CHAPTER 4

DISCUSSION AND CONCLUSIONS
4.1 Discussion

Breast cancer accounts for 23% of all newly occurring cancers in women worldwide and represents 13.7% of all cancer deaths. It is the most frequent cancer in both developed and developing regions as well as the most frequent cause of cancer death [1].

Though lot of effort has gone into uncovering the genetic drivers responsible for breast cancer initiation and progression [362], the important role of tumor microenvironment is increasingly being recognized. The tumor microenvironment consists of different cell types that interact with each other and influence tumor initiation, growth and metastasis. In breast cancer, the tumor–stroma interactions are dynamic networks which take place between epithelial cells and the microenvironment consisting of stromal cells that include fibroblasts, innate and adaptive immune cells, adipocytes, vasculature and specialized mesenchymal cells [363, 364]. Immune cells represent a major component of the tumor microenvironment and consist of monocytes/macrophages, neutrophils, eosinophils, mast cells and lymphocytes [124]. Tumor-infiltrating immune cells were originally regarded as cytotoxic to the tumor cells; however, current findings support that such tumor-associated leukocytes have a dual role and can either contribute to cancer initiation, proliferation, metastasis due to immune tolerance, or can result in tumor suppression [365, 366]. The tumor associated macrophages (TAMs) represent the largest population of these immune cells and have been ascribed a dual role depending on the phenotype and secreted factors [124]. A vast amount of secretome including cytokines and growth factors is released both by the cancer and cells in the tumor microenvironment. The ECM in tumor microenvironment contains the secretome, constituted by proteins, receptors, proteoglycans and adhesive molecules as well as a milieu of secreted proteins including cytokines, chemokines, growth factors, angiogenesis factors and proteases at its surroundings [364, 367].
In the present studies, the TGF-β1-ROS-ATM-CREB pathway responsible for the increased migration of tumor cells was identified by studying the *in vitro* interaction of macrophage secretome and the tumor cells. This was achieved by employing MCM and MφCM treatment to tumorigenic but noninvasive epithelial breast cancer cell line, MCF7 and invasive breast cancer cell line, MDA-MB-231. These two cell lines differ in their invasive nature as well as in several aspects including the p53 status, caspase 3 expression and estrogen receptor status [368]. Though both cell lines showed decreased clonogenic ability with MφCM treatment, the mechanism behind this and other effects related to migration turned out to be dramatically different. Smaller merging colonies resulting in increased migration in one cell line (MCF7) and large multinucleated cells resembling senescent phenotype in the other (MDA-MB-231).

Cellular senescence is one of the many links between aging and cancer [369]. Though senescent cells fail to proliferate, they remain metabolically active and may secrete cytokines including IL-6, IL-8, IL-1α and IL-1β [370, 371]. Though a senescent phenotype was induced by MφCM in invasive MDA-MB-231 cells, increased secretion of pro-inflammatory cytokines was not observed. But since only a limited number of cytokines were assessed, secretion of other factors leading to this phenotype cannot be ruled out. In contrast, the present study has identified that the network of secreted cytokines are the major players in the interaction of macrophages with MCF7 cells resulting in EMT responses.

The next quest was to identify this cytokine network between macrophages and tumor cells responsible for the observed changes. Pro-inflammatory cytokines like TNF-α, IL-1β and IL-6 were detected in the MφCM. Though TGF-β1 was not secreted by both monocytes and macrophages, the interaction of other pro-inflammatory cytokines with cancer cells resulted in TGF-β1 secretion. Interestingly, the pro-inflammatory cytokine cocktail present in
MϕCM increased apoptosis only in MCF7 cells as opposed to MDA-MB-231 (ER/PR negative cell line). TNF-α has been shown to decrease ER dependent gene expression and cell survival [372] (Lu et al 2006) and induce apoptosis in MCF-7 cells [373, 374]. On the other hand, MDA-MB-231 cells express higher levels of anti-apoptotic proteins as demonstrated by these studies as well as by others [375, 376]. The Bcl-2 family of proteins comprises a number of related proteins whose expression has been shown to regulate apoptosis [377]. This family includes antiapoptotic members (Bcl-2, Mcl-1 and Bcl-XL) and proapoptotic members (Bax, Bid, Bad etc) whose individual expression and heterodimerization with each other regulate the sensitivity of cells to apoptosis. Bcl-2 overexpression inhibit apoptosis, whereas, a predominance of Bax over Bcl-2 accelerates cell death upon apoptotic stimuli [378]. This differential effect observed between the two cell lines could thus be combination of these factors like status of ER expression as well as the balance in pro and anti apoptotic proteins which alters their response to pro-inflammatory cytokines.

Though apoptosis was increased in MCF7 cells with MϕCM treatment, involvement of pro-apoptotic proteins Bax, Bad, truncated Bid, Bim and Puma were ruled out. Since Bax levels did not change, TNF-α induced apoptosis could have been through a RIP dependent mechanism [379]. In addition, PARP cleavage was also observed in these cells. Though MCF7 cells are caspase 3 deficient, TGF-β1 has been known to induce PARP cleavage as an independent event dissociated with cell apoptosis [380]. MϕCM induced apoptosis in MCF7 cells might be inititated by TNF-α itself through a RIP dependent mechanism or could be the effect of the induced TGF-β1. Both cytokines together also could have increased the susceptibility of MCF7 cells to undergo apoptosis.
Induction of various cytokines by TNF-α has been reported in different cell types: TGF-β1 in lung fibroblasts [381], IL-6 and TGF-β2 in breast cancer cells [382], IL-8 in endothelial cells [383], IL-1 and IL-6 in cardiac fibroblasts [384]. In the current study, there was no detectable secretion of TGF-β1 in the supernatant of MCF7 although about 30% of MCF7 cells were positive for intracellular TGF-β1. However, with MφCM treatment of MCF7 cells, there was increased secretion of TGF-β1 as well as intracellular accumulation in presence of Golgi plug™. This upregulation seems to be at the transcriptional level as observed by an increase in mRNA of TGF-β1 and TGF-βRII and specific to TGF-β1 as there was no increase in mRNA of TGF-β2, TGF-β3 and TGF-βRI. The levels of TGF-β1, TGF-β2, TGF-βRI, TGF-βRII mRNA have been correlated with increasing invasive ability of a panel of breast cancer cell lines, with MCF7 having the lowest levels [385]. TNF-α produced by the activated macrophages accelerated TGF-β1 driven EMT in colon carcinoma [386] and enhanced recruitment of TAMs generated EMT-promoting microenvironment by increasing expression of TGF-β, PDGF and EGF in the mouse MMTV-PyMT mammary tumor model (in which the expression of the Polyoma Virus Middle T antigen oncogene is driven by the Mouse Mammary Tumor Virus promoter) [120].

TGF-β shows powerful cytostatic activity in normal mammary epithelial cells (MECs), but this ability is frequently inactivated in malignant MECs and leads to the acquisition of oncogenic activity in developing and progressing mammary tumors [153, 387]. This switch in TGF-β function is referred to as the “TGF-β Paradox”. This is supported by a variety of genetic and epigenetic events that ultimately underlie the adverse prognosis associated with elevated TGF-β production in developing mammary carcinomas [388].

Induction of TGF-β1 by MCF7 cells was associated with the downstream events of increase in oxidative stress, DNA damage and CREB mediated survival signaling. This paradox of increased apoptosis in a group of cells and redox, DNA damage mediated survival
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signaling in the remaining cells seems to be characteristic of TGF-β1. This has also been
demonstrated in hepatocytes [389] and the choice of cells to undergo apoptosis or EMT
responses depended on the cell cycle stage. TGF-β1 induced apoptosis in cells synchronized
at G2/M phase and EMT responses in unsynchronized cells and cells at G1/S phase of the cell
cycle [385]. Data from the present studies further confirm that apoptosis as well as EMT
responses can be observed at the same time in a population of breast cancer cells, though
changes in specific cell cycle stages were not studied. The gene expression signatures
associated with the TGF-β signaling has been linked to the acquisition of EMT and stem cell-
like phenotypes by breast cancer cells [390, 391].

A significant increase in EGF receptors was also observed along with TGF-βRII in
MCF7 cells following MϕCM treatment. Activation of these receptors resulted in activation
of SAPK/JNK and ERK 1/2 MAP kinase as compared to untreated cells and those treated
with MCM. However an appreciable change in phosphorylation of p38 was not observed with
MϕCM treatment of MCF7 cells. TGF-β1 signals through complexes of type II (TRII) and
type I (TRI) receptors. Upon ligand binding, TRII receptors phosphorylate and activate the
TRI receptors, which then activate regulatory Smads such as Smad2 and Smad3 via
phosphorylation [145]. Phosphorylated Smads form complexes with regulatory Smads,
translocate into the nucleus and regulate the transcription of TGF-β1 target genes. Smad4
cooperates with other transcription factors, such as FoxH1, Mixer, Runx-related proteins and
E2F, as well as transcriptional co-activators (e.g., p300 and CBP) and co-repressors (e.g., SKI
and SnoN, pro-oncoproteins) in the regulation of target genes [392, 393]. TGF-β1 signaling
also activates signal transducers other than Smads, such as ERK1/2 MAP kinases, p38 MAP
kinase (p38 MAPK), PI3 kinase and Rho-like GTPases [394, 395]. Synergistic signaling by
EGF and TGF-β1 could have resulted in enhanced SAPK/JNK and ERK1/2 phosphorylation.
The combined effect of these two ligands on activation of ERK1/2 and subsequently MMP-
9 function resulting in increased cell migration has been demonstrated [396]. Thus the present studies clearly demonstrate macrophage mediated upregulation of TGF- β1 signaling in MCF7 cells.

In addition to activation of MAPK pathways, there was an increase in redox signaling in MCF7 cells as demonstrated by augmentation in ROS and RNS generation. Increased ROS and RNS production was observed only in MCF7 cells and not in MDA-MB-231 cells following MφCM treatment again showing that this could be specific downstream effect of TGF- β1. ROS and RNS play important roles in regulation of cell survival. ROS include radical species such as superoxide (O$_2^-$) and hydroxyl radical (HO'), along with non-radical species such as hydrogen peroxide (H$_2$O$_2$). ROS arise as a by-product of mitochondrial oxidative phosphorylation, oxygen metabolism and NADPH/NADPH oxidase (NOX) [397, 398]. RNS include nitric oxide (NO') and peroxynitrite (ONOO−) and are generated through specific nitric oxide synthase isoenzymes (reviewed in [399]).

In general, moderate levels of ROS/RNS function as signals to promote cell proliferation and survival, whereas severe increase of ROS/RNS induces cell death. The high metabolic rate of cancer cells drives their intracellular ROS up to an intermediate level, resulting in a shift in redox balance. ROS can also activate STAT3, MAPK and PI3K signaling pathways triggering secretion of other growth factors that can induce cell proliferation, aggressiveness phenotype and apoptosis inhibition in the estrogen-responsive breast cells. [400].

Boudreau and colleagues have demonstrated that in both normal and metastatic breast epithelial cells, TGF-β treatment resulted in NOX-dependent superoxide production in the plasma membrane [175]. In contrast, studies have also demonstrated that expression of iNOS and secretion of NO antagonized TGF-β1 induced apoptosis and EMT in hepatocytes [401].
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This again highlights the differences in TGF-β1 mediated signaling in normal and malignant cells (hepatocytes vs breast adenocarcinoma).

This oxidative stress resulted in DNA damage response signaling only in MCF7 cells as compared to MDA-MB-231 cells. The generation of ROS and RNS elevates the probability of oxidative DNA lesions. ATM is regarded as the major regulator of the cellular response to DNA double strand breaks (DSBs). Furthermore, ATM can also be activated directly by oxidative stress independent of DSBs by a mechanism distinct from MRN/DSB-dependent activation [402]. The observed ATM activation following MϕCM treatment could thus be directly activated by oxidative stress in a MRN independent pathway or as a result of MRN dependent DNA damage response. The basal level expression of ATM was higher in MDA-MB-231 cells which remained unchanged with the treatments. Phosphorylation of histone H2AX is performed by kinases of the PI3K family DNA-PK and ATM [403]. In the untreated and MCM treated MCF7 cells, there was no labeling of γ-H2AX. However, following MϕCM treatment, there was an increased formation of intense γ-H2AX-foci intense foci indicating complex DNA damage. γ-H2AX as well as other components of the DNA repair pathways are detectable in close vicinity to the DSBs soon after DNA damage [404]. The associated conformational changes of these proteins in turn recruit transducer and effector proteins responsible for the DNA damage response signaling. The earliest event in this process is histone poly (ADPribosylation), in which the poly (ADP-ribose) polymerases (PARP1, PARP2 and PARP3) catalyze the formation of poly (ADP-ribose) (PAR) and their covalent linkage to lysines in core histone proteins [405, 406]. PARP cleavage, a characteristic feature of apoptosis was observed only in MϕCM treated MCF7 cells. This again highlights the dichotomy of responses observed following TGF-β1 induction. PARP cleavage is typically associated with apoptosis, whereas pATM or γ-H2AX could result in DNA damage response and survival. This co-existence of PARP cleavage with pATM and γ-
H2AX could be similar to the parallel induction of apoptosis as well as EMT responses in different groups of cells by TGF-β1.

Along with redox signaling and DNA damage responses, MCF7 cells treated with MϕCM also displayed higher FL2/FL1 ratio of mitochondrial membrane potential. This basal level ratio was higher in MDA-MB-231 cells which did not further change on MϕCM treatment indicating membrane hyperpolarization. Subpopulations of cells with significant stable variations in intrinsic ΔΨm have been described within primary mammary tumor and in both primary and metastatic colonic tumor [407]. This hyperpolarization could be due to mitochondrial biogenesis which has been reported to increase in response to DNA damage. DNA topoisomerase II-targeting anticancer drugs like doxorubicin, mitoxantrone and etoposide, known inducers of DNA damage, also upregulate the abundance of mitochondria [408]. ROS may perturb mitochondrial homeostasis through two opposing effects. First, ROS can damage mitochondria leading to the production of more ROS and more defective mitochondria via a vicious cycle. Second, ROS can also induce mitochondrial biogenesis through a DNA damage/ATM/AMPK pathway and therefore ameliorate the ROS-mediated vicious cycle. The results presented in this dissertation support the second phenomenon. Cells with increased mitochondrial membrane potential as well as those with increased nitric oxide exhibited phenotypic properties consistent with promotion of tumor cell survival and expansion including secretion of angiogenic factors [200, 407]. These results strongly suggest that the increased ΔΨm could play an important role in the increased invasion observed in MCF7 cells following treatment with MϕCM. These changes of hyperpolarization instead of hypopolarization (characteristic of apoptotic cells) of mitochondrial membrane potential along with no changes observed in expression of Bax/Bcl-2 family members not only confirms Bax independent cell death mechanism but reaffirms the dichotomy of responses in the cells.
In response to DNA damage or extracellular signals, expression of several transcription factors like CREB, NF-κB, c-fos and c-jun allows the cells to overcome stressful or deleterious environment [264-267]. In the present studies, DNA damage response along with MAPK signaling was observed in MφCM treated MCF7 cells. This resulted in significant increase in pCREB and total CREB in these cells in contrast to MDA-MB-231 cells where basal level expression of CREB was higher and was unaffected by macrophage treatment. CREB (cyclic AMP (cAMP) response element binding protein) belongs to the basic/leucine zipper (bZIP) superfamily of transcription factors, which include CREB and the closely related factors CREM (cAMP response element modulator) and ATF-1 (activating transcription factor 1) [409]. Studies have shown that CREB, a 43 kDa bZip transcription factor plays an important role in cell differentiation, survival, proliferation, development, cell cycle progression and glucose metabolism [288, 303, 410, 411]. Canonical activation of CREB occurs in response to cAMP, which induces PKA-dependent Ser-133 phosphorylation [412]. The phosphorylation of CREB on Ser-133 promotes recruitment of additional proteins or co-activators like CBP [277] and p300 [413]. In addition to its regulation by metabolic and growth signals, CREB is also a target of the DNA damage response [266, 414]. CREB is activated by cAMP, growth factors, hormones, retinoids, cytokines and prostaglandins via multiple signaling pathways, including the cAMP/protein kinase A, PI3K/Akt, extracellular signal-regulated kinase (ERK)/p90 ribosomal S6 kinase and p38/mitogen- and stress-activated protein kinase pathways [274, 276]. cAMP may as well support the metastatic activity of TGF-β1 on triple-negative breast cancers. Triple-negative breast cancers from which MDA-MB-231 cells were derived, show higher metastatic potential, where the TGF-β/Smad3 pathway is more active [415].

Increased CREB stability as a result of phosphorylation of CREB on ser-133 has been reported [416]. We also observed increased total CREB expression in MCF7 cells following
MφCM treatment whereas the basal levels were high in MDA-MB-231 cells. These results implicate the stabilization of total CREB due to phosphorylation on ser-133. These data also confirm earlier report on differences between these two cell lines in terms of total CREB expression [417].

The term epithelial to mesenchymal transition (EMT) describes a multi-step event during which cells lose numerous epithelial characteristics and gain the properties typical for mesenchymal cells. Transitions in cell phenotype from epithelial to mesenchymal (EMT) or mesenchymal to epithelial (MET), play a crucial role during embryonic development and tumorigenesis and require complex changes in gene expression, cell architecture and migratory and invasive behavior. Studies on human and mouse tumors suggest that the same molecular processes that drive developmental EMT are reactivated in the tumor cell to drive tumor progression towards invasive metastatic carcinomas [418].

A marked increase in vimentin expression as well as in vitro migration was found in MCF7 cells treated with MφCM whereas MDA-MB-231, a highly invasive cell line had higher basal level expression of vimentin which did not further increase with the treatment. A rapid increase in expression of mesenchymal markers vimentin and fibronectin has been demonstrated after TGF-β treatment of HMECs [418]. One of the essential molecules for formation and maintenance of the epithelial phenotype is the adhesion molecule E-cadherin (encoded by Cdh1) which is typically located at cell-cell adhesion junctions. Loss of E-cadherin is consistently observed during EMT and is currently regarded as a hallmark of EMT [419]. At the same time, up regulation of Snail, Slug, vimentin and fibronectin leads to acquisition of motility and invasive properties and allows the cells to migrate through the extracellular matrix and form metastases at distant sites [420]. EMT regulators like the transcription factors SNAIL/SLUG and TWIST, the homeobox protein SIX1 along with
interconnecting signaling pathways including Wnt, TGF-β and other growth factors are implicated in mammary development and in breast cancer [421, 422].

The results discussed so far thus implicate that the pro-inflammatory cytokine cocktail found in MϕCM induce a TGF-β1/ROS/ATM/CREB signaling axis in MCF7 cells. This effect seems to be differential and not observed in MDA-MB-231 cells probably due to a difference in estrogen receptor status or due to higher expression of anti-apoptotic proteins. To confirm if indeed this is the signaling pathway induced by macrophages resulting in EMT responses and increased migration, multiple approaches were followed. (1) Neutralization of pro-inflammatory cytokines TNF-α, IL-1β and IL-6 in MϕCM (2) Use of ROS scavenging agents like NAC or specific inhibitors of iNOS or ATM (3) Use of ATM knock down cells. The ability of all these agents to block this pathway was tested in two endpoints assays. These were (1) MϕCM induced pCREB expression (2) MϕCM induced increased migration in MCF7 cells. All three treatments of antibody neutralization, inhibitor treatment as well as ATM KD were very effective in decreasing MϕCM induced CREB phosphorylation as well as increased migration confirming that this indeed is the pathway activated by macrophages that resulted in EMT responses in MCF7 cells.

The growth and progression of breast tumor cells depend not only on their malignant potential, but also on the multidirectional interactions of secreted substances (secretome), including extracellular matrix (ECM), produced by all the cell types including tumor, stroma, endothelial cells and immune cells within the local microenvironment. A permissive tumor microenvironment is required for successful progression and metastasis of tumor cells [423]. The mixture of multiple proteins and peptides released either from tumor or host cells constitute the secretome and are crucial for the communication between the tumor cells and their microenvironment [424]. The identification of proteins or peptides released into the
medium of tumor cells or immune cells cultured \textit{in vitro} is the most common method for determining a secretome. However, the secretory pattern of cells \textit{in vitro} might be different from the \textit{in vivo} secretome. In the present study, the MCM and M\(\Phi\)CM were employed to study the tumor-immune cell interaction.

Apart from the pro-inflammatory cytokines detected by ELISA, attempts were made to identify other key players in M\(\Phi\)CM. This was carried out by 1D and 2D electrophoresis of MCM and M\(\Phi\)CM in which a differential pattern of secreted proteins was observed. When these proteins in CM were concentrated by ultrafiltration, they did not absorb onto IPG strips and remained immobile in the electric field during IEF in contrast to proteins that were concentrated by ammonium sulphate precipitation. This indicated the presence of lipid components in the CM when they were concentrated by ultrafiltration. Cancer cells and TME are known to communicate with each other not only via direct contact (by adhesion factors) but also by secreted paracrine factors (released factors) such as secreted proteins (cytokines and pro-angiogenic factors), nucleic acids and extracellular vesicles (EVs) [425]. Among the released factors, EVs represent a new paradigm of intercellular communications [426]. EVs have a size range of 50 to 1000 nm and are further categorized into microvesicles/apoptotic bodies, membrane particles, exosome like vesicles and exosomes based on their size, origin and molecular composition [427]. Exosomes are multivesicular body-derived vesicles of 50 to 100 nm in diameter and were first described as such by Johnstone \textit{et al.}, in 1987 [428]. These vesicles contain a wide range of functional proteins, mRNAs and miRNAs and are actively secreted via exocytosis from almost all cell types including dendritic cells, lymphocytes and tumor cells [429]. Though exosomes were purified from both MCM and M\(\Phi\)CM, they did not play any role in the M\(\Phi\)CM induced effects observed in MCF7 cells. This was confirmed by two ways: (1) Similar uptake of labeled exosomes obtained from both MCM and M\(\Phi\)CM by MCF7 cells; (2) The effect of EFM\(\Phi\)CM treatment in clonogenic assay.
was similar to MφCM. These results conclusively demonstrated that only soluble factors and not exosomes that played a major role in macrophage induced EMT responses of MCF7 cells in this study.

In addition to proinflammatory cytokines in MφCM, MALDI/TOF analysis identified six upregulated proteins in MCM and eleven upregulated proteins in MφCM. The proteins identified in MCM were moesin, plastin-2 isoform 16, glucose-6 phosphate isomerase isoform 1, actin, aldolase A and ferritin light polypeptide and they play a functional role in cell-cell recognition and signaling, cell motility and cytoskeleton regulation.

The proteins identified in secretome of macrophages were matrix metalloproteinase 1 (MMP-1) preproprotein, annexin V, GAPDH, chitinase 3 like protein, plasminogen activator inhibitor 2, myoferlin, plastin 2, CLK-3 isoform, metalloproteinase 9 (MMP-9) preproprotein and nitric oxide synthase. The majority of these proteins play a functional role of tissue remodeling, breakdown of extracellular matrix, membrane trafficking and cell migration. Even though many secreted proteins still remain to be determined, the biological activities of the proteins identified so far have provided us with a glimpse of the biological processes that can be initiated by proteins present in the tumor microenvironment.

The functional activity of MMP-1 and MMP-9 proteins identified in macrophage secretome was confirmed through zymography. A clear band was observed in MφCM corresponding to the molecular weight of the MMP-1 and MMP-9. MMPs are a family of structural and functionally related endopeptidases. They are secreted as inactive zymogens and are activated by other activated MMPs or serine proteases outside the cell (e.g trypsin, plasmin, kallikrein) [430]. Increased expression and activity of MMP-2 and -9 in tumors has been associated with the degradation of basement membranes, an essential step in tumor invasion and with the tumor grade [431] as well as reduced survival in breast cancer patients.
Studies have also demonstrated a basal level difference in expression of MMP-1, MMP-3 and MMP-13 in breast cancer cell lines. The highly invasive MDA-MB-231 cell line with a higher level of MMP-1, MMP-3 and MMP-13 expression may play a key role in the invasiveness of these cells through basement membranes [433].

Apart from MMPs, the other proteins identified by MALDI-TOF in MφCM also had functional role in actin binding, regulation of cytoskeleton rearrangement and cell migration and invasion. Chitinase-3-like protein 1 (CHI3L1) identified in MφCM, also known as YKL-40, is a glycoprotein secreted by activated macrophages, chondrocytes, neutrophils and synovial cells. It plays a role in the process of inflammation and tissue remodeling and has been linked to activation of the Akt pro-survival (anti-apoptotic) signaling pathway and promotion of angiogenesis through VEGF-dependent and independent pathways [434]. Elevated level of YKL-40 has been suggested as a biomarker of disease severity as it correlated strongly with stage and outcome of various types of cancer [435].

Another protein PAI-1 present in MφCM, is a physiological inhibitor of urokinase-type plasminogen activator (uPA), a serine protease involved in the promotion of cellular de-adhesion, migration/invasion and activation of plasmin from plasminogen [436, 437]. Increased levels of this protease are associated with a poor prognosis of breast cancer [438].

Myoferlin, also present in MφCM is a member of the ferlin family of proteins that participate in plasma membrane fusion, repair and endocytosis, vesicle trafficking and cell motility [439, 440]. Myoferlin also participates in the stabilization of several receptor tyrosine kinases [441].

L-plastin was differentially upregulated in MφCM. A number of experiments performed with macrophages and polymorphonuclear neutrophils (PMN) point to a role for
L-plastin in regulating integrin mediated adhesion [442, 443]. From many in vitro and in vivo studies, there are indications that L-plastin plays a role in tumor cell motility [444].

The protein Dynamin was identified in exclusively upregulated in MφCM. A large GTPase dynamin, is required for endocytic vesicle formation and regulates the actin cytoskeleton [445]. This has been suggested to be a novel antimitotic drug target for the treatment of cancer because the methyl ammonium bromide (MiTMAB) dynamin inhibitors exclusively block the abscission phase of cytokinesis, inhibits cell proliferation and reduce viability [446].

Since all identified proteins point toward cytoskeletal reorganization, the expression of some proteins with such functions like ezrin, radixin and moesin were studied in MCF7 cells treated with MφCM. A significant increase in cytoskeleton associated proteins ezrin, radixin and moesin expression was observed in MCF7 cells with MφCM treatment as compared to MCM treatment. Similar MφCM induced upregulation of these proteins has also been reported in [447]. ERM (ezrin, moesin and radixin) belongs to a larger protein family, known as FERM (4.1 protein, Ezrin, Radixin, Moesin) [448, 449]. These proteins are cytoskeleton associated and have traditionally been known as molecules involved in maintaining the integrity and morphology of cells. They are also involved in the regulation of the migration of the cells and organizing the ruffling of the membrane. They have also been suggested to be candidate molecules in directional cell movement including that of cancer cells [450]. Ezrin has been shown to co-operate with c-Src in mammary cancer cells and regulate cell-cell contact and migration [451]. Moesin acts as a potential epithelial-mesenchymal transition (EMT) marker in breast and pancreatic cancer, and the expression level of moesin is linked to tumor size, invasion, and differentiation of oral squamous cell
carcinoma [452]. The expression level of radixin is found to be significantly increased in colon tumor tissues [453].

Though *in vitro* studies employing either conditioned media or co-culture can give a glimpse of the interaction that occur in the tumor microenvironment, the ultimate test of the hypothesis is validation of these markers in clinical samples. So taking clues from the *in vitro* study, expression of a set of chosen markers were validated in benign and malignant breast cancer. Pathologists have known for decades that breast cancer cannot be described as a single disease and heterogeneity based on tumor morphology, location, grade and lymph node metastasis, expression of hormone and growth factor receptors is well documented. However, in addition to heterogeneity between tumors, it is becoming increasingly appreciated that a high degree of molecular and morphological heterogeneity exists even within tumors, further complicating the development of therapeutic strategies and our understanding of disease progression. Through the use of gene expression profiling and other genomics approaches, the complexity and heterogeneity of breast cancer has been confirmed and emerging evidence has indicated that the tumor microenvironment plays a pivotal role in driving tumor heterogeneity.

*ONCOMINE*, a cancer microarray database and web-based data-mining platform aimed at facilitating discovery from genome-wide expression analyses. Differential expression analyses comparing breast cancer with respective normal tissues as well as a variety of cancer subtypes and clinical-based and pathology-based analyses was studied for iNOS, p53, H2AX and CREB. The data base constitutes of many studies which compare markers expression between different carcinoma subtypes. iNOS and p53 mRNA was significantly upregulated in invasive breast carcinoma as compared to normal breast tissue. Studies in the database for H2AX expression show a fold change of 2.588 (p<0.0001) between normal and invasive breast carcinoma (TCGA Breast Statistics, 2010) and fold
change of 1.226 (p=0.017) in adenocarcinoma and ductal breast carcinoma. CREB mRNA was significantly upregulated in invasive breast carcinoma in data sets as compared to normal breast tissue.

In the present study, the expression of iNOS, p53, γ-H2AX, pCREB and CREB were found to be crucial for increased migration in ER/PR positive MCF7 cells. Hence these markers were chosen for IHC analysis. CD68, a macrophage marker also was studied in these samples. However the labeling with the antibody clone (KP-1) seemed to be non specific and hence was not included in further analysis.

Invasive ductal carcinoma not otherwise specified (IDC NOS) was found to be the most common type in Indian population (88%) followed by infiltrating lobular carcinoma (3.7%), colloid carcinoma (1.1%) and ductal carcinoma in situ (DCIS) (1.1%) and metaplastic types (0.9%) [454]. In the present studies, 24 benign fibroadenoma samples and 91 IDC samples were studied for expression of these markers.

The incidence of breast cancer has been consistently increasing and it is estimated that it has risen by 50% between 1965 and 1985 [455]. The rise in incidence of 0.5-2% per annum has been seen across all regions of India and in all age groups but more so in the younger age groups (<45 years). More than 80% of Indian patients are reported to be younger than 60 years of age [6]. In this study, the age of IDC patients ranged from 28-77 years with a mean of 58.10±10.48 years. The demographics recorded show that patients in the study group were largely representative of overall population of breast cancer patients. The peak age frequency was in the age category of 56-70 years at the time of diagnosis. In Indian population, the average age of breast cancer patients is reported to be 50–53 years in various population-based studies [456]. While the majority of breast cancer patients in western countries are postmenopausal and in their 60s and 70s, the picture is quite different in India.
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with pre-menopausal patients constituting about 50% of all patients [457]. In this study, 37.5% patients were below 55 yrs age and 62.5% patients were above 55 yrs age.

The majority of breast cancers diagnosed in the study were grade III cancers (47%) with grade I and grade II cancers representing 16% and 37% of the cases. Reports also suggest that in Indian population the majority of patients are detected with grade III disease [458]. Grade of cancer is an important prognostic factor and is used as one of the components of the Nottingham Prognostic Index (NPI).

The tumor size was recorded in each case and there was a wide range from 5 mm to 90 mm with a mean of 36 mm. The cancers were divided into size groups according to the TNM staging system. The majority of tumors in the group were T2 cancers (20-50 mm). Though the average tumor size in Indian population is reported to be 54 mm and this presentation is similar in many other developing countries [459]. In this study, the patient’s tumor size significantly correlated with the patient’s age at diagnosis (p=0.0288) and tumor stage (p=0.0031), as patients detected with advanced stage had larger tumor size.

The frequency of distribution of IDC patients according to pathological TNM staging was 5.8%, 58.1% and 36.1% for Stage I, Stage II and Stage III respectively. This was consistent with data from Indian breast cancer patients where in Stage I: 1–8%; Stage II: 23–58%; Stage III: 29–52 % patients were recorded [460]. The data from the present studies also correlated with the Mumbai’s breast cancer statistics which had reported around 7% patients with stage I, 57% patients with stage II and 28% patients with stage III cancer. In the present work, the patient’s stage correlated with tumor size (p=0.0305) and LN metastasis positivity (p<0.0001). The advanced stage samples were LM Mets positive.

Though not used in the NPI, lympho-vascular vessel space invasion (VSI) is recognised as an independent prognostic indicator. The presence of VSI is important when
making decisions regarding adjuvant therapy. In this study, 28.2% of IDC samples were VSI positive. The patient’s data show that VSI positivity was associated with high grade tumors (p=0.0406).

Lymph node status is established as the single most significant prognostic factors in invasive breast cancers [461]. The lymph node status was positive in 59% of IDC samples in the study and it correlated well with the stage of the tumor. The pathological feature of this study group conformed to expectations and standards set by previous studies in breast cancer [462].

In this study group, 61.36% of the IDC samples were ER positive and 68% were PR positive. ER status has been established to be an important factor in breast cancer correlating to pathological features and overall survival. Estrogen (ER) and progesterone receptors (PR) are found positive in only 20-45% of Indian patients. ER-positive rates were reported to be lower in Indian patients than those in western countries as not all patients in India undergo hormonal receptor testing as evident from the study in Delhi which showed only 35.5% of patients had receptor testing [463]. At TMH Mumbai, the ER+ status was found in 33% and PR+ in 46% of patients [458]. Comparatively, in this study higher percentage of ER positive samples was noted. In the present dissertation, the ER positive tumors were significantly related to low grade tumors (p=0.0124) and smaller size tumors (p=0.0436). These results agree with previously published work [30, 464, 465] and it is now widely agreed that ER status is an independent prognostic marker of disease outcome. The results also demonstrated a significant relationship between ER positive and PR positive cancers (p<0.0001) confirming that progesterone receptors expression could be induced by estrogen [466]. As the PR gene transcription is regulated by estrogen, PR expression has been considered to be a marker of functioning ER [467]. PR expression was significantly associated with low grade tumors (p=0.0042). This study showed an inverse correlation between ER positive and Her2
positive tumors (p<0.0001). This was in concordance with the previous studies [468], though it is suggested that this relationship or predictive power of ER status or Her2 status is more apparent in the elderly patients [469]. When considering PR correlation with other receptors, it was clear that it was significantly associated with ER (p<0.0001) and like ER it was also negatively correlated to Her-2 (p< 0.0001).

In this study, it was seen that the majority of the tumors were ER/PR double positive (56.8%) or ER/PR double negative (32.95%). The other 2 groups, ER+ve/PR-ve (4.54%) and ER-ve/PR+ve (5.68%) were too small. In larger studies, it has been suggested that ER/PR double positive cancers are more responsive to hormonal therapy and hence have a better outcome when compared to ER+ve/PR-ve cancers. This again is based on the theory that PR represented a functioning ER signaling system [470].

It is generally accepted that Her2/neu over expression is a marker of increased tumor aggressiveness [471] and in many studies Her2/neu over-expression has been related to a poorer overall survival. In this study, Her2/neu was over expressed in 40.69% of the cases. As with the previous receptors, the correlation of Her2/neu expression with the other pathological features of the tumors was also studied and the over expression of Her2/neu was not significantly associated with any of the clinico-pathological feature. It is widely reported that Her2/neu over expression is inversely associated with ER/PR expression [472]. The results from the present study are in agreement with a significant inverse relationship between Her2/neu expression with ER (p<0.0001) and PR (p<0.0001) status.

ER, PR and Her-2/Neu receptor negative breast cancer (triple negative), is biologically aggressive cancer phenotype which is resistant to conventional cytotoxic chemotherapy treatment and is associated with reduced survival compared to other subtypes of breast cancer [473]. In this study, 8.53% samples were triple negative cancer.
In this dissertation, expression of iNOS, p53, γ-H2AX, pCREB and CREB was studied in benign fibroadenoma and IDC samples. iNOS was the first NOS isoform implicated in the macrophage-mediated tumor killing process and as a consequence this isoform has been at the center of attention for study of its expression in cancer. In this study, 23 benign fibroadenoma samples and 91 breast cancer samples were analyzed using IHC for the expression of iNOS. The samples were scored according to the cytoplasmic location of the iNOS. The significant outcome of the present study has been to identify differences between iNOS expression in benign fibroadenoma and malignant samples with varying stages and grades of breast tumors.

The iNOS scores were significantly higher in malignant samples as compared to benign fibroadenoma group (p=0.0001). Strong iNOS expression was seen in 47.82% of benign fibroadenoma samples as compared to 87.3% of malignant tumors. Differences in iNOS expression between benign and malignant samples suggest a role for NO in breast carcinogenesis. The present study has demonstrated increased expression of iNOS in malignant tumors as compared to benign samples. Expression of NOS has been reported in malignant tissue derived from gynecological, breast, central nervous system, gastric and colorectal tumors and its role in cancer progression is suggested [186, 187, 474]. Reports on the activity of NOS in breast cancer have been conflicting, with different or completely opposing findings being reported. Most authors reported the presence of NOS activity in carcinoma [186, 475-477]. Among these, some reported higher NOS activity in malignant than in benign breast tissue [186, 476, 477]. A positive correlation between NOS activity and tumour grade is also reported [186, 476, 477] whereas others have also reported the reverse [478]. These paradoxical results may be explained by the fact that at low concentrations, nitric oxide has a tumor promoting effect, whereas at higher concentrations, it has antitumour activity. Although Loibl et al had demonstrated that none of the benign lesions were positive
for iNOS, in the present study, the benign samples were iNOS positive although with low expression. These results draws parallel with an ovarian cancer study which showed that though a majority of malignant samples had NOS activity, iNOS was also detected at lower levels in non cancer samples [479].

A significant association of iNOS expression was not observed with other clinico pathological characteristics of IDC samples in this study. Previous reports indicated that elevated NOS2 expression may be linked to a high grade and poor prognosis in breast cancer [480, 481] and also to poor outcome in other human epithelial cancers. There was a statistically significant higher expression of iNOS in IDC as compared to the benign fibroadenoma samples. Although our hypothesis was that iNOS may be a marker to detect malignant disease and metastasis, these results with limited number of samples demonstrate that it would be useful to differentiate benign and malignant tumors. In this study, there was no association between iNOS positivity and hormone receptor expression. Larger sample size is needed to confirm any such association as there are chances that it can be missed in studies with smaller sample size.

NO has proangiogenic activities [207] and also promotes carcinogenesis through the inactivation of wild-type p53 function, by either causing loss of DNA-binding activity [482] and/or selecting for mutant p53 [483]. In the current study, p53 expression status in benign and malignant samples was evaluated. Although p53 antibody used in this study could not differentiate wild-type p53 from mutant p53, previous studies have indicated that p53 protein detected by immunohistochemistry is representative of mostly mutant p53 along with accumulation of p53 and is an indicator for a loss of p53 tumor suppressor function. The p53 accumulation is caused by both mutations and protein-protein interactions [484]. High coincidental expression of iNOS and p53 protein was observed (spearsman r coefficient=0.3461, p=0.0025). Rajnkova et al., reported that increased expression of iNOS
may promote gastric cancer progression by providing a selective growth advantage to tumor
cells with non-functioning p53 [485]. Thus p53 protein accumulation may also be important
event to enhance breast carcinogenesis. Mutations in the tumor suppressor gene p53 are
present in 18%–25% of primary breast carcinomas [486]. p53 expression was studied in 22
benign fibroadenoma and 76 IDC samples. Similar % of samples from both groups were
positive for p53 (77.27% of benign fibroadenoma and 75 % of IDC samples). The mean p53
score in in fibroadenoma samples was 2.227±0.349 and that of malignant samples was
4.625±0.502. Although p53 expression was higher in IDC samples, this was not found to be
statistically significant.

Studies have reported that p53 mutation does not impact the outcome of early breast
cancer and that the evidence is not strong enough for p53 status to be recommended as a
routine marker in clinical practice [487]. In contrast to this, some studies have indicated that
abnormal p53 immunohistochemical expression, or p53-positive status, was associated with
more aggressive tumor features, a higher tumor grade, negative estrogen and progesterone
receptor (ER/PR) status and the more aggressive basal subtype [488]. Breast tumors
expressing high levels of p53 are more frequently ER-negative and PR-negative. They are
also associated with a high proliferation rate, high histological and nuclear grades, aneuploidy
and poorer survival. Consistent with earlier reports, in this study, higher p53 expression was
seen along with negative estrogen (p=0.0384) and progesterone receptor (p=0.0079) status. A
high p53 level is frequently observed in tumors over-expressing as Her-2/neu [489]. In
accordance to this, higher p53 expression was seen in Her2/ neu positive samples (p=0.0095).
The mean p53 scores of patients with ER+PR+Her2+ was 2.3±3.27, ER+PR+Her2− was
3.15±4.06, ER−PR−Her2+ was 7.25±4.39 and ER−PR−Her2− was 1±1. p53 expression scores
were highest in ER−PR−Her2+ group whereas ER−PR−Her2− group showed lowest p53
expression. A co-existence of HER2/neu over-expression and p53 protein accumulation has
been suggested to be a strong prognostic molecular marker in breast cancer [490]. Insignificant association of p53 expression with age, tumor size, grade, stage, LM mets and VSI was observed.

p53 is a key player in the tumor suppressive DNA damage response (DDR) and is mutationally inactivated in approximately 50% of human cancers. Cellular responses to DNA damage are mediated through highly conserved DNA damage checkpoint mechanisms that are important for tumor suppression by arresting cell cycle progression, or evoking cellular senescence and apoptosis [491]. As a result of DNA DSBs in eukaryotic cells, the serine amino acid at position 139 of the H2AX proteins is phosphorylated in response to DNA damage [232]. Detection of $\gamma$-H2AX foci has been used as a biomarker for aging and cancer, as a biodosimeter for drug effects and radiation exposure in chemo- and radiotherapy respectively [492, 493]. The aim of the study was to assess the expression of $\gamma$-H2AX in a cohort of 23 benign fibroadenoma and 82 IDC patients and correlate its expression with clinico-pathological parameters.

Nuclear $\gamma$-H2AX staining was observed in tumor cells. All of the benign fibroadenoma samples were positive for $\gamma$-H2AX expression while 77% of IDC samples were positive for $\gamma$-H2AX labeling. Though the IDC samples showed higher mean $\gamma$-H2AX expression scores, it was statistically insignificant from $\gamma$-H2AX expression scores of benign fibroadenoma samples. Similar differences in expression of $\gamma$-H2AX has been observed in a study with benign nevus and malignant melanoma cases [494].

As DNA damage gradually accumulates during lifetime, both the likelihood of oncogenic transformation as well as tissue dysfunction and degeneration increases with age. The increasing trend in the $\gamma$-H2AX expression score was seen with increase in patient’s age, although not differing statistically. The median $\gamma$-H2AX scores of different groups viz. age
Discussion

25-40, 41-55, 56-70 and above 70 were 3, 3.5, 4 and 6 respectively. No direct significant association was observed with γ-H2AX expression and tumor size, grade, stage, LN Mets and VSI.

The γ-H2AX expression scores were highest in triple negative (ER-PR-Her2+) group whereas triple positive (ER+PR+Her2+) group showed lowest γ-H2AX expression. This was in accordance with reported data that triple negative breast cancers display more endogenous γ-H2AX expression [495] and that his subset of cancers has a higher incidence of aberrations in components of the DNA damage repair pathway [495, 496].

Studies have shown that CREB may act as a positive or negative transcription regulator in various human benign and malignant conditions [410, 497-499]. It has been reported that CREB may act as a positive transcription regulator of aromatase and hence increased estrogen synthesis in breast cancer cells [356]. However, despite the extensive work on aromatase, little information is available on the expression and role of CREB in human breast cancer. Kovach et al had reported that total CREB and phosphorylated CREB (pCREB) proteins were both significantly elevated in hepato cellular carcinoma versus normal liver [500]. In resting cells, CREB exists in an unphosphorylated state that is transcriptionally inactive but can bind to DNA. Upon activation, CREB becomes phosphorylated, which induces its transcriptional activity by promoting its interaction with the 256-kDa coactivator protein CREB binding protein (CBP).

The aim of this study was to investigate the correlation of pCREB and CREB expression between benign fiboadenoma and IDC together with clinico-pathological characteristics of IDC samples. In the current study, CREB expression was studied in 22 benign fibroadenoma and 82 IDC samples. The median pCREB score in benign fibroadenoma samples was 7.33 whereas median pCREB score in malignant samples was
The pCREB score distribution in benign fibroadenoma and malignant group did not differ significantly. The median score in benign fibroadenoma was 2 as compared to that of 4 in malignant samples. CREB score distribution in benign fibroadenoma and malignant group differed significantly (Mann Whitney U test, p value = 0.0499). A significant correlation between p53 expression and CREB expression in IDC samples was also observed (Spearsman r coefficient = 0.2670, p=0.0277).

Though an insignificant association was observed with tumor grade and patient’s age with pCREB and CREB expression, a significant association with tumor size was seen with CREB. An inverse correlation was also observed with pCREB expression and tumor TNM staging of IDC samples (p=0.0487). The median pCREB scores of Stage I tumor was 9.16, Stage II tumor was 7.11 and Stage III tumor was 6.66. The highest pCREB expression was seen in Stage I samples whereas Stage III samples showed the lowest pCREB expression. An insignificant difference in CREB scores distribution was observed when CREB scores of IDC patients in different TNM staging groups were compared. The median CREB scores of Stage I tumor was 5, Stage II tumor was 3 and Stage III tumor was 4.5.

CREB and pCREB expression was not correlated with lymph node metastasis, VSI and hormone receptors levels in IDC samples. This may be attributed to the small sample size that has limited the ability to derive statistically significant results and correct for differences in subgroup characteristics.

The findings reported herein emphasize that macrophages can induce change in tumor cells resulting in increased migratory properties. Macrophage mediated pro-inflammatory cytokines induce TGF-β1 further driving the ROS/ATM/CREB signalling axis in tumor cells ultimately resulting in increased migration. This has been conclusively proven in this dissertation by blocking the pathway at every step which ultimately leads to abrogation of
MφCM induced pCREB as well as migration. The effect of these macrophages seems to be differential since the highly invasive MDA-MB-231 cells already having a high basal level of this signaling axis seems to be tolerant to further induction.

The complexity of cell-cell interaction by soluble factors is further exemplified by the fact that apart from the pro-inflammatory cytokines that induce TGF-β1/ROS/ATM/CREB signaling axis, there are many proteins present in the secretome of the macrophages. One interesting fact that emerges from this study is the identification of proteins involved in breakdown of extracellular matrix, membrane trafficking, cytoskeletal re-organization and cell migration in macrophage secretome further strengthening our hypothesis that macrophages secreted factors result in increased migration of tumor cells.

The crucial players of this macrophage–tumor cell interaction identified from the *in vitro* study were also subjected to validation in clinical samples. The most significant result from this study is that iNOS and CREB expression were significantly correlated with the malignancy of disease. There was also a significant positive correlation of p53 with iNOS as well as CREB in malignant samples. Expression of pCREB was significantly but inversely associated with the staging of the IDC samples and p53 expression was significantly associated with expression of hormone receptors.

The findings reported in this dissertation emphasize the importance of soluble factors or cytokines in the tumor microenvironment. It also reiterates the role of cytokine signaling network between the macrophages and the tumor cells. The study provides framework for targeting the stromal factors for effective control of tumor growth and metastasis. The proteomic studies gives a glimpse into the complexity of the tumor microenvironment and have given an insight of the many different proteins present in the tumor microenvironment that could affect the behavior of the cancer cells apart from the major players. This
knowledge of the stromal factors can also lead to predictive prognostic assays as well as improvement of cancer therapeutics. The potential markers, iNOS and CREB were significantly overexpressed in malignant breast cancer as compared to the benign disease. It would be interesting if these markers can be used along with the existing set of diagnostic markers that can determine course of therapy as well as responsiveness to different drugs. As seen from the in vitro study, some of these markers are promising targets for cancer therapeutics. Additional data with survival status of patients as well as greater sample size can confirm if these markers can be used for prognosis as well.
4.2 Summary

Macrophages and tumor cells mutually influence each other’s behaviour in majority of cancers, with the tumor cell attracting macrophages and sustaining their survival and they, in turn, producing a myriad of factors to promote or regulate tumor growth and angiogenesis. The main finding of this study is that the pro-inflammatory cytokine cocktail of TNF-α, IL-1β and IL-6 secreted by macrophages induce secretion of TGF-β1 in MCF7 cells. This results in dichotomy of responses with apoptosis in a fraction of cells and activation of MAPK pathway, increase in redox signalling and DNA damage response, in the remaining cells. All these events triggered CREB mediated survival signaling inducing EMT responses. Blocking of all the upstream events resulted in abrogation of MφCM induced pCREB expression and migration further confirming our hypothesis. Interesting information that emerged from these studies is that some breast cancer cell types could be refractory to the effect of macrophage induced signaling that resulted in increased migration.

Apart from the proinflammatory cytokines, many other crucial players were identified in macrophage secretome that also could contribute to EMT changes in MCF7 cells. All these proteins play a functional role in tissue remodeling, breakdown of extracellular matrix, membrane trafficking and cell migration.

The significance of these markers identified from the in vitro study was also validated in clinical benign fibroadenoma and IDC samples. In IDC, the patient’s tumor size correlated with the patient’s age at diagnosis (p<0.05) and tumor stage (p<0.01). The stage of tumor correlated with tumor size (p<0.01) and LN metastasis positivity (p<0.0001). The patient’s data also showed that VSI positivity was associated with high grade tumors (p<0.05).
ER+ tumors were significantly related to low grade tumors (p<0.01) and smaller size tumors (p<0.01). PR expression was significantly associated with low grade tumors (p<0.01). ER expression correlated significantly with PR (p<0.0001) and negatively correlated to HER-2/neu expression in IDC samples (p<0.0001).

A statistically significant higher expression of iNOS and CREB was observed in IDC samples as compared to the benign fibroadenoma samples. A significant association of iNOS expression was not observed with other clinico pathological characteristics of IDC samples in this study. High coincidental expression of iNOS and p53 protein accumulation was observed (spearsman r coefficient=0.3461, p=0.0025). Although the p53 expression was higher in IDC samples, this was not found to be statistically significant. A higher p53 expression was seen along with negative ER (p=0.0384) and PR (p=0.0079) status. A higher p53 expression was seen in Her-2/neu positive samples (p=0.0095).

Though the IDC samples showed higher mean γ-H2AX expression scores, it was statistically insignificant from γ-H2AX expression scores of benign fibroadenoma samples. No direct significant association was observed with γ-H2AX expression and tumor size, grade, stage, LN Mets and VSI.

The CREB score distribution in malignant and benign fibroadenoma group differed significantly (Mann Whitney U test, p=0.0499). A significant correlation between p53 expression and CREB expression in IDC samples was observed (spearsman r coefficient =0.2670, p=0.0277).
In summary, this comprehensive study of macrophage-tumor interactions may provide new diagnostic markers as well as unique therapeutic opportunities directed at the microenvironment to improve patient response to therapies and improve survival rates.

**Figure 52: Schematic representation of the effect of MφCM on MCF7 cells.** Pro-inflammatory cytokines TNF-α, IL-1β and IL-6 along with MMP-1, -9 and other proteins secreted by macrophages induce secretion of TGF-β1 in MCF7 cells. This results in apoptosis in a fraction of cells. In the remaining cells, there is activation of ROS/ATM/CREB signalling axis resulting in EMT responses.
4.3. Conclusions

The major conclusions of the present dissertation are as follows:

- Macrophage conditioned medium (MΦCM) containing elevated levels of cytokines TNF-α, IL-1β and IL-6 had a differential effect on non-invasive (MCF7) and highly invasive (MDA-MB-231) breast cancer cell lines.
- MΦCM induced the secretion of TGF-β1 and IL-6 in MCF7 cells.
- MΦCM decreased colony numbers in both the cell lines but it was associated with increase in apoptosis, ROS and RNS generation only in MCF7 cells.
- MΦCM had a differential effect on mitochondrial membrane potential in MCF7 and MDA-MB-231 cell lines with hyperpolarization in the former and no significant effect on latter.
- Activation of MAPK pathway and upregulation of EGFR observed in MCF7 cells with MΦCM treatment.
- Increased phosphorylation of ATM and H2AX as well as PARP cleavage following MΦCM treatment was observed in MCF7 cells and not in MDA-MB-231 cells.
- Increased phosphorylation of CREB and stabilization of total CREB protein was observed in MCF7 cells following treatment with MΦCM. In contrast, expression of pCREB was higher in MDA-MB-231 cells which did not change further with MΦCM treatment.
- Neutralization of TNF-α, IL-1β and IL-6 in MΦCM or pre-treatment with inhibitors of ROS, RNS or DNA damage as well as ATM knockdown abrogated the MΦCM induced expression of pCREB as well as migration through a transwell insert.
• Apart from pro-inflammatory cytokines, numerous proteins were identified by MALDI-TOF analysis which had a functional role of tissue remodeling, breakdown of extracellular matrix, membrane trafficking and cell migration.

• Bioinformatics analysis demonstrated transcript level upregulation of proteins like iNOS, p53, H2AX and CREB in invasive breast cancer as compared to normal breast tissue.

• In clinical samples, iNOS and CREB expression were significantly higher in IDC as compared to benign fibroadenoma.

• The expression of p53 was positively correlated with iNOS and CREB in IDC.

• p53 expression was associated with hormone receptors in IDC.

• pCREB expression in IDC was inversely associated with the staging of the tumor.

• iNOS and CREB can be potential diagnostic biomarker to detect invasive ductal carcinoma.

Figure 53: Summary chart of in vitro observations in MCF7 cells with MφCM treatment.
4.4 Future directions

A logical extension of this work would require the following:

- Identification of the key players in the TGF-β1-ROS-ATM-CREB signaling pathway induced by MφCM in MCF7 cells.
- Studying the interaction of SMAD pathway and TGF-β receptors with key transcription factors such as ATF2, Jun, JunB, JunD, Sp1, Sp3, Fos, Mixer, Runx etc.
- Study of the effect of TFG-β1 on the cell cycle regulation in the synchronized cells.
- Elucidation of the downstream mediators of CREB signaling by silencing CREB or by inhibition of its phosphorylation.
- To study the role of in vitro differentiated macrophages of M1 and M2 phenotypes from peripheral blood monocyte on MCF7 and MDA-MB-231 cell line.
- To further identify the macrophage mediated signaling pathway in tumor bearing mouse models and to study the CREB signaling under in-vivo conditions.
- The study of clinical markers such as chemokine receptors CXCR4; chemokine ligands CCL2 and 5; growth factors EGF, HGF, IGF and TGF-β; immunosuppressive Treg cells markers FOXP3 or micro RNA such as miR-497, miR-373 in light of their value as prognostic or predictive factors. To study their expression and its correlation with clinic-pathological parameters as well as 5 years survival data to identify their potential for integration into clinical practice.