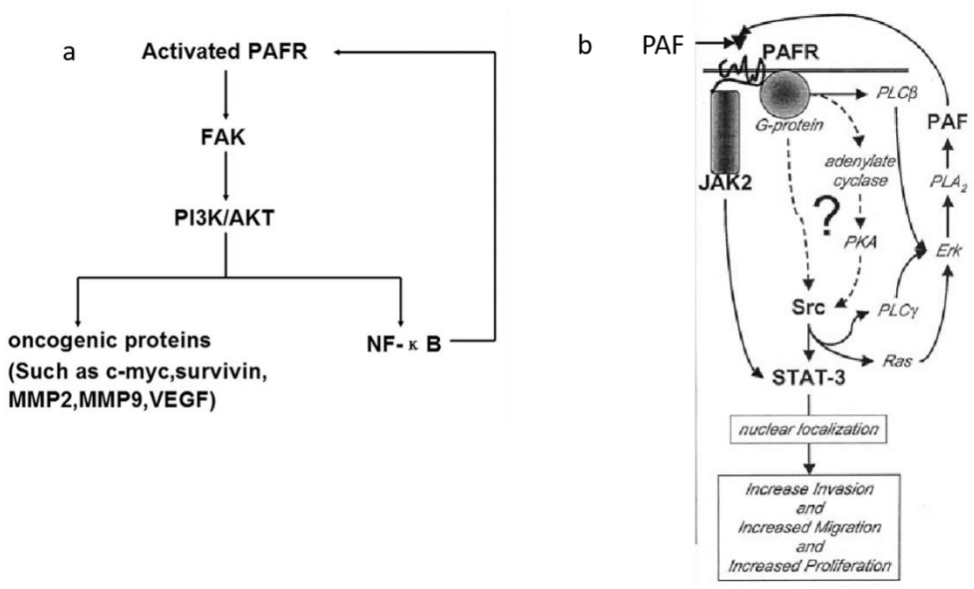


Figure 12: Pathways of PAF induced transformation.

References:

Anandi, V.L., Ashiq, K.A., Nitheesh, K., and Lahiri, M. (2015). Platelet-activating factor promotes motility in breast cancer cells and disrupts non-transformed breast acinar structures. Oncology reports.

Bennett, S.A., Leite, L.C., and Birnboim, H.C. (1993). Platelet activating factor, an endogenous mediator of inflammation, induces phenotypic transformation of rat embryo cells. Carcinogenesis *14*, 1289-1296.

Bussolati, B., Biancone, L., Cassoni, P., Russo, S., Rola-Pleszczynski, M., Montrucchio, G., and Camussi, G. (2000). PAF produced by human breast cancer cells promotes migration and proliferation of tumor cells and neo-angiogenesis. The American journal of pathology *157*, 1713-1725.

Chao, W., and Olson, M.S. (1993). Platelet-activating factor: receptors and signal transduction. The Biochemical journal *292 (Pt 3)*, 617-629.

Chen, J., Lan, T., Zhang, W., Dong, L., Kang, N., Zhang, S., Fu, M., Liu, B., Liu, K., Zhang, C.*, et al.* (2015). Platelet-activating factor receptor-mediated PI3K/AKT activation contributes to the malignant development of esophageal squamous cell carcinoma. Oncogene *34*, 5114-5127.

Debnath, J., and Brugge, J.S. (2005). Modelling glandular epithelial cancers in three-dimensional cultures. Nature reviews Cancer *5*, 675-688.

Deo, D.D., Axelrad, T.W., Robert, E.G., Marcheselli, V., Bazan, N.G., and Hunt, J.D. (2002). Phosphorylation of STAT-3 in response to basic fibroblast growth factor occurs through a mechanism involving platelet-activating factor, JAK-2, and Src in human umbilical vein endothelial cells. Evidence for a dual kinase mechanism. The Journal of biological chemistry *277*, 21237-21245.

Evans, D.G., Shenton, A., Woodward, E., Lalloo, F., Howell, A., and Maher, E.R. (2008). Penetrance estimates for BRCA1 and BRCA2 based on genetic testing in a Clinical Cancer Genetics service setting: risks of breast/ovarian cancer quoted should reflect the cancer burden in the family. BMC cancer *8*, 155.

Hebner, C., Weaver, V.M., and Debnath, J. (2008). Modeling morphogenesis and oncogenesis in three-dimensional breast epithelial cultures. Annual review of pathology *3*, 313-339.

Kim, H.A., Kim, K.J., Seo, K.H., Lee, H.K., and Im, S.Y. (2012). PTEN/MAPK pathways play a key role in platelet-activating factor-induced experimental pulmonary tumor metastasis. FEBS letters *586*, 4296-4302.
Melnikova, V., and Bar-Eli, M. (2007). Inflammation and melanoma growth and metastasis: the role of platelet-activating factor (PAF) and its receptor. Cancer metastasis reviews *26*, 359-371.
Prescott, S.M., Zimmerman, G.A., Stafforini, D.M., and McIntyre, T.M. (2000). Platelet-activating factor and related lipid mediators. Annual review of biochemistry *69*, 419-445.
Ramos-Nino, M.E. (2013). The role of chronic inflammation in obesity-associated cancers. ISRN oncology *2013*, 697521.
Robert, E.G., and Hunt, J.D. (2001). Lipid messengers as targets for antiangiogenic therapy. Current pharmaceutical design *7*, 1615-1626.
Sounni, N.E., and Noel, A. (2013). Targeting the tumor microenvironment for cancer therapy. Clinical chemistry *59*, 85-93.
Vidi, P.A., Bissell, M.J., and Lelievre, S.A. (2013). Three-dimensional culture of human breast epithelial cells: the how and the why. Methods in molecular biology *945*, 193-219.
Welcsh, P.L., and King, M.C. (2001). BRCA1 and BRCA2 and the genetics of breast and ovarian cancer. Human molecular genetics *10*, 705-713.
Zhu, T., Gobeil, F., Vazquez-Tello, A., Leduc, M., Rihakova, L., Bossolasco, M., Bkaily, G., Peri, K., Varma, D.R., Orvoine, R.*, et al.* (2006). Intracrine signaling through lipid mediators and their cognate nuclear G-protein-coupled receptors: a paradigm based on PGE2, PAF, and LPA1 receptors. Canadian journal of physiology and pharmacology *84*, 377-391.