Progression and Metastasis of Lung Cancer - A Study of Predominant Cellular Interactions in Tumor Microenvironment

THESIS SUBMITTED FOR THE AWARD OF

DOCTOR OF PHILOSOPHY

IN

BIOTECHNOLOGY

BY

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CERTIFICATE

This is to certify that the work contained in this thesis entitled,**"*Progression and Metastasis of Lung Cancer - A Study of Predominant Cellular Interactions in Tumor Microenvironment*"**, is the bonafide research work of ***Mr. Asif Amin*** and is worthy of consideration for the award of Doctor of Philosophy in Biotechnology.

Prof. G.N Lone
(Co-Supervisor)

Prof. Raies A. Qadri
(Supervisor and Head of the Department)

DECLARATION

The work entitled, **"*Progression and Metastasis of Lung Cancer - A Study of Predominant Cellular Interactions in Tumor Microenvironment*"**, presented in this thesis embody the original work done by me for the Doctor of Philosophy (Ph.D.) degree in the Department of Biotechnology at University of Kashmir, Srinagar. This work has not been submitted in part or in full for any other degree or diploma.

Asif Amin

To my parents whose prayers and love sustained me throughout

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Though many have not been mentioned, none is forgotten

Asif Amin

Contents

ABBREVIATIONS

BSA	Bovine serum albumin
CAF	Cancer associated fibroblast
CBB	Coomassie Brilliant Blue
CCM	Co-culture conditioned medium
CM	Conditioned medium
CSC	Cancer stem cell
CSF	Colony Stimulating factor
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle's medium
DTT	Dithiothreitiol
ECL	Enhanced chemiluminescence
ECM	Extra Cellular Matrix
EDA	Extra domain A
EDB	Extra domain B
EDTA	Ethylenediamine tetra acetic acid
EGF	Epithelial growth factor
EGFR	Epithelial growth factor receptor
ELISA	Enzyme linked immunosorbent assay
EMT	Epithelial-mesenchymal transition
FGF	Fibroblast growth Factor
FITC	Fluorescein isothiocyanate
FN	Fibronectin
h	hours
HGF	Hepatocyte growth factor
HIF	Hypoxia Inducing Factor
HPLC	High-performance liquid chromatography
HRP	Horse radish peroxidise
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IP	Immunoprecipitation
Iκ-B	Inhibitor of kappa B

LB	Luria Broth
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
M	molar
MAPK	Mitogen-activated protein kinase
MDSC	Myeloid Derived Supressor cell
MHC	Major Histocompatibility Complex
min	minutes
MMP	Matrix metalloprotease
MSC	Mesenchymal stem cells
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF-κB	Nuclear factor-kappa B
NK cell	Natural Killer cell
NSCLC	Non–small cell lung cancer
OPD	Ortho-phenylene diamine
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PDGF	Platlet derived growth factor
PGE2	Prostaglandin E2
RANKL	Receptor activator of nuclear factor kappa-B
RPMI	Roswell Park Memorial Institute
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TAM	Tumor-associated macrophage
TBABH	Tetra-butyl ammonium borohydride
TEMED	N, N, N’, N’- Tetramethylethylenediamine
TGF-β	Transforming growth factor beta
Th cell	T helper cell
TIL	Tumor Infiltrating Lymphocyte
TLR	Toll like Receptor
TMB	3, 3’, 5, 5’ tetra methyl benzidine
TNF-α	Tumor necrosis factor alpha

Treg	T- regulator cells
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor receptor

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SUPPLEMENTARY DATA

The heterotypic view of cancer envisions solid tumors as ecosystems consisting of abnormal epithelial tumor cells and a plethora of cell types collectively referred to as stromal cells. In these ecosystems, innate immune cells are highly represented and most abundant among these are macrophages. Macrophages commonly designated as Tumor associated macrophages (TAMs) in the tumor microenvironment, originate from circulating pool of monocytes and play a critical role in orchestrating and promoting tumor growth. The acquisition of tumorigenic properties by TAMs relies upon a complex interplay between them and tumor cells. In this study, we demonstrated through co-culture experiments that such properties of TAMs are shaped up by the tumor-derived secretory signals which favor their tumor promoting phenotypes. Co-culture of model human monocytes (THP-1) with model human lung carcinoma cells (A549) enabled THP-1 cells to secrete tumor-promoting cytokines and therefore enhanced proliferation, migration and invasiveness of A549 cells. We demonstrated that A549 cells consistently secrete EDA-containing Fibronectin that mediates the pro-inflammatory response from THP-1 monocytes in a paracrine manner. Ablation of such responses by the treatment of THP-1 cells with TLR-4 blocking antibody implicated Fibronectin-TLR4 axis in tumor-associated inflammation and suggests a paradigm wherein lung carcinoma cell derived EDA-containing Fibronectin drives a pro-inflammatory and pro-metastatic tumor microenvironment. Intriguingly, it was also observed that the EDA-containing Fibronectin from A549 cells promotes their migration, invasion and anchorage independent growth in an autocrine fashion. Finally, we showed that the EDA-containing Fibronectin imparts metastatic capacity to A549 cells through the activation of epithelial–mesenchymal transition program. Collectively this study identifies a mechanism of communication between lung carcinoma cells and monocytes and reveals a novel oncogenic role of lung cancer derived EDA-containing Fibronectin in the establishment or promotion of pro-inflammatory and pro-metastatic tumor microenvironment.

The hallmarks of cancer comprise various biological capabilities including sustained proliferative signaling, evasion from growth suppressors, resistance to cell death, replicative immortality, enhanced angiogenesis, pro-invasive and pro-metastatic properties, reprogramming of energy metabolism and evasion from immune destruction. Underlying these hallmarks are genome instability, which generates the genetic diversity that expedites their acquisition, and inflammation, which fosters multiple hallmark functions (Hanahan and Weinberg, 2011; Hanahan and Coussens, 2012). In addition to genetically altered malignant cells, tumors contain a repertoire of recruited distinct cell types collectively referred to as "stromal cells", whose dynamic interactions profoundly contribute to the acquisition and development of hallmark capabilities. These recruited cells including immune cells, cancer associated fibroblasts, lymphatic endothelial cells and angiogenic vascular cells together with the extracellular matrix components constitute the tumor microenvironment. There is growing evidence indicating that these stromal cells play a critical role in the progression of various cancers, including lung cancer. Through the production of cytokines, chemokines, growth factors and matrix degrading enzymes, accessory stromal cells help tumor growth and metastatic dissemination by disrupting basement membrane, supporting blood vessel formation and attracting tumor cells to distant sites. In turn tumor cells through direct cell-cell interactions as well as by the production of secreted factors activate the stromal cells, eventually developing permissive niches that, in return, promote tumor cell survival and proliferation. Thus the dynamic and reciprocal interactions between tumor cells and the cellular components of tumor microenvironment orchestrate events critical to tumor evolution towards metastasis.

It is now well known that the tumors are capable of both evading and exploiting the immune surveillance mechanisms and can thrive alongside a competent immune system. The mutated premalignant tumor cells on their way to giving rise to a tumor acquire the abilities like withstanding the chronically inflamed microenvironment, evading immune recognition and suppressing the immune reactivity, collectively referred to as the immune hallmarks of cancer (Cavallo *et al*., 2011). The inflammatory cells present within the tumor microenvironment polarize the host immune responses towards specific phenotypes favoring tumor

growth and progression. There are increasing evidences which point out at the role of chronic innate immune cells like macrophages and neutrophils in tumor development. Therefore in contrast to erstwhile point of view which suggested that host immune system was protective against cancer, it is now very explicit that the innate immune cells facilitate the progression and metastasis of tumors (Hanahan and Coussens, 2012).

Infiltration by immune cells is a hallmark of most of the solid tumors. The stroma of a full-blown tumor becomes infiltrated with many different leukocyte populations including monocytes/macrophages, natural killer cells, dendritic cells, neutrophils, eosinophils, basophils, (Lin and Pollard, 2004). Tumor infiltrating macrophages, also referred to as tumor educated or tumor-associated macrophages (TAMs) are key regulators of the complex interplay between a tumor and the immune system. TAM-tumor cell interaction results in the release of a variety of cytokines, chemokines and growth factors from macrophages which in turn facilitate the recruitment of additional inflammatory cells and hence amplifying the inflammatory cascade within the tumor microenvironment. The pro-tumorigenic functions of TAMs rely mainly on the proliferative effects of some pro-inflammatory cytokines, such as TNF-α, IL-6, and IL-11 (Putoczki *et al*., 2013), which have been shown to enhance proliferation of lung, liver, and gastric, cancer cells via NF-kB and STAT-3 pathways (Grivennikov *et al*., 2010). Recent evidence also suggests that TAM-derived inflammatory insults act on tissue stem cells to promote tumorigenesis. TAMs through direct cell–cell interactions with cancer stem cells involving Thy1 and Eph4A receptors have also been reported to induce activation of NF-kB, which in turn sustains the cancer stem cell state (Lu *et al*., 2014). TAMs also favor tumor growth by producing immunosuppressive factors such as IL-10, TGF-β, and prostaglandin E2 (PGE2) which help to recruit immunosuppressive T regulatory cells (TREG) to the tumor microenvironment (Ojalvo *et al*., 2009). Additionally, TGF-β is a potent inducer of EMT and promotes the invasive and metastatic behavior of tumor cells. In summary, factors produced by TAMs stimulate tumor cell survival, proliferation and motility, thereby emphasizing the close link between the inflammatory mediators and tumor progression.

Mounting evidences suggest that the acquisition of TAM specific properties by tumor infiltrating monocytes is clearly dependent upon the tumor-derived signals which may vary across different tumor types. The cancer secretome harbors most of the tumor derived signals and includes the proteins released from cancer cells, either through classical or non-classical secretory pathways. These include proteins like adhesion molecules, growth factors, shed receptors, cytokines, proteases etc which can act both locally as well as systematically. Presumptively, the cancer secretome includes all the proteins that are present in the interstitial fluid of a tumor mass (Celis *et al*., 2005). Under *in vitro* conditions it is better envisaged as the group of proteins identified in the conditioned medium of cancer cell lines (Kulasingam and Diamandis, 2008). However owing to the heterotypic nature of tumor microenvironment, cancer cell secretome encompasses proteins that are released from all the participating cells and act in an autocrine or paracrine manner, resulting in the acquisition of a milieu favoring tumor progression and metastasis. In this context, the present study was undertaken to understand the lung cancer cell derived secretory signals that mediates their reciprocal interactions with the monocytes/macrophages and in turn potentiate tumor growth and dissemination.

1.1 Lung Cancer

Lung cancer is the single most devastating cause of cancer-related deaths in developed as well as developing countries (Jemal *et al*., 2011). The high mortality associated with this disease is due to the late diagnosis in majority of the cases (Herbst *et al*., 2008; Youlden *et al*., 2008). Smoking is the major risk factor in lung cancer. Other risk factors include exposure to radon and asbestos, occupational exposures, hormones imbalance and genetic factors (Darby *et al*., 2006; Ganti *et al*., 2006; Ferlay *et al*., 2010; Bouchardy *et al*., 2011). Lung cancer remains a major health concern in Kashmir valley and constitutes about 10% of all cancers (Wani *et al*., 2014). In 2010, the number of lung cancer cases registered at SKIMS, the main oncology facility in the valley was at record high and surpassed the number of esophageal cancer cases, the most prevalent cancer here. Despite advances in diagnostic and treatment modalities, there has been little improvement in survival rates over the past three decades (Siegel *et al*., 2012; Luqman *et al*., 2014). Evidently there is urgent need to understand the mechanistic details of lung cancer pathogenesis and devise strategies for its effective prevention. Insight into the cellular interplay in lung tumor microenvironment holds a great promise in unravelling therapies for lung cancer in particular and other carcinomas in general.

1.2 Tumor Microenvironment

Most of the cancers are carcinomas, originating from epithelial tissues. The epithelial tissue consists of sheet-like organization encapsulated by the basement membrane that separates the epithelial cells from surrounding immune cells, fibroblasts, and cells of vasculature like endothelial cells and pericytes and extracellular matrix, together called as stroma. During the advanced stages of tumor growth, the basement membrane barrier is breached, thus exposing the genetically deranged and proliferating tumor cells to reactive stroma, so termed because of immune cell infilitrations, active angiogenesis and numerous proliferating fibroblasts secreting various factors that are supportive and permissive for the cancerous growth. Thus carcinomas can be depicted as highly complex societies comprising in addition to parenchymal malignant cells, a heterogenous population of non-parenchymal stromal cells including fibroblasts, immune cells and the cells of microvasculature as well as extracellular matrix

components often referred to as tumor microenvironment (Elenbaas and Weinberg, 2001) (Figure 1.1). Cells of tumor parenchyma and the stroma engage in extensive cross talk that profoundly enhances the tumor progression and metastasis.

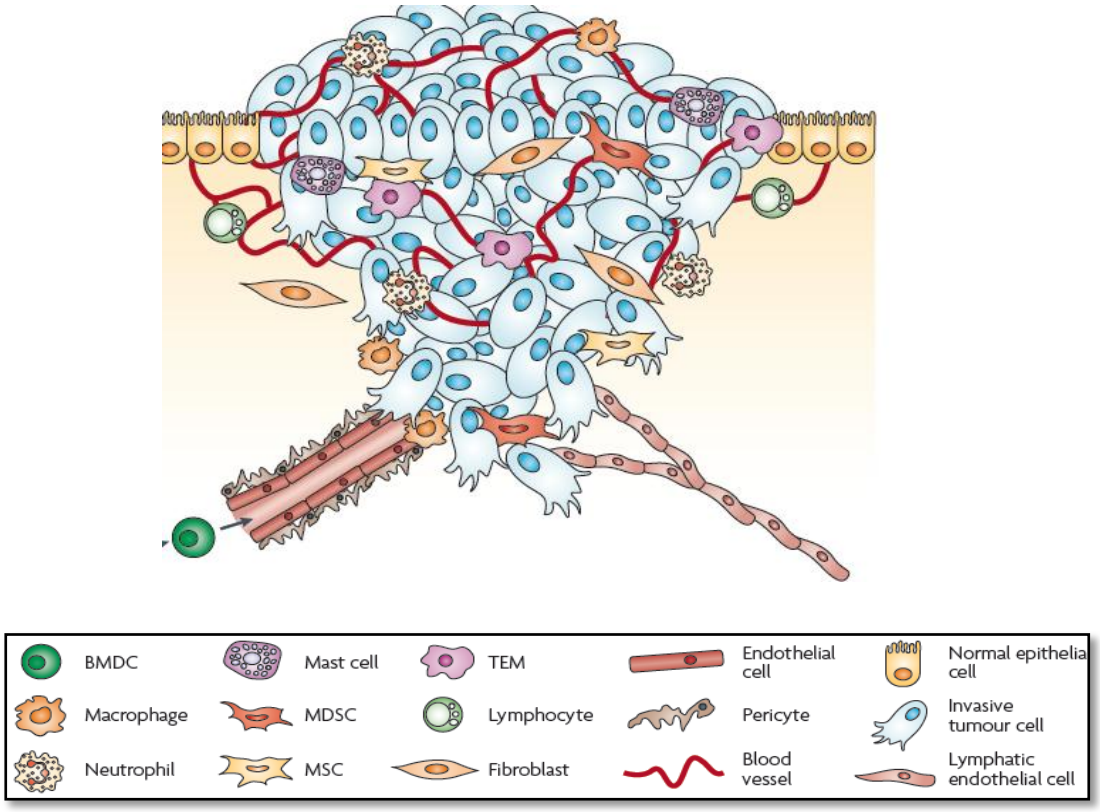


Figure 1.1 Various cellular populations in primary tumor microenvironment *(Adapted from Joyce and Pollard, 2009)*

The intricate interactions of the malignant cells with cellular and acellular components influence tumor growth by altering angiogenesis, suppressing or subverting host immune responses, modulating extracellular matrix and secreting signaling molecules which inturn act on cellular populations to further alter the composition of cellular and acellular microenvironment (Liotta and Kohn, 2001). These complex interactions among the various cell types in tumor microenvironment contribute to the development and expression of certain

hallmark capabilities necessary for tumor growth and metastatic dissemination (Hanahan and Weinberg, 2011) (Figure 1.2). The contribution of stromal cells to cancer development is not limited to primary growth but also involves the establishment of pre-metastatic niches at distant organs (Hanahan and Weinberg, 2011). A better understanding of this ecosystem and the interplay between various cellular members can provide new and better targets of therapeutic implications.

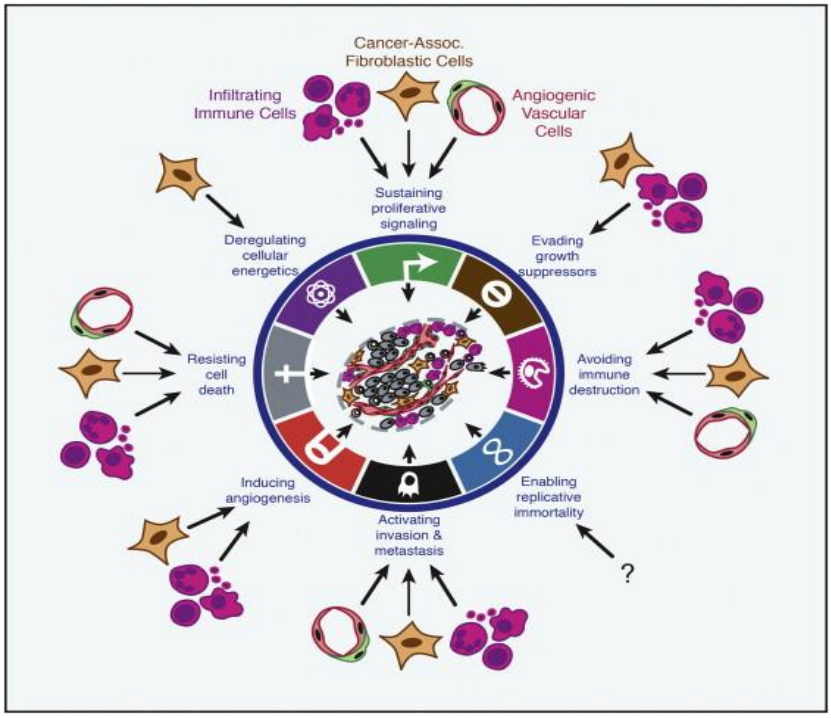


Figure 1.2 Multifactorial contributions of recruited stromal cells to the hallmarks of cancer *(Adapted from Hanahan and Coussens, 2012)*

1.3 Key cellular members of tumor microenvironment

1.3.1 Monocytes/Macrophages: *The inflammatory immune infiltrates*

The interaction between cancer and the immune cells is now well documented. In fact immune reaction to tumors is now recognized as the seventh hallmark of cancer (Zitvogel *et al*., 2006; Mantovani, 2009; Pages *et al*., 2010). The tumor microenvironment contains the cells of both innate (macrophages, dendritic cells,

neutrophils, natural killer cells and myeloid-derived suppressor cells) and adaptive (B and T lymphocytes) immune system, the content being variable with the type of tumors and patients. These cells communicate with cancer cells either directly or through the production of cytokines and chemokines that can have strong implications in the progression and metastasis of a tumor. Immune cells are hypothesized to promote tumor development by triggering inflammatory responses. Inflammation results in the generation of reactive oxygen and nitrogen species that in turn cause mutations in the adjacent epithelial cells (Meira *et al.*, 2008). The presence of cytokines and growth factors in the microenvironment further stimulates the survival and proliferation of these epithelial cells now referred to as 'initiated cells'. Macrophages are considered central to this type of immune response and work in concert with other immune cells in the tumor microenvironment.

Macrophages are the most common and frequently found immune infiltrates seen in both primary and secondary tumors (Bingle *et al*., 2002), where they exhibit a distinct phenotype and are termed tumor-associated macrophages (TAMs). TAMs originate from monocytes which enter tumors through blood vessels, starting from early-stage tumor nodules that are beginning to vascularize to late-stage tumors that are invasive and metastatic (Lewis and Pollard, 2006). Studies in animal models suggest proactive roles of TAMs in promoting angiogenesis and metastatic dissemination (Condeelis and Pollard, 2006). Recruitment of macrophages to the primary tumor sites is associated with poor prognosis in most of the tumors (Scholl *et al*., 1994). Various clinical studies also make a strong case that macrophages promote tumorigenesis. In one meta-analysis, it has been reported that over 80% of studies show a correlation between macrophage density and poor patient prognosis (Bingle *et al*., 2002). A strong association between poor patient survival and increased macrophage density has been observed in thyroid, lung, and hepatocellular cancers (Ryder *et al*., 2008; Zhu *et al*., 2008). In contrast, some studies correlate the increased patient survival with high macrophage densities particularly in pancreatic cancers (Kim *et al*., 2008). In terms of contributions to the tumor progression, TAMs promote inflammation, matrix remodeling, tumor cell invasion, angiogenesis and seeding at distant sites (Condeelis and Pollard, 2006). TAMs produce a number of factors like fibroblast growth factor,

hepatocyte growth factor, epidermal growth factor and transforming growth factor that directly promote growth of tumor cells. The paracrine signaling loop involving CSF-1 (colony-stimulating factor 1) and EGF produced by tumor cells and macrophages respectively has been directly correlated with the metastatic dissemination of the tumor cells (Wyckoff *et al*., 2004). Indeed in human breast cancer patients, overexpression of CSF-1 is often associated with poor prognosis (Scholl *et al*., 1994). Intriguingly removal of the gene encoding CSF-1 in the polyoma middle T (PyMT) oncoprotein mouse model of breast cancer through a homozygous null mutation greatly reduced the macrophage density in tumors and in turn slowed down the tumor progression and metastasis (Lin *et al*., 2006).

1.3.1.1 TAM phenotypes

TAMs are classified into M1 and M2 types. M1 or classically activated macrophages are activated and regulated by T_H1 cytokines like interferon-γ, and TNF-α. These are capable of priming antitumor responses, possess potent antigen presenting ability and produce high levels of proinflammatory cytokines such as TNF-α., IL-6, IL-1, and IL-12 (Mosser and Edwards, 2008) (Table 1.1). Exposure to T_H2 cytokines, IL-4 and IL-13 in tumor microenvironment induces TAMs to develop into polarized or alternatively activated M2 macrophages (Qian and Pollard 2010). M2 macrophages are known to foster a pro-tumorigenic state by initiating proliferation, angiogenesis, tissue remodeling (DeNardo *et al*., 2011) and express anti-inflammatory cytokine IL-10. These cells have poor antigen-presenting capability, produce factors that suppress T-cell proliferation and activity. The presence of M2 macrophages correlates with poor survival in patients with breast cancer, non-small lung cancer and Hodgkin's lymphoma (DeNardo *et al*., 2011). The phenotype of TAMs is plastic, however those responsible for initiation of tumors resembles the M1 type (Gordon, 2003). As the tumors progress towards malignancy, the macrophage phenotype changes from M1 to the one that resembles M2 type and promote tissue formation (Joyce and Pollard, 2009).

1.3.2 Fibroblasts: *The Populous Residents*

Fibroblasts are non-epithelial, non-vascular and non-inflammatory cells of the connective tissue involved in the deposition of the extracellular matrix, regulation of epithelial differentiation and wound healing (Tomasek *et al*., 2002). Fibroblasts associated with carcinomas are referred to as "cancer associated fibroblasts", "Peri-tumoral Fibroblasts", "reactive stromal fibroblasts", "activated fibroblasts" or "myofibroblasts" (Mueller and Fusenig, 2004) and constitute the preponderant stromal cell type in most types of human carcinomas (Sappino *et al*., 1988). CAFs can be derived from local fibroblasts as well as from the population of various progenitor cells including endothelial cells, bone marrow derived cells (BMDCs) through endothelial-to-mesenchymal transition and from cancer cells through epithelial-to-mesenchymal transition (Xing *et al*., 2010). Cancer associated fibroblasts are mostly recognized by their expression of α-smooth muscle actin (Sugimoto *et al*., 2006) and others like fibroblast-specific protein 1, fibroblast activation protein (FAP) and PDGF receptor α/β (Ostman, 2004). CAFs are active players in tumor stroma and promote cancer cell proliferation, angiogenesis, invasion and metastasis. The role of CAFs in malignant progression has largely been defined by Xenograft studies in mice wherein CAFs mixed with epithelial cancer cells showed more competency than normal fibroblasts in promoting tumorigenesis.

The tumor promoting abilities of the CAFs can be attributed to the various secretory effectors like growth factors and cytokines including SDF-1, HGF, EGF, TGF-β, PDGF, VEGF matrix metalloproteinases and (Table 1.1). It has been shown in a breast cancer model that CAFs play important role in the progression of invasive tumors to metastatic tumors via TGF-β dependent mechanism. Co-cultures of fibroblasts and tumor cells significantly increased the levels of fibroblast derived TGF-β resulting in enhanced migration of tumor cells *in vitro* and malignant behavior *in vivo* (Stuelten *et al*., 2010). Increased expression of TGF-β correlates with the accumulation of fibroitic desmoplastic tissue, as shown in an experimental model of pancreatic carcinoma (Lohr *et al*., 2001). In another study, SDF-1 secreted by the fibroblasts has been shown to stimulate the growth of MCF breast cancer cells as well as initiating the recruitment of the bone

marrow derived endothelial progenitor cells (Orimo *et al*., 2005). In a study by Wang *et al* (Wang *et al*., 2009) fibroblast derived HGF has been implicated in inducing the gefitinib resistance of lung cancer cells harboring EGFR-activating mutations. Bartling *et al* (Bartling *et al*., 2008) studied the impact of fibroblasts in altering the responsiveness of lung cancer cells to chemotherapeutic agents paclitaxel and cisplatin. Conditioned medium from WI-38 fibroblasts impaired H358 cell death induced by paclitaxel but not by cispatin via Erk ½ and Akt kinase pathways. A Cross-species functional characterization of mouse and human lung CAFs linked the secretion of CLCF1 and IL-6 from CAFs to the tumor progression *in vivo* (Vicent *et al*., 2012). In a recent study CAFs have been reported to modulate the self-renewal and proliferation of cancer stem cells *in vitro* and *in vivo* (Liao *et al*., 2010; Giannoni *et al*., 2010).

In addition to promoting the proliferating and angiogenetic abilities, CAFs have been implicated in enhancing the migratory and invasive properties of the tumor cells. When activated, Fibroblasts secrete a variety of ECM degrading Proteases like MMPs allowing cancer cells to cross tissue barriers (Stetlor *et al*., 1993). MMP3 cleaves the extracellular domain of E-cadherin, thus promoting the epithelial cells to undergo EMT (Lochter *et al*., 1997). MMP-1 also promotes cancer cell migration and invasion by cleavage and activation of tethered-ligand receptor PAR1. Besides these, fibroblast secreted growth factors such as TGF-β, HGF and FGF have been shown to be important stimuli for EMT (Muller *et al*., 2002; Thiery, 2002). CAFs are also known to promote migration of tumor and related stromal cells. Under stimulation from tumor cells, CAFs express various isoforms of fibronectin that significantly induces their migration as well as that of the tumor cells (Mhawech *et al*., 2005; Schor *et al*., 2003). Furthermore fibroblasts appear to provide favorable conditions for tumor cell proliferation at metastatic sites. Olaso and co-workers (Olaso *et al*., 1997) found that during the hepatic metastasis of melanoma, several factors secreted by the melanoma cells activate hepatic stellate cells to a myofibroblast like state to support the growth of metastasing tumor cells.

Table 1.1 Summary of major tumor promoting growth factors, chemokines, cytokines and proteases related to various stromal cellular populations in the tumor microenvironment.

Growth Factors	**Cells**
EGF	Fibroblasts, monocytes/macrophages
HGF	Fibroblasts, monocytes/macrophages
PDGF	Fibroblasts, monocytes/macrophages
FGF	Fibroblasts, monocytes/macrophages
TGF-β	Fibroblasts, monocytes/macrophages
VEGF	Fibroblasts, monocytes/macrophages
Chemokines	
SDF-1	Fibroblasts
CLCF1	Fibroblasts
CCL-5	MSCs
RANKL	Tregs
Cytokines	
IL-1	Fibroblasts, monocytes/macrophages
IL-6	Fibroblasts, monocytes/macrophages
IL-10	monocytes/macrophages
IL-12	monocytes/macrophages
TNF-α	monocytes/macrophages
Proteases	
MMP-1	Fibroblasts
MMP-2	Fibroblasts, monocytes/macrophages
MMP-3	Fibroblasts
MMP-9	monocytes/macrophages
MMP-11	Fibroblasts
MMP-14	Fibroblasts
MMP-19	monocytes/macrophages
uPA	Fibroblast, monocytes/macrophages

1.3.3 T-cells: *Quenchers of anti-tumor immune responses*

Another immune cell type most commonly found in tumors is T cell. T cells are classified into two types as αβ and γδ on the basis of the T cell receptors they express. αβ T cells are further classified into $CD8^+$ cytotoxic T cells and $CD4^+$ T helper cells which include TH1, TH2, TH17, T regulatory cells (Tregs) and natural killer T cells (NKTs). T cells possess disparate functions and may exert tumor promoting or tumor suppressing effects. For example infiltration of $CD8^+$ T cells in tumors of colon, pancreas, head and neck correlates with better survival (Talmadge *et al*., 2007). On the other hand breast cancers with high $CD4^+/CD8^+$ and TH2/ TH1 T cell ratios show poor prognosis (Kohrt *et al*., 2005). Various subsets of T cells including $CD8^+$T cells, TH1 cells, TH2 cells, TH17 and Tregs have been shown to promote progression and metastasis of many tumors. Of particular importance among these in suppressing anti-tumor immune responses is T regulatory cells (Tregs). Tregs constitute a subset of $CD4^+$ T-cell population that constitutively express high affinity interleukin receptor CD25, CTL antigen-4 (CTLA-4), glucocorticoid-induced tumor necrosis factor (GITR), and the lineage-specific transcription factor FOXP3. Tregs in their physiological capacity act to suppress responses to self antigens, thus preventing autoimmunity (Hori *et al*., 2003). Since most of the tumor cells express self antigens, thus it follows that tumors may evade immune detection by activating Tregs based pathways. These observations are well supported by the studies that show increased infilitration of Tregs in several tumors correlates with poor survival (Bates *et al*., 2006). Tumor infilitating Tregs also express many soluble mediators like RANKL that stimulates metastasis in many tumors (Tan *et al*., 2011).

1.3.4 Myeloid-derived suppressor cells (MDSCs) and Mesenchymal stem cells (MSCs): *The Undifferentiated metastasis promoters*

Myeloid-derived suppressor cells comprise the partially differentiated myeloid progenitors found in tumor stroma and possess remarkable tumor-promoting activity like their more differentiated derivatives (Murdoch *et al*., 2008). These cells are characterized by the co-expression of macrophage marker CD11b and neutrophil marker Gr1 respectively and have been shown to suppress adaptive

immune responses by blocking the functions of $CD8^+$ T cell and NK cells and at the same time promote generation of Tregs (Qian and pollard, 2010). MDSCs promote tumor growth and malignant progression and show significant increase in the bone marrow, spleen and blood of several cancer patients (Ostrand, 2008). MDSCs have also been shown to mediate resistance against various angiogenic inhibitors (shojaei *et al*., 2007; Priceman *et al*., 2010).

MSCs are non-hematopoietic bone marrow derived multipotent cells that differentiate into osteoblasts, chondrocytes, adipocytes and those that form muscles (Pittenger *et al*., 1999). MSCs are present in abundance in primary tumors and contribute to invasion and metastasis. This ability of MSCs has been investigated through a xenograft model of breast cancer in which MSCs when coinjected with breast cancer cell lines enhanced the metastatic potential of these breast cancer cells (karnoub *et al*., 2007). The inductive effect of MSCs on the cancer cells was found to be mediated by a paracrine signalling loop involving CCL5 secreted by MSCs and its receptor on cancer cells.

1.3.5 Endothelial cells: *Responders to angiogenic switch*

Endothelial cells comprise the stromal constituents responsible for tumor-associated vasculature and are accordingly depicted as the responders to the "angiogenic switch". Angiogenic switch results due to presence of variety of pro-angiogenic stimuli in the tumor microenvironment, the process involving the concerted action of endothelial, perivascular, immune and other stromal cells. The pro-metastatic intratumoral milieu modulates endothelial cell paracrine secretions, transforming the endothelial cell phenotype from quiescent and disease-inhibitory to dysfunctionally activated and metastasis stimulatory. Consequently, the resulting vasculature is irregular, dilated, chaotic, leaky with dead ends and hence tumor promoting, these being attributable to aberrant expression of VEGF in the tumor microenvironment (Nagy *et al*., 2007).

Most of the tumors are characterized by the high expression of VEGF, a potent angiogenic and permeability enhancing factor. VEGF is a critical endothelial cell survival factor and stimulates their proliferation, migration and vascular branching (Carmeliet and Jain, 2011). The expression of VEGF in tumor cells is strongly induced by hypoxia formation in the tumor microenvironment, HIF-1α (Hypoxia

Inducible Factor) being a key regulator of VEGF under these conditions (Semenza, 2003 and Liu *et al*., 1995). Besides hypoxia, various oncogenes including mutant ras, HER-2 and bcr-abl induce the expression of VEGF in tumor cells. VEGF induces the expression of anti-apoptotic proteins BCL-2 and survivin in endothelial cells, thereby mediating resistance against apoptosis induced by chemotherapy (Fukuda and Pelus, 2006; Nor *et al*., 1999; Samuel *et al*., 2011). This can be viewed as a tumor cell mechanism to maintain a conducive microenvironment, as the tumor associated endothelial cells and not the normal endothelial cells withstand such apoptotic insults. The bidirectional cross-talk between endothelial and tumor cells induces reciprocal growth factor exchange that positively correlates with vascular expansion and metastatic potential of the tumor. Indeed various studies suggest the role of endothelial cells in regulating the angiogenic factor expression by tumor cells. In a study by Buchanan *et al* (Buchanan *et al*., 2012), HMEC-1, microvascular cells when co-cultured with MDA-MB-231 breast cancer cells, significantly increased the expression of angiogenic stimulators, VEGF and ANG2 (Angiopoietin 2) in these breast cancer cells. Besides angiogenic factors, a variety of ligands like Neuropilin, Robo and Notch displayed by endothelial cells engage a various receptors on cancer cells and trigger a network of interconnected signalling cascades including those that promote angiogenesis and inflammation (Pitroda *et al*, 2012) leading to cancer invasion and metastasis.

1.3.6 Cancer stem cells (CSCs): *Initiators of the macabre process*

Originally identified in hematological malignancies and tumors of brain and breast, these have been hypothesized to constitute a set of specialized cells responsible for initiation, maintenance, progression and drug resistance inherent to tumors. Alternatively referred to as “cancer initiating cells” or “tumor initiating cells”, CSCs resemble normal stem cells by sharing the functional attributes of self renewal, extensive proliferation, drug resistance and ability to give rise to a differentiated progeny. Experimentally CSCs are defined by their ability to form tumors similar to their origin when implanted in immunodeficient mice (Bonnet and Dick, 1997) and are functionally identified by expression of CD44 or CD133 and aldehyde dehydrogenase (ALDH), exclusion of florescent dyes like

Hoeschst33342 and Rhodamine (SP phenotype) and ability to form spheres in serum- free growth medium (Mannelli and Gallo, 2012; Qiu *et al*., 2012; Dou *et al*., 2007). In clinical settings, CSCs have been implicated in imparting resistance to the variety of cancer therapy regimens including chemotherapy and radiotherapy. Inherent abilities of CSCs that make them resistant to chemo and radiotherapy include high expression of drug transporters, more active DNA repair mechanisms and apoptotic resistance (Signore *et al*., 2013). CSCs have been found to express high levels of ABC transport proteins, notably ABCG2, that confers cross resistance to several structurally unrelated chemotherapeutic drugs (Morrison *et al*., 2011). Another ABC transporter, ABCB1 expressed by CSCs mediates resistance against apoptosis induced by chemotherapeutic drugs besides blunting the complement mediated lysis. Dysregulation of various signalling molecules like EGFR, PI3 Kinase and several non-coding regulatory RNAs (miRNAs) (Guessous *et al*., 2010) have recently been found to impart chemoresistence to these cell types.

1.4 Roles of Tumor Associated Macrophages (TAMs) in Tumor Progression

Macrophages confer various extrinsic traits that enhance tumor incidence, progression and metastasis. These include tumor growth, tumor cell invasion, migration, and intravasation, tumor angiogenesis, immune suppression and metastasis as discussed in the following sections (Figure 1.3).

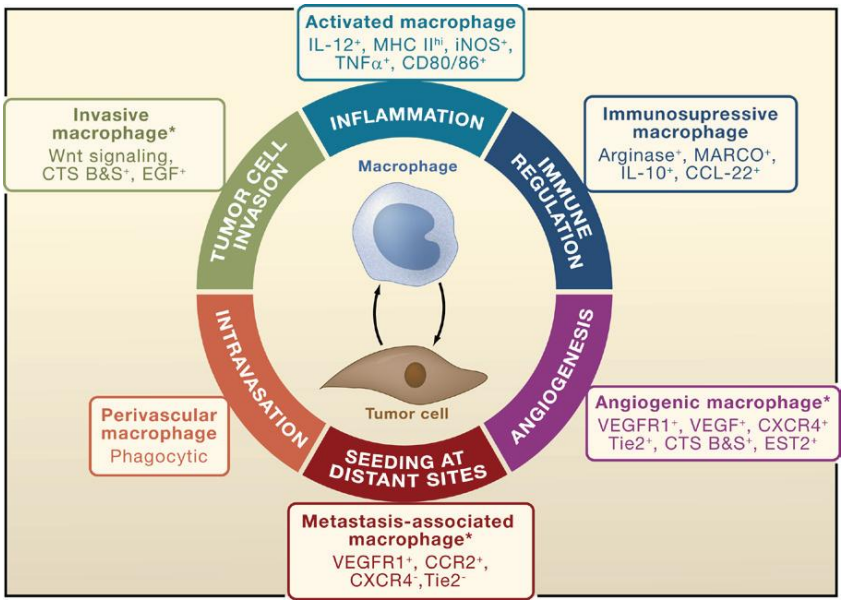


Figure 1.3 Various traits of malignancy conferred upon by different sub-populations of macrophages (*Adapted from Qian and Pollard, 2010*)

1.4.1 Tumor Growth

The rate of tumor cell proliferation has been shown to correlate positively with TAM infiltration as evaluated by the levels of MIB-1 in breast carcinomas (Tsutsui *et al*., 2005), Ki67 in endometrial carcinomas (Wang *et al*., 2005), or mitotic index in renal cell carcinoma (Hamada *et al*., 2002). TAMs express a variety of factors including, hepatocyte growth factor, platelet-derived growth factor, TGF-β1, EGF and basic fibroblast growth factor (Lewis and Murdoch, 2005) that stimulate tumor cell proliferation and survival (Goswami *et al*., 2005; O'Sullivan *et al*., 1993) (Figure 1.4). This dependence of tumor cells on macrophage derived signals has been confirmed by depletion studies in experimental tumors *in vivo* (Polverini *et al*., 1987). *In vitro* when co-cultured with tumor cells, macrophages secrete various factors that stimulate tumor cell proliferation (Vannetten *et al*., 1993; Hewlett *et al*., 1979). The production of such factors like TGF-β1 by macrophages in wounds may also help to explain the efficient growth of tumors (e.g., tumors induced by injections of the Rous sarcoma virus) at sites of wounding or tissue injury (Sieweke *et al*., 1990).

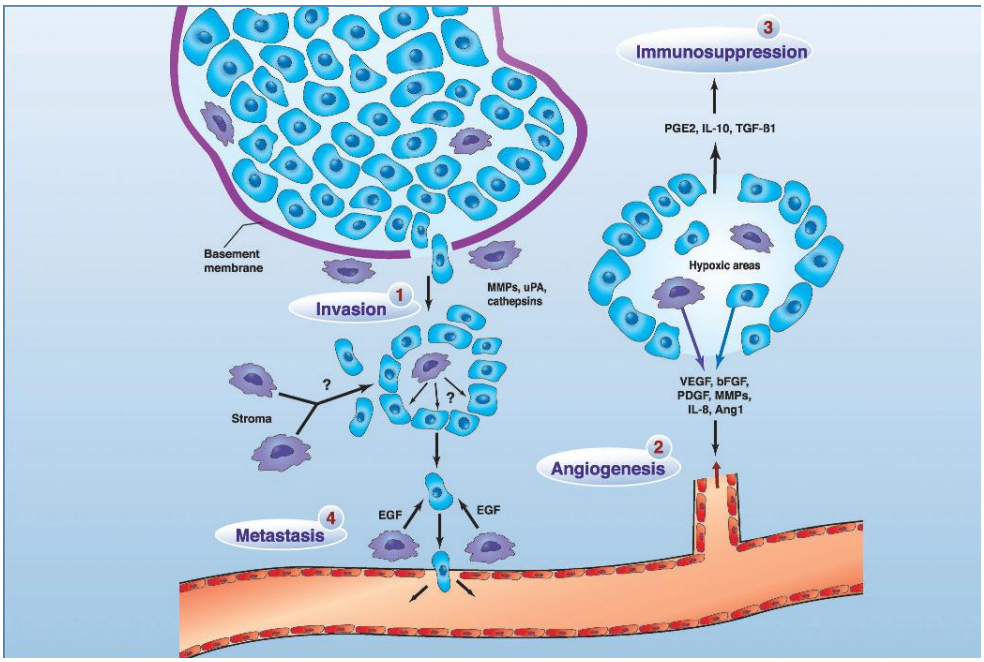


Figure 1.4 TAMs and the process of tumor progression *(Adapted from Lewis and Pollard, 2006)*

1.4.2 Tumor Cell Invasion, Migration, and Intravasation

With the help of intravital imaging and studying PyMT-induced mammary tumors, the process of tumor cell migration and invasion has been shown to be dependent on macrophages (Condeelis and Pollard, 2006). During the development of early-stage lesions, macrophages are present in the areas of basement membrane breakdown and invasion (Lin *et al*., 2001) with high expression of proteolytic enzymes like cathepsin B (Gocheva *et al*., 2010) and Urokinase/Plasminogen activator (uPA) (Almholt *et al*., 2005). A study by Hagemann *et al* (Hagemann *et al*., 2004) has demonstrated that co-culturing macrophages with tumor cells enhances the invasive properties of tumor cells through TNF-α and matrix metalloproteinases (MMPs) dependent pathways. Thus TAMs represent the key that unlocks the gate to allow tumor cells to escape. The interaction between TAMs and cancer cells in tumors is also reciprocal. While tumor cells synthesize CSF-1, that stimulates macrophages to move, macrophages

produce epidermal growth factor (EGF), which expedites the migration of tumor cells (Wyckoff *et al*., 2004) (Figure 1.4). Inhibition of either the EGF or CSF-1 signaling pathways results in inhibition of migration and chemotaxis of both cell types. Experimental systems involving *in vitro* collagen overlay assay (Condeelis and Pollard, 2006), mammary epithelial organoid culture system (DeNardo *et al*., 2009), or co-culture (Green *et al*., 2009) points at the role of macrophages in inducing tumor cell migration and thus provides an evidence that macrophages and tumor cells are sufficient for mediating EGF-CSF-1 paracrine interaction. Other molecules that have been showed to be involved in the macrophage induced stimulation of invasiveness include Wnt5a acting through the noncanonical pathway (Pukrop *et al*., 2006) in organoids and TNF-α via NF-κB in co-culture (Hagemann *et al*., 2004).

1.4.3 Tumor Angiogenesis

Angiogenesis, involving formation of new blood vessels from the existing vasculature is now widely recognized as a process indispensible for the growth and spread of tumors. Compelling evidence suggests that TAMs play an important part in regulating angiogenesis. In most of the tumors, there is a dramatic enhancement of vascular density from the benign-to-malignant transition, a process referred to as the angiogenic switch (Hanahan *et al*., 1996). Cells of the mononuclear phagocytic lineage cells and macrophages in particular, are major contributors to this process (Zumsteg and Christofori, 2009). The indispensability of macrophages to angiogenic switch has been studied well in PyMT-induced mouse mammary tumors. Depletion of macrophages in such tumor models by null mutation of Csf1 gene provides evidence for the requirement of these cells to angiogenic switch. This observation is further substantiated by the premature accumulation of macrophages into hyperplastic lesions and a dramatic early angiogenic switch due to overexpression of CSF-1 in wild-type mice. These data strongly argue for the role of the angiogenic switch in regulating the malignant transition and for macrophages to be important players in this regulation (Lin and Pollard, 2007). Such studies also demonstrate the significant role played by macrophages in vascular remodeling, as tumors progress to late carcinoma stages (Lin *et al*., 2006). TAMs express VEGF almost exclusively and can also make it bio-available through the production of MMP-9, which releases VEGF from

extracellular depots. VEGF is also induced by hypoxia, a process being dependent on HIF-1, that regulates the transcription of a large panel of genes associated with angiogenesis, including VEGF (Cramer *et al*., 2003; Murdoch *et al*., 2008). TAMs also release a variety of other potent proangiogenic cytokines and growth factors including TNF-α, IL-8, and bFGF. Additionally, they synthesize elevated levels of angiogenesis-modulating enzymes such as MMP-2, MMP-7, MMP-9, MMP-12, and cyclooxygenase-2 (Sunderkotter *et al*., 1991; Klimp *et al*., 2001). These enzymes act upon extracellular matrix and basement membrane, and are known to stimulate endothelial cell proliferation and migration, which in turn can support the tumor angiogenesis (Nishizuka *et al*., 2001).

1.4.4 Immune suppression

Contrary to macrophages from healthy tissues, which are capable of presenting tumor-associated antigens, lysing tumor cells, and stimulating the anti-tumor functions of T cells and NK cells, TAMs in the tumor microenvironment lack these activities, leaving the host incompetent to mount an effective anti-tumor immune response. A number of tumor-derived factors, like cytokines, chemotactic molecules, growth factors and proteases are known to influence the normal function of macrophages (Elgert *et al*., 1998; Sunderkotter *et al*., 1991; Benbaruch, 2006). For example, tumor cell secreted IL-6, IL-4, IL-10, TGF-β1, MDF, and PGE2 inhibit the cytotoxic activity of TAMs (Elgert *et al*., 1998; Sunderkotter *et al*., 1991). Additionally TGF- β1, IL-10, and PGE2 also suppress the expression of MHC class II molecules in macrophages locally as well as at the distant sites like spleen and peritoneum, thereby limiting the ability of TAMs to present tumor-associated antigens to T cells (Elgert *et al*., 1998). Thus, tumors undergo immunoediting to down-regulate macrophage functions that are potentially dangerous to the tumor even in circumstances where recognizable tumor antigens are presented. Another important aspect of tumor cell mediated functional alteration of macrophages is the marked decrease in expression of IL-12, a cytokine known to stimulate both the proliferation and cytotoxicity of T cells and NK cells. Hypoxia in the tumor microenvironment is another element contributing to the suppression of antitumor activity of TAMs as it stimulates the release of the potent immunosuppressive factors, PGE2 and IL-10. These factors impede the development of immune cells by acting on the early stages of their

development from primitive pluripotent stem cells (Elgert *et al*., 1998; Wojtowicz-Praga, 1997). In addition, they act on TAMs to reduce their cytotoxicity activity toward the tumor cells (Zeineddine *et al*., 1999). Hypoxia also compromises the ability of macrophages to phagocytose dead or dying cells and present antigens to T cells. This occurs by the reduction in surface expression of CD80, a co-stimulatory molecule required for the full activation of T-cell responses to antigenic peptides. Hypoxia can also enhance the direct cytotoxicity of macrophages by up-regulating release of TNF-α. As mentioned above, TAMs also up-regulate the expression of MMP-7 in hypoxic areas of tumors (Burke *et al*., 2003). MMP-7 is known to cleave the Fas ligand from neighboring cells, making tumor cells not only less responsive to chemotherapeutic agents, such as doxorubicin (Mitsiades *et al*., 2001), but also resistant to lysis by NK and T cells (Fingleton *et al*., 2001).

1.4.5 Metastasis

Mounting evidences point at the role of TAMs in the regulation of metastasis (Figure 1.4). Early establishment of metastases in a number of tumor types have been correlated with high numbers of TAMs in such tumors (Hanada *et al*., 2000). TAMs help both in the release of metastatic cells from the primary tumor as well as in the establishment of secondary tumors at distant sites. Intravital imaging in PyMT-induced murine mammary tumors and xenografts of rat breast cancer cells has defined the role of TAMs in promoting the motility of tumor cells away from main body of the tumors as well as extravasation of tumor cells into the blood vessels. TAMs have also been shown to express the lymphatic endothelial growth factor, VEGF-C, suggesting that they may also promote dissemination of tumor cells by stimulating the formation of lymphatic vessels in tumors (Schoppmann *et al*., 2002).

1.5 Clinical Significance of Macrophages in tumors

In most of the solid tumors, high density of cells expressing macrophage-associated markers have generally been found to be associated with a poor clinical outcome (Komohara *et al*., 2014; Zhang *et al*., 2012) (Figure 1.5). However there are contradicting data for lung, stomach, prostate, and bone, with both positive and negative outcome associations being reported (Zhang *et al*., 2012), possibly

related to the type and stage of cancer evaluated, (Buddingh *et al*., 2011; Fujiwara *et al*., 2011) or to the type of analysis performed such as quantitation of stromal versus intratumoral macrophages. This discrepancy may also arise due to the use of different macrophage markers. For example in murine macrophages CD68 is a specific marker and in combination with F4/80, identifies a majority of tumor-associated macrophages. However in humans, CD68 is widely expressed by granulocytes, dendritic cells, fibroblasts, endothelial cells, and some lymphoid subsets (Gottfried *et al*., 2008). The use of CD68 for association studies in this context is therefore of variable utility. This is also quite explicit in human non-small-cell lung cancer, where detection of the macrophage scavenger receptors CD163 and CD204, but not CD68, yields correlations with negative outcome (Chung *et al*., 2012; Hirayama *et al*., 2012). Both CD163 and CD204 are also associated with activation of macrophages toward an alternative or tumor-promoting and immunosuppressive phenotype, besides representing more selective macrophage biomarkers. Accordingly, significant correlations between CD163/CD204 and negative outcomes have been reported across multiple tumor types (Komohara *et al*., 2014). Unlike most populations of tumor-associated macrophages that possess pro-tumor and immunosuppressive properties (Biswas and Mantovani, 2010), macrophages in human colorectal cancer have been found to be functionally and phenotypically anti-tumor (Edin *et al*., 2012). Thus these studies collectively support the presumption that repolarizing macrophages toward an anti-tumor phenotypic state, either by impeding activities or signals that drive pro-tumor polarization or by delivering exogenous signals that enhance anti-tumor polarization, could act as an alternative and perhaps more efficacious approach to block macrophage recruitment, even though these activities and responses are all dynamically regulated *in vivo*.

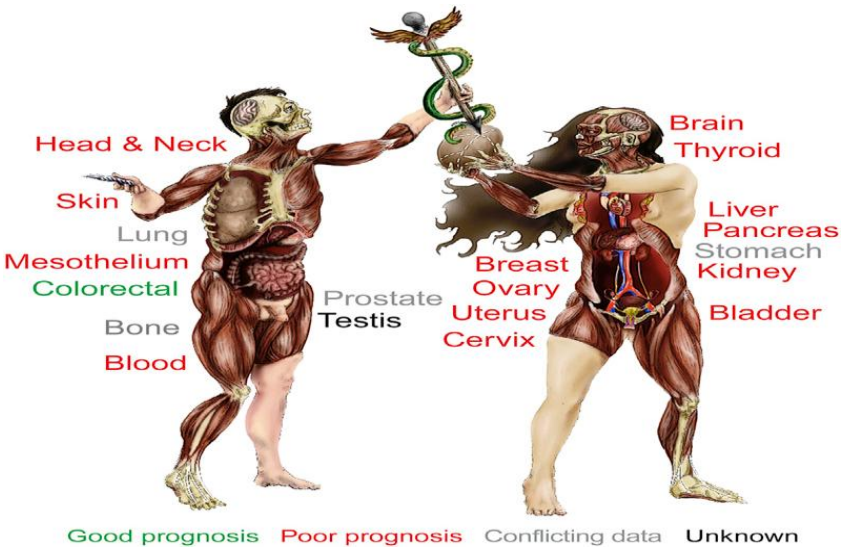


Figure 1.5 Clinical Implications of Macrophage Density in various tumors *(Adapted from Ruffell and Coussens, 2015)*

1.6 Prominent tumor-promoting cytokines in tumor microenvironment

Most of the neoplastic lesions are characterized by the infiltration of numerous types of immune cells of both the arms of immune system. Such responses were often conceived as anti-tumoral, however their paradoxal role came to fore in the ensuing decade. By now there are compelling evidences that suggest the tumor promoting effects of tumor-associated inflammatory responses. Activated macrophages are central to these types of immune responses and work in concert with other immune cells in the tumor microenvironment (Balkwill, 2006). Tumors also exploit infiltrating immune cells to generate inflammatory microenvironment e.g., metastatic cells produce certain factors that activate myeloid cells to induce the secretion of IL-6 and TNF-α (Kim *et al*., 2009). In turn the inflammatory cytokines present in the microenvironment confer growth promoting effects on tumor cells and facilitate invasion and metastasis through the activation of EMT programme in tumor cells (Qian and Pollard, 2010).

1.6.1 Tumor Necrosis Factor-alpha (TNF-α)

TNF-α is one of the main inflammatory mediators that has been implicated in carcinogenesis, due to its participation in chronic inflammatory diseases (Popa *et*

al., 2007). The effect of TNF- α appears to be more potent in the early stages of tumor formation including angiogenesis and invasion, compared to the tumor progression (Szlosarek *et al*., 2006).While TNF-α is a prototypical proinflammatory cytokine, evidence suggests its double-edged role in carcinogenesis. The high concentrations of this cytokine have been shown to induce an anti-tumoral response in a murine model of sarcoma (Havell *et al*., 1988). However low and sustained TNF-α levels in tumor microenvironment can induce a tumor promoting phenotype (Balkwill, 2006). This tumor promoting mechanism of TNF-α is based on reactive oxygen species (ROS) and reactive nitrogen species (RNS) generation, both of which can induce DNA damage, thereby facilitating tumorigenesis (Woo *et al*., 2000). TNF-α mediated inflammation has been identified to be intimately associated with tumor development and metastasis**.** A study by Kwong *et al* (Kwong *et al*., 2009) explored TNF-α associated tumorigenesis using an organoid of normal human ovarian epithelial cells exposed to a prolonged TNF-α dose. This model demonstrated generation of a precancerous-like phenotype with structural and functional changes, such as tissue disorganization, epithelial polarity loss, cell invasion, and over-expression of cancer markers. In tumor microenvironment, TNF-α can induce cancer invasion and metastasis associated with epithelial-mesenchymal transition (EMT). TNF-α induced EMT is characterized by the acquisition of mesenchymal spindle-like morphology and increased expression of N-cadherin and fibronectin with a concomitant decrease of E-cadherin and Zona occludin-1 (Wang *et al*., 2013). The role of TNF-α in tumor promotion is also evidenced by the observation that prolonged TNF-α exposure can enhance the proportion of cancer stem cell phenotypes in oral squamous cell carcinoma, increasing their tumor forming sphere ability, stem cell-transcription factor expression, and thus tumorigenicity (Lee *et al*., 2012).

1.6.2 Interleukin-6 (IL-6)

IL-6 is another proinflammatory cytokine with a typical protumorigenic effect. Elevated levels of IL-6 have been detected in the serum of patients with systemic cancers as compared to healthy controls or patients with benign diseases. IL-6 plays a key role in promoting proliferation and inhibition of apoptosis. IL-6

activates the JAK/STAT signaling pathway by binding to its receptor, IL-6Rα and coreceptor gp130. Various studies have highlighted the role of IL-6/JAK/STAT signaling pathway in cancer initiation and progression. Through the activation of genes involved in cell cycle progression and suppression of apoptosis, IL-6 can directly protect tumor cells from apoptosis. IL-6 partners with TNF-α and contributes to obesity-mediated tumor promotion in hepatocellular carcinoma (HCC) (Park *et al*., 2010). Mice deficient in IL-6 develop much less HCC in response to chemical pro-carcinogen DEN. This is further substantiated by the observation that gender-biased production of IL-6 accounts for the much higher HCC load in males as compared to females (Naugler *et al*., 2007). High levels of circulating IL-6 have been related with risk factors of HCC including obesity, cirrhosis and hepatosteatosis (Wong *et al*., 2009). IL-6 has also been shown to act as an autocrine growth factor for tumors (Baffet *et al*., 1991). Another mechanism by which IL-6 can induce tumorigenesis is hypermethylation of tumor suppressor genes as well as the hypomethylation of retrotransposon long interspersed nuclear element-1 (LINE-1) as has been observed in oral squamous cell cancer lines (Gasche *et al*., 2011). IL-6 also accentuates tumor development by promoting conversion of non-cancereous cells into tumor stem cells. IL-6 secretion under low attachment culture conditions upregulate Oct4 gene expression by activating the IL 6R/JAK/STAT3 signaling pathway (Kim *et al*., 2013).

1.6.3 Transforming growth factor-β (TGF-β)

TGF-β is a pleiotropic cytokine, with immune-suppressing and anti-inflammatory properties. Under physiological conditions, TGF-β plays an important role in embryogenesis, cell proliferation, differentiation, apoptosis, adhesion, and invasion (Santibanez *et al*., 2011). There are three isoforms of TGF-β including TGF-β1, TGF-β2, and TGF-β3 all of which are known to bind the cognate type II receptor (TGF-β RII), inducing phosphorylation of type I TGF-β receptor (TGF-β RI), and in turn leading to the formation of a heterotetrameric complex that activates SMAD-dependent transcription (Massague, 2008). SMAD transcription factors comprise of a serine and threonine-rich linker region that connects two MAD (mothers against dpp) homology regions. Differential phosphorylation of these amino acid residues activates various cellular functions like cytostatic

effects, cell growth, invasion, extracellular matrix synthesis, cell cycle arrest, and migration (Matsuzaki, 2013). Under TGF-β receptor activation, differential phosphorylation of SMAD2 and SMAD3 promotes their translocation into the nucleus, where they form a complex with SMAD4, further bind to DNA, associate with other transcription factors, and induce gene expression (Massague, 2008). The role of TGF-β in cancer is dual in nature and paradoxical, varying according to cell type and stage of tumorigenesis. During early stages, TGF-β acts as a tumor suppressor, inhibiting cell cycle progression and promoting apoptosis. However at later stages, TGF-β enhances invasion and metastasis by inducing epithelial-mesenchymal transition (EMT) programme (Morrison *et al*., 2013). In the context of cancer inhibition, TGF-β acts a tumor suppressor by upregulating cyclin-dependent kinase inhibitor (CKI) p21 and downregulating c-Myc (Malliri *et al*., 1996). Using a conditional TGF-β RII knock-out mice model, Guasch *et al* found that highly proliferative epithelia (such as rectal and genital) developed spontaneous squamous cell carcinomas and furthermore showed accelerated carcinoma progression, Ras mutations, and apoptosis reduction (Guasch *et al*., 2007) suggesting that a deficient TGF-β pathway contributes to tumorigenesis. There are firm evidences demonstrating that TGF-β signaling changes are consistent with cancer progression. High levels of TGF-β 1 mRNA and protein have been observed in the carcinomas of stomach, lung, colorectal and prostate (Bierie and Moses 2006). Furthermore, TGF-β receptor deletion or mutations have been associated with invasive and advanced grades of colorectal, prostate, breast, and bladder cancer (Levy and Hill, 2006).

1.7 Fibronectin in cancer pathogenesis

Fibronectin (FN) is a multifunctional glycoprotein found in the extracellular matrix of tissues and blood plasma. It is expressed by a variety of cell types and plays a key role in cell adhesion and migration. Indeed, fundamental processes such as embryogenesis, wound healing, maintenance of tissue integrity and homeostasis depend on interactions between cells and the fibronectin in the extracellular matrix (Hynes, 1990). The indespensibility of fibronectin to such processes was conclusively demonstrated by lethal effect of the murine *FN*-null mutation i.e., mice lacking *FN* died from severe defects in embryonic development (George *et al*., 1993). In humans, the clinical significance of FN is evidenced by recent data demonstrating that certain types of glomerulopathy result from mutations in the *FN* gene (Castelletti *et al*., 2008). Studies in human colon, lung, breast, and prostate cancer showed that carcinoma cells are submerged in a microenvironment containing abundance of extracellular matrix (ECM) proteins including such as Fibronectin (Pupa *et al*., 2002). Fibronectin is known to promote tumor growth and progression through its participation in the development of an ideal microenvironment (Kaspar *et al*., 2006; Jerhammar *et al*., 2010). Intriguingly, the amount of fibronectin mRNA in stroma has been found to be 7–13 times higher in carcinomas, as compared to normal tissues (Moro *et al*., 1992).

1.7.1 Fibronectin structure

Fibronectin is a high molecular weight dimeric glycoprotein consisting of two similar or identical subunits of 220–250 kDa that are held together by two disulphide bonds near their carboxyl-termini. Each dimer consists of two nearly identical polypeptide chains and three types of homologous repeating modules termed types I, II and III. The 15 Type III modules constitute the largest part of the FN polypeptide and are clustered in the central part of the protein (Figure 1.6).

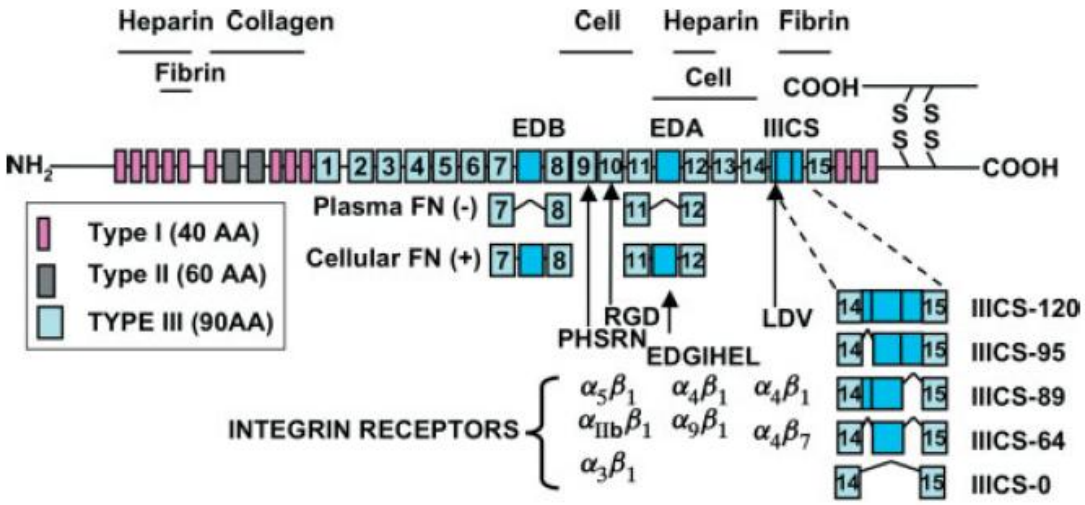


Figure 1.6 Fibronectin primary structure *(Adapted from White et al., 2008)*

Fibronectin is an excellent model to study gene function regulated by alternative splicing. Fibronectin is produced by transcription of a single gene, comprising 47 exons that span over 90 kbp in the genome. All the Type III domains of fibronectin are encoded by two exons excluding the EDA (Extra-domain A), EDB (Extra-domain B) and the ninth Type III domain, each of which is encoded by a single exon. Thus multiple Fibronectin mRNAs, and subsequently multiple protein isoforms, arise due to alternative splicing within a single pre-mRNA (Figure 1.7). The EDA and EDB exons can be included or excluded from *FN* mRNA (Kornblihtt *et al*., 1984; Schwarzbauer *et al*., 1983). However the Type III connecting segment (IIICS) element (also termed the variable, or V, region) undergoes a more complex splicing pattern: it can be completely included (V120) or excluded (V0), or partially included, according to the species. Inclusion of the alternatively spliced regions occurs during embryonic development and decreases significantly after birth and with ageing (Chauhan *et al*., 2004). However, under certain circumstances in adult life such as tissue fibrosis, tissue repair, and angiogenesis, embryonic splicing pattern is temporally re-established. For example inclusion of the EDA and EDB domains occurs during skin wound healing, where expression of both these domains gets increased in cells present at base of the wound (Ffrench-Constant *et al*., 1989). The re-appearance of EDA cFN has been also observed in lung fibrosis, prior to the appearance of collagen.

Alternative splicing of EDA and EDB is regulated by members of the family of splicing regulator serine- and arginine-rich proteins termed SR proteins through characteristic mechanisms. In case of EDA exon, regulatory sequences are located within the exon itself, whereas in case of EDB, distinct regulatory sequences are located within the exon and within the downstream intron.

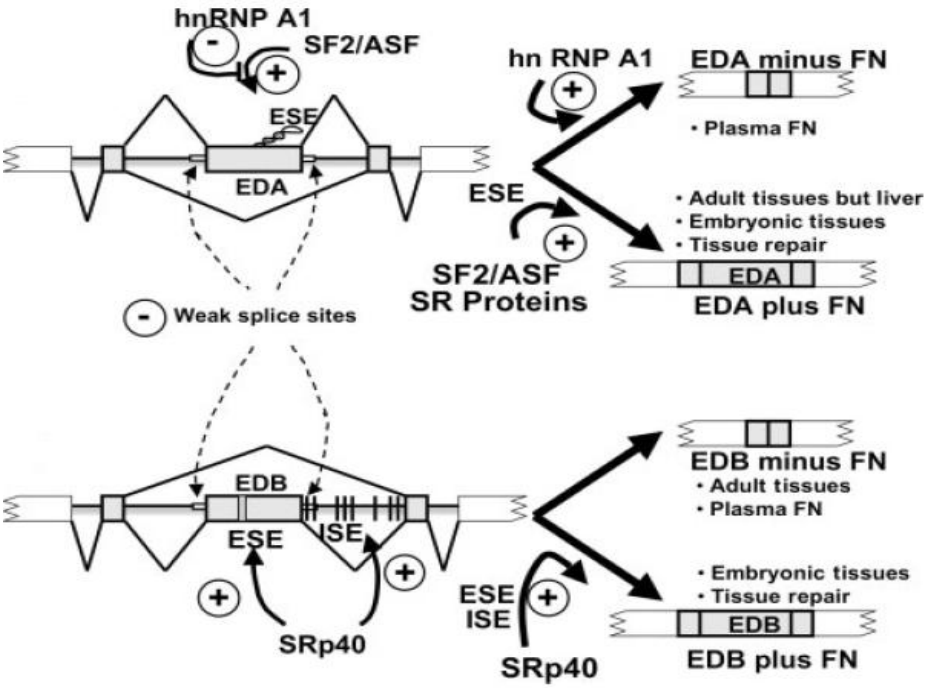


Figure 1.7 Mechanisms of EDA and EDB alternative splicing *(Adapted from White et al., 2008)*

1.7.2 EDA/EDB in health and disease

EDA plays an important role in various cellular processes including cell adhesion, matrix assembly (Guan *et al*., 1990), dimer formation (Peters *et al*., 1990), wound healing (Clark *et al*., 1983), protein secretion (Wang *et al*., 1991), cytokine-dependent matrix metalloproteinase expression (Saito *et al*., 1999), cell differentiation, cycle progression and mitogenic signal transduction (Manabe *et al*., 1999). Similarly, EDB is thought to participate in many processes such as organogenesis, angiogenesis, cell proliferation, matrix assembly and healing of bone fractures (Astrof *et al*., 2004; Matuskova *et al*., 2006). Both EDA and EDB exons are indispensible to embryonic development, with simultaneous deletion of

EDA and EDB exons in mice leading to embryonic lethality (Astrof *et al*., 2007). The significance of correct EDA splicing is also strongly supported by the observation that mice lacking the EDA exon displayed a considerably reduced lifespan (Muro *et al*., 2003). Conversely elevated levels of EDA cFN are found in affected tissues and plasma of patients with certain disorders, such as rheumatoid arthritis, psoriasis, scleroderma, diabetes and cancer, although the functional role of EDA cFN in these disease states is still obscure.

1.7.3 EDA in the pathogenesis of Lung fibrosis, Scleroderma and Atherosclerosis

Tissue fibrosis is a pathological condition that occurs when myofibroblast differentiation and their ultimate removal gets dysregulated. One such threatening form of fibrosis in the lung is idiopathic pulmonary fibrosis (IPF). IPF is largely untreatable and patients ultimately die due to persistent ECM deposition in the lung, culminating in progressive respiratory failure (Thannickal *et al*., 2004; White *et al*., 2003). EDA domain of Fibronectin has been suggested to play a functional role in the *in vitro* differentiation of fibroblasts into myofibroblasts (Serini *et al*., 1998). In IPF, EDA cFN deposition occurs in the regions of active fibrosis before collagens (Kuhn and McDonald, 1991), which correlates with the increased expression of markers of fibroblast activation α-smooth muscle actin (α-SMA). The relationship between EDA cFN and myofibroblast differentiation *in vivo* has been recently demonstrated using the well-described intratracheal bleomycin model of lung fibrosis in mouse. In $EDA^{-/-}$ mice receiving intratracheal bleomycin, lung fibrosis was completely suppressed, suggesting that EDA cFN is necessary for the development of pulmonary fibrosis (Muro *et al*., 2008). These observations correlated with diminished activation of latent TGF-β as well as decreased lung fibroblast responsiveness to active TGF-β. Intriguingly, $EDA^{-/-}$ lung fibroblasts may undergo TGF-β induced myofibroblast differentiation, but only when plated on a matrix containing EDA cFN (Muro *et al*., 2008). The observation that EDA cFN is important for latent TGF-β activation and myofibroblast differentiation supports the previously recognized crucial role of TGF-β and the ECM in lung fibrosis. Recently, a study by Bhattacharya *et al* (Bhattacharya *et al*., 2014) investigated the role of EDA FN in scleroderma, an autoimmune disease characterized by hardening of skin and

multiple organ fibrosis. It was shown that EDA FN induces fibrotic responses by acting as an endogenous ligand of TLR-4.

There are also abundant evidences that link FN and its isoforms to the development of atherosclerosis. Atherogenesis involves a series of events such as recruitment of blood monocytes to the arterial intima, their maturation to tissue macrophages, lipid accumulation leading to foam cell formation and smooth muscle cell migration from the arterial wall (Ross, 1999). Interestingly, lipid accumulation and foam cell formation does not occur without stable monocyte interactions with tissues, suggesting the importance of specific ECM-dependent signalling (Wesley *et al*., 1998). Fibronectin lacking EDA and EDB is present in considerable amounts in the normal arterial wall. However, in atherosclerotic lesions and in experimental models, there is a marked increase in total and EDA cFN adjacent to smooth muscle cells (Glukhova *et al*., 1989). One possible role for EDA cFN in this context may be activation of toll-like receptor-4 (TLR-4) (Okamura *et al*., 2001), thereby triggering nuclear translocation of NF-κB, a molecule central to inflammation and atherogenesis (Xu *et al*., 2001).

1.8 Epithelial-Mesenchymal Transition

Epithelial–mesenchymal transition (EMT) is a developmental programme that enables a polarized epithelial cell to undergo multiple biochemical changes including enhanced migratory abilities, invasiveness, elevated resistance to apoptosis and enhanced production of extracellular matrix components, all peculiar to a mesenchymal cell phenotype (Kalluri and Neilson, 2003). EMT, is being prominently implicated as a means by which transformed epithelial cells can acquire the abilities to invade and disseminate (Klymkowsky and Savagner, 2009; Polyak and Weinberg, 2009; Yilmaz and Christofori, 2009). During EMT, cancer cells acquire fibroblast-like properties, thus losing their defined cell–cell and cell–extracellular matrix contacts (Thiery *et al*., 2010). At the molecular level EMT is characterized by down regulation of E-cadherin and cytokeratins, the process being controlled by a group of transcription factors referred to as epithelial–mesenchymal transition regulators (EMTRs): SIP1 (Comijn *et al*., 2001) Slug (Hajra *et al*., 2002; Bolos *et al*., 2003), Snail (Batlle *et al*., 2000), Twist (Yang *et al*., 2004), ZEB1 (Hartwell *et al*., 2006; Eger *et al*., 2005) and

E12/47 (Perez-Moreno *et al*., 2001). These transcriptional regulators are aberrantly expressed in a number of tumor types as well as in the experimental models of carcinogenesis. When ectopically over-expressed, some of them have been found to elicit metastasis (Micalizzi *et al*., 2010; Taube *et al*., 2010). The cell biological traits evoked by such transcription factors include loss of adherens junctions and associated conversion from a polygonal/epithelial to a spindly/fibroblastic morphology, expression of matrix-degrading enzymes, increased motility, and heightened resistance to apoptosis. Several of these transcription factors can directly repress E-cadherin gene expression, thereby depriving neoplastic epithelial cells of this key suppressor of motility and invasiveness (Peinado *et al*., 2004). In the context of tumor microenvironment, increasing evidence suggests that heterotypic interactions of cancer cells with the associated stromal cells can induce expression of the malignant cell phenotypes that are known to be overseen by a set of EMT-inducing transcriptional regulators (Karnoub and Weinberg, 2006–2007). Furthermore, cancer cells at the invasive fronts of certain carcinomas can be seen to have undergone an EMT, suggesting that these cancer cells are subject to microenvironmental stimuli distinct from those received by cancer cells located in the cores of these lesions (Hlubek *et al*., 2007).

1.9 Cellular interplay in Lung cancer microenvironment

The metastatic potential of non-small cell lung cancer cells has been shown to be associated with their interactions with various cellular populations within the tumor microenvironment, which primarily comprises of Tumor associated macrophages (TAMs) and cancer-associated fibroblasts (CAFs) (Choe *et al*., 2013). Non–small cell lung cancer (NSCLC) cells recruit a variety of stromal cells cell types to the tumor microenvironment that can promote or inhibit tumorigenesis by mechanisms that have not been fully unraveled. In turn, stromal cells alter the profile of tumor cell secreted proteins, including those required for tumor growth and metastasis. In order to define the interactions between lung cancer cells and the stromal cells that promote lung tumorigenesis, Zhong *et al* (Zhong *et al*., 2008) co-cultured a lung adenocarcinoma cell line derived from $Kras^{LA1}$ mice (LKR-13) with stromal cell lines including MHS (macrophage), MLg (fibroblast), MEC (endothelial cell), and characterized the secreted proteins

in co-cultures. Such co-culturing of lung adenocarcinoma cell line (LKR-13) with either of stromal cell (macrophage, endothelial cell, or fibroblast) enhanced stromal cell migration, increased LKR-13 cell proliferation, induced endothelial tube formation, and regulated the secretion of proteins involved in proliferation, angiogenesis, inflammation, and epithelial-to-mesenchymal transition. The proteomic approach used in this study implicated CXCL1 and IL-18 in NSCLC development. Genetic and pharmacological ablation of CXCL1 and IL-18 revealed that stromal cell migration, LKR-13 cell proliferation, and LKR-13 cell tumorigenicity required the concerted action of both these proteins. It was concluded that stromal cells enhance the LKR-13 cell tumorigenicity partly through their effects on the secretome of LKR-13 cells. In another study, Kim *et al*., (Kim *et al*., 2009) investigated the role of cancer cell secreted factors that directly activate myeloid cells particularly macrophages. A biochemical screen was conducted to identify the factors secreted by metastatic carcinomas that activate macrophages. Conditioned medium from metastatic cells, especially LLC, induced higher amounts of IL-6 and TNF-α secretion than Conditioned medium from non-metastatic cells. The activation was found to occur through Toll-like receptor family members TLR-2 and TLR-6. Biochemical purification of LLC conditional medium (LCM) led to identification of the extracellular matrix proteoglycan versican, which has been found to be upregulated in many human tumors including lung cancer (Isogai *et al*., 1996; Pirinen *et al*., 2005), as a macrophage activator. By activating TLR-2: TLR-6 complexes and inducing TNF-α secretion by myeloid cells, versican strongly enhances LLC metastatic growth. This study explains the ability of cancer cells to depose the host innate immune system to generate an inflammatory microenvironment congenial for metastatic growth.

2.1 MATERIALS

2.1.1 Chemicals and Reagents

DMEM, RPMI-1640, LPM and fetal bovine serum (FBS) were purchased from Gibco (USA). Synser, Pancerin and DCCM-1 were obtained from Biological Industries (Kibbutz, Israel). Human TNF-α, IL-6 and TGF-β were procured from BD Biosciences (San Diego, USA). Collagen coated Transwell inserts were purchased from Corning (UK). Bovine serum albumin (BSA), 3,3'-diaminobenzidine tetrahydrochloride (DAB), 3,3',5,5'-Tetramethyl benzidine (TMB), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), LPS (0111:B4) and dimethyl sulfoxide (DMSO) were purchased from Sigma–Aldrich Co. (St. Louis, USA). Trizol was purchased from Ambion (Carlsbad, USA). PVDF membrane for immunoblotting was obtained from Millipore (Bengaluru, India). Plasmid miniprep kit, Gel extraction kit, PCR purification kit and nuclease-free water were purchased from Qiagen (Hilden, Germany). cDNA synthesis and Micro BCA^{TM} (Bicinchoninic acid) protein assay kits were procured from Thermo Scientific Ltd. (Rockford, USA). Protein G- Agarose beads were obtained from Calbiochem (Sweden). Cytoscan-LDH cytotoxicity Assay kit, was purchased from G-Biosciences (USA). The reagents used in electrophoresis and immunoblotting were purchased from Sigma Chemical Co. (St. Louis, USA). Chemicals used in the preparation of buffers and other solutions were of analytical grade, and unless otherwise stated were obtained from Amresco (Ohio, USA).

2.1.2 Cell lines

Human lung adenocarcinoma cell line (A549), human colon adenocarcinoma cell line (Caco-2) and human monocytic leukemia cell line (THP-1) were kindly provided by Dr. Ayub Qadri, National Institute of Immunology, New Delhi. The other cell lines such as Hela (human cervical adenocarcinoma), HT-29 (human colorectal adenocarcinoma), IMR-32 (human neuroblastoma), T47D (human ductal carcinoma) used in the study were procured from National centre for cell science (NCCS), Pune.

2.1.3 Antibodies

Mouse anti-human Fibronectin polyclonal antibody was purchased from Thermo Scientific Ltd. (Rockford, USA). Mouse anti-human EDA (IST-9) monoclonal and Rabbit anti-human Transferrin polyclonal antibodies were obtained from Abcam (Cambridge, UK). Epithelial-Mesenchymal Transition antibody sampler kit was purchased from Cell Signalling and Technology (Boston, USA). Either Horse-radish peroxidase (HRP) conjugated anti-mouse IgG or IR-labelled anti-mouse IgG, both procured from Thermo Scientific Ltd. (Rockford, USA) were used as Secondary antibodies. The Alexa Fluor secondary antibody used for immunoflorescence was also purchased from Thermo Scientific Ltd. (Rockford, USA). Mouse anti-Human TLR-2 monoclonal blocking antibody (CD282) and mouse anti-Human TLR-4 (CD284) monoclonal blocking antibodies were obtained from Invivogen (San Diego, USA)

2.2 Methods

2.2.1 Maintenance of cell lines

Cell lines such as A549, Caco-2, HT-29, Hela, T47D and IMR-32 were cultured in DMEM supplemented with 10% FBS whereas THP-1 was maintained in RPMI supplemented with 10% FBS. Both the media supplements were also supplemented with 100 U/ml penicillin and 100 mg/ml streptomycin. Cells were cultured under a humidified 5% CO_2 atmosphere at 37°C in incubator. The medium was changed every 2 days or until the cells became confluent and then used for experimentation. For co-culturing and secretome analysis, cells were cultured in low protein serum free medium (LPM).

2.2.2 Co-culture and conditioned medium obtention

Human lung carcinoma cells, A549 were co-cultured with human monocytic leukemia cell line, THP-1 in selected ratios of 1:1, 1:5, 1:10, 1:20 and 1:40. Briefly, A549 cells were seeded in a 24-well plate, allowed to grow overnight in 10% FBS supplemented DMEM. Thereafter, the medium was removed and cells were washed thrice with serum free medium (DMEM or RPMI-1640). Subsequently, the cells were co-cultured with varying ratios of THP-1 cells in a LPM for various time points. After co-culture, the conditioned medium designated

as co-culture conditioned medium (CCM) was collected and centrifuged at 4000 g for 30 min to remove any cell debris and stored at -80 °C until use. Control consisted of A549 or THP-1 cells (higher number of both cell types used for co-culture) cultured in low protein serum free medium without any admixed cell population and the conditioned medium collected thereof was referred to as homotypic conditioned medium (CM).

2.2.3 Cell proliferation assay

MTT assay was employed to evaluate the role of macrophages in promoting tumor cell proliferation. Cell suspension containing 2 $\times 10^4$ A549 cells per well was seeded into a 96-well microtiter plate. After 24h of seeding, cells were cultured in co-culture conditioned medium obtained after co-culture of A549 and THP-1 in various ratios. Controls consisted of cells treated with homotypic conditioned medium or unconditioned medium (LPM only). Each treatment was tested in triplicate. The cells were incubated at 37°C in a humidified incubator with 5% CO_2 for 24h. After 24h, media was removed and MTT solution was added to the cells at a concentration of 0.1 mg/ml followed by incubation for 4h at 37°C in dark. Then the supernatant was removed and an equal volume of DMSO was added to dissolve the formazan crystals. The absorbance was measured at 565 nm against the background absorption at 650 nm in a microplate reader (Bio-Tek Instruments, USA).

2.2.4 Wound healing assay

A549 cells were plated in 30 mm dishes and grown upto 90% confluency in DMEM supplemented with 10% FBS. The media was then removed and the monolayer was scratched with a 200 ml pipette tip, washed twice with PBS to remove detached cells and photographed (t=0). Cells were then incubated in co-culture conditioned medium or homotypic conditioned medium for 24 h. Then the wounds were observed and photographed (t=24). The assay was repeated three independent times.

The percentage of wound closure was estimated by the formula as:

Percentage Wound closure = [1- (wound area at t=24)/ wound area at t=0 x 100%], where t=24 is the time after wounding and t=0 is the time immediately after wounding.

2.2.5 Invasion assay

The polycarbonate filter inserts (8 mm pore size, Corning) precoated with Matrigel were pre-incubated in DMEM supplemented with 1% FBS for 2h before the cells were plated. Tumor cells (5×10^4) in 200 µl of co-culture conditioned medium or homotypic conditioned medium were seeded in the upper chamber. Then 500 µl DMEM supplemented with 10% FBS was added to the lower chamber as a chemotactic agent. After 24h incubation, non-migrating cells in the upper chamber of the filters were removed using cotton swabs. The cells that migrated and adhered to the other side of the filter were fixed in 3.7% formaldehyde for 20 min, stained with crystal violet and counted per five fields or quantitated by measuring absorbance at 570 nm after elution of crystal violet with 10% acetic acid.

2.2.6 Cytokine quantification

TNF-α, IL-6 and TGF-β levels were quantified from culture supernatants using commercially available kits following manufacturer's instructions. Briefly, a 96-well microplate (Maxisorp, Genetix) was coated with 50 µl capture antibody (diluted 1: 250 in 100mM carbonate buffer, pH 9.5) and kept overnight at 4°C. Then plate was washed 3 times with PBS-Tween (PBST) and blocked with PBS-BSA-1% (100µl/well) for 1h at 37°C. After washing, samples were added to each well and the plate was incubated for 1h at 37°C. Subsequently, the plate was washed and incubated with detection reagent mix (detection antibody + avidin-HRP) diluted 1: 250 in PBS-BSA 1%. After 1h of incubation, the plate was washed and the enzyme activity determined by adding freshly prepared substrate solution containing TMB/TBABH/H_2O_2 (50 µl/well). The reaction was stopped with 50 µl of 2N H_2SO_4 and the absorbance was read at 450 nm (or as advised in manufacturer's instructions). All the assays were performed in triplicate.

2.2.7 Gelatin zymography

Gelatin zymography was performed to get an insight into the effect of co-culture on the expression of MMP-9. The conditioned medium was mixed with 5x sample buffer (lacking β-mercaptoethanol and DTT) and the mixture loaded on 10 % SDS-PAGE. After completion of the run, gel was washed two times with wash buffer (2.0% Triton X-100 in dH_20) for 20 minutes each. Finally the gel was incubated in incubation buffer (50 mM Tris-HCl, pH 8.0, 10 mM CaCl2, 0.02% NaN_3) in a sealed container at 37°C overnight. Gelatinase activities were visualized by staining the zymograms with Coomassie Brilliant Blue G250 (0.25% Coomassie Brilliant Blue G250, 30% acetic acid and 10% methanol), followed by destaining in acetic acid-methanol-dH_20 (1:5:5).

2.2.8 Xenograft Model

A group of five BALB/c mice of same sex and age were used in this study. Mice were immunized with A549, Lung adenocarcinoma cell line. This was done by subcutaneously injecting 5×10^6 A549 cells suspended in sterile Phosphate buffer saline per mouse. Before immunization, retro-orbital bleeding was carried out to obtain pre-immune sera. After fifteen days a booster dose of same number of cells was given intraperitoneally and bleeding carried after a week to obtain the anti-sera.

2.2.9 Flow cytometry

In order to evaluate the specificity of anti-sera, 2×10^5A549 and other cancer cell lines were suspended in 200 µl of DMEM-10 and added to each well of 96-well microplate. This was followed by centrifugation at 1500 rpm for 5 min and incubation with pre-immune or anti-sera for 2h at 4°C. After incubation with primary antibody, cells were washed with PBST three times. Finally incubation with rabbit anti-mouse FITC conjugated secondary antibody was carried out for 1h at 4°C in dark, followed by three washings with PBST and finally the samples were analyzed using BD FACS caliber™.

2.2.10 ELISA

To screen anti-sera for reactivity against the proteins in conditioned medium, 100 µl of conditioned medium was used to coat the wells of 96-well microplate overnight at 4°C. Blocking (100 µl/well) was carried with 1% BSA for 1h, followed by two washings with PBST. Subsequently, the plate was incubated with Pre-immune or anti-sera (100 µl/well, used at 1:500 dilution) at 37ºC for 1h. After three washings with PBST, HRP-antimouse (100 µl/well) was added to each well and incubated for 1h. The assay was developed using OPD as a substrate (100 µl/well). Finally, the reaction was stopped with 50 µl of 2N H_2SO_4 and the absorbance was read at 450 nm.

2.2.11 Secretome preparation

For secretome preparation, equal cell numbers of each type (A549, Caco-2, THP-1) were platted and cultured in standard culture medium (DMEM or RPMI-1640 supplemented with 10% FBS) until reaching sub-confluency and then washed 3 times with serum free medium (DMEM or RPMI). Subsequently the cells were cultured in medium supplemented with 1% FBS, LPM, Synser, DCCM-1 or Pancerin for secretome analysis. After 48h of incubation, conditioned media was collected, centrifuged, filtered to remove cellular debris and stored at -80ºC until use.

2.2.12 Cell Death (LDH) Assay

Lactate dehydrogenase activity was measured to evaluate the extent of cell death in various media formulations used for secretome analysis. The assay quantitatively measures a stable cytosolic enzyme lactate dehydrogenase which is released upon cell lysis. The released LDH is measured with a coupled enzymatic reaction that results in the conversion of a tetrazolium salt (Iodonitrotetrazolium) into a red color formazan. Briefly 50 µl of the samples were pippetted in 96-well plate and 50 µl of substrate mixture added to it. The reaction was incubated in dark at 37°C for 20 min and finally stopped with stop solution. The absorbance was measured at 500 nm using a micro plate reader (Biotek instruments, USA).

2.2.13 SDS-PAGE

SDS-PAGE was carried out using the Laemmli buffer system under reducing conditions. The resolving gel was polymerized in a Hoefer or BioRad Protean-3 mini gel apparatus for 30-45 min. The thickness of the gel was 1.5 mm. The stacking gel prepared afresh was layered on top of the resolving gel and allowed to polymerize for 15-20 min. 30- 40 µg of samples (total protein extract or conditioned medium) to be analyzed were mixed with 5x Laemmli sample buffer and placed in a heating block at 100°C for 5 min before loading into wells. Electrophoresis was carried out at a constant current of 30 mA. The gels were stained using CBB or silver nitrate.

2.2.14 Western Blot

The samples to be analyzed were separated on 10% or 12% SDS-PAGE and transferred to a PVDF membrane by semi-dry blotting for 1.5h at a constant current of 250 mA, using a semi-dry transfer apparatus (Siplast, UK). The transfer of proteins was ascertained by staining the PVDF membrane with Ponceau-S (1X). The membrane was blocked overnight at 4ºC with 1% BSA prepared in PBS and subsequently probed with the appropriate primary antibody at recommended dilutions and time points, followed by three washings with PBST. Finally the membrane was incubated in HRP or IR-labelled labeled secondary antibody for 1h. After three washings with PBST, the membrane was developed using Enhanced Chemiluminescence reagents or scanned on an Infrared imager, Odyssey Licor (Licor Biosciences, USA).

2.2.15 LC/MS/MS Analysis

The analysis was carried out at Proteomics facility of Centre for Cellular and Molecular platforms (CCAMP), National centre for Biological Sciences, India. According to the protocol, samples were subjected to ingel digestion as per the protocol of Shevchenko et al. Digested peptides were reconstituted in 15 µL of the 0.1% formic acid and 1 µL of the same injected on column and subjected to Standard 70 min RPLC-MS/MS analysis with Collision induced dissociation as the fragmentation method. The generated data was searched against identity using Mascot 2.4 as search engine on proteome discoverer 1.3. The data was searched

against Uniprot Swiss-Prot database (non redundant database with reviewed proteins). Minimum of two high confident peptides was used as a prerequisite to identify the proteins. 12.5 fmoles of Standard BSA digest was analyzed at the beginning and end of sequence to check the performance of the instrument. The taxonomy selection was Homo sapiens.

2.2.16 RNA isolation and cDNA Preparation

RNA was isolated using TRIzol reagent according to manufacturer's instructions. Briefly 1 ml of TRIzol was added to the cells grown in 30 mm culture dish followed by homogenization using a pipette. Thereafter 1ml of sterile water was added and the mixture incubated at room temperature for 5-15 min. The mixture was centrifuged at 12,000 g for 15 min and supernatant collected, to which 70% isopropanol was added. The supernatant was further centrifuged at 12,000 g for 10 min to obtain the RNA pellet which was washed two times with 75% ethanol. The integrity of RNA was checked on 1% agarose gel. In succession to RNA isolation and subsequent quantification, RNA was reverse transcribed to cDNA using reverse transcriptase and oligo-dT primers in a final volume of 20 µl. The steps followed were as:

Step 1

Reaction mixture 1

1. RNA = 2 µl (1 µg)
2. Oligo-dT = 0.5 µl (0.1 µM)
3. Nuclease free H_20 = 10 µl

Programme: 70°C - 10 min; 4°C - ∞

Step 2

1. Reaction mixture 1 = 12.5 µl
2. 5x buffer RT buffer = 4 µl
3. DTT = 2 µl
4. dNTP's = 1 µl
5. RT = 0.5 µl

Programme: 37°C - 60 min; 4°C - ∞

2.2.17 Polymerase Chain Reaction

After the successful preparation of cDNA, a PCR reaction of 25 cycles was set with following programme for the amplification of EDA and EDB exons respectively.

- Initial denaturation: 94 °C / 10 min
- Denaturation: 94 °C / 30 sec
- Annealing: (as indicated on a primer set)/ 30 sec
- Extention: 72 °C / 30 sec
- Final extention: 72 °C / 7 min

The primers used for evaluating the expression status of EDA or EDB are as:

EDA

Forward primer 5**…**AGTCAGCCTCTGGTTCAGAC**...**3

Reverse primer 5**…**CTTCAGGTCAGTTGGTGCAG**...**3

EDB

Forward primer 5**…**ACACTGTCAAGGATGACAAGGA**...**3

Reverse primer 5**…**TTGGTGAATCGCAGGTCAGT**...**3

Primers used for cloning of EDA are as:

Forward primer 5**…** **GAATTC**AGTCAGCCTCTGGTTCAGAC**...**3

ECORI

Reverse primer 5**…** **GGATCC**CTTCAGGTCAGTTGGTGCAG**...**3

BAMHI

PCR amplified DNA was mixed with 6X loading dye and the sample was electrophoresed on an Agarose gel, and its size was determined relative to the mobility of DNA ladder.

2.2.18 Extraction of plasmid DNA (pGEX-4T2)

Bacteria were cultured overnight at 37°C in LB supplemented with the appropriate antibiotic (Ampicillin 100 µg/ml). Small-scale preparation of DNA for analysis, cloning and transformation was carried out using miniprep kit according to the manufacturer's instructions. pGEX-4T2 is IPTG inducible and carries a GST tag.

2.2.19 Restriction Digestion and Ligation

Digestion of the amplified fragment and pGEX-4T2 vector was carried using BamHI and EcorI Thermo Scientific FastDigest enzymes along with the prescribed universal FastDigest Green reaction buffers. The amplicon was subjected to purification using PCR purification kit prior to digestion. Once the purified product was confirmed on an agarose gel, the double digestion reaction was set up for both the amplicon and isolated plasmid. The digestion was carried for 10-15 min at 37°C. Both the Digested Products were run on an agarose gel, the band corresponding to fragment and digested plasmid was cut and purified using gel extraction kit. Again the purified products were checked on an agarose gel and subsequently quantified using a Nanodrop (Thermo, USA).

The plasmid and amplicon were mixed in a ratio of 1:3 in presence of T4 DNA Ligase. Two tubes ligation and relegation tubes were prepared with latter containing the digested plasmid only. The reaction was set up overnight at 16°C.

2.2.20 Preparation of competent *E. coli* strains

E. coli (laboratory stocks) were inoculated in 5 ml Luria Broth (LB) and incubated overnight for 16-20 hours at 37°C by shaking at 240 rpm. Next day, 1 ml overnight culture was diluted in 100 ml LB and allowed to grow in a shaking incubator at 37^0C until an OD (at 600 nm) of 0.3-0.4 absorbance units, was reached. The culture was kept on ice for 10 min. The cells were centrifuged at 5000 rpm for 10 min at 4°C. The supernatant was discarded, the cell pellet was resuspended in 20 ml sterile $CaCl_2$ (100mM) and left on ice for 30 min. Then the

cells were centrifuged at 5000 rpm for 5 min at 4°C. The supernatant was removed followed by addition of 4 ml sterile $CaCl_2$ (100mM). The cells were used immediately for transformation or stored at -80°C by addition of 15% glycerol for future use.

2.2.21 Transformation of competent *E. coli* cells and subsequent screening

1-5 µl (~100 µg) of plasmid DNA was added to 50 µl competent bacteria and incubated on ice for 30 min. The bacteria were subjected to heat shock for 45 seconds at 42°C and then chilled on ice for 10 min. Following this, the entire mix was spread on agar plate containing the appropriate antibiotic (Ampicillin 100µg/ml). Agar plates were put inverted in a 37°C incubator for 16-20 hours. Ampicillin resistant clones were picked up and plasmids were purified using plasmid purification kit and digested with same restriction enzymes as used for cloning the insert to confirm the presence of insert. This was further confirmed by using recombinant plasmid as template for the amplification of EDA insert.

2.2.22 Protein expression and purification

Constructs pGEX4T2 (GST-EDA) and the parental control plasmid pGEX4T2 were transformed into the BL21-DE3 pLysS host strain and fusion protein expression was induced for 16h at 37°C with different concentrations of IPTG viz 100 mM, 110 mM and 130 mM. Bacteria were collected at 5,000g and cells were lysed by incubation for 30 min in a buffer containing 50 mM Tris-Cl, 5 mM EDTA, 50 mM NaCl, lysozyme (0.2 mg/mL) and protease inhibitor cocktail, followed by sonication (10 Sec pulses -10 Sec rest) for 1 min. Pre-cleared lysates were subjected to affinity chromatography using GSH beads (50% slurry) by batch purification method as per standard conditions and as recommended by the manufacturer. Fusion protein was eluted from the beads using glutathione buffer containing 50 mM Tris-Cl, 10 mM reduced glutathione (Sigma) and SDS-PAGE was used to assess the purity of the protein. The eluted protein was passed through the endotoxin removal column before its use in cell culture.

2.2.23 Depletion of Fibronectin in conditioned medium

Protein G-Agarose beads were washed three times with PBS and centrifuged to pellet at 1000 rpm. For immobilization, mouse anti-human Fibronectin polyclonal

antibody diluted in PBS was incubated with beads overnight at 4°C. Following washing, ~ 1 ml of conditioned medium was added to the antibody-beads complex and incubation carried overnight at 4°C. The Fibronectin depleted conditioned medium was passed through endotoxin removal columns before its use in cell culture and was designated as FN dep CM**.** The efficiency of depletion was checked by immunoblotting.

2.2.24 In-Cell Western blotting

THP-1 cells were seeded in 24-well tissue culture plates and grown overnight in RPMI supplemented with 10% FBS. After washing with serum free medium, cells were incubated in conditioned medium containing Fibronectin or medium supplemented with recombinant Fibronectin for 24h. This was followed by blocking with 1% BSA in PBS for 1 h and incubation in mouse anti-human Fibronectin antibody overnight at 4°C. After three washing with PBST, cells were incubated in anti-mouse IR-labelled secondary antibody for 1h and the plate was scanned on an Infrared imager, Odyssey Licor (Licor Biosciences, USA).

2.2.25 TLR Blocking Assay

THP-1 cells were incubated with 5 μg/ml mouse anti-human TLR-2 or TLR-4 monoclonal blocking antibody along with isotype control for 1h. Thereafter, the cells were washed 3-5 times with serum free medium to wash off any free antibody. Subsequently, cells were incubated in conditioned medium containing Fibronectin or medium supplemented with recombinant EDA and immunoassayed for cytokines.

2.2.26 Soft agar colony formation assay (Clonogenic assay)

Anchorage independent growth in soft agar was used to assess the tumorigenic potential imparted by secretory Fibronectin and its associated EDA *in vitro*. The soft agar assay was performed in 6-well plates containing two layers of Agar. The bottom layer consisted of 0.8% agar in 1 ml of DMEM supplemented with 10% FBS. Cancer cells ($1x10^4$/well) were placed in the top layer containing 0.4% agar in the same medium as the bottom. Cells were cultured for 14 days under different conditions and colonies were photographed and counted per four fields under a microscope.

2.2.27 Immunofluorescence

Cancer cells were seeded in 24 well tissue culture plates and grown overnight in DMEM supplemented with 10% FBS. After washing with serum free medium, cells were incubated in conditioned medium containing Fibronectin or medium supplemented with recombinant EDA for 24h. Then the cells were fixed with 4% formaldehyde and permeablized with PBS containing 0.1% Triton X-100 for 30 min. This was followed by blocking with 1% BSA in PBS for 1h and incubation in primary antibody overnight (or as recommended) at 4°C. After washings with PBST three times, cells were incubated in secondary anti-mouse IgG antibody conjugated with Alexa Fluor for 1h, observed under Evos cell imaging system (Life Technologies, USA) and photographed.

3.1 Interaction between lung carcinoma cells (A549) and monocytes (THP-1) enhances the Proliferation of lung carcinoma cells

Compelling evidences suggest that there is a strong association between poor survival and increased macrophage density in various cancers, thus pointing at the role of macrophages in tumor cell proliferation and survival. However there is conflicting data for lung, stomach and prostate cancers, where both positive and negative outcome associations have been reported (Zhang *et al*., 2012). In order to evaluate the role of macrophages in promoting tumor cell proliferation, human lung carcinoma cells, A549 were co-cultured with human monocytes, THP-1 to mimic the actual tumor microenvironment scenario. The two cell types (A549 and THP-1) were co-cultured in varying ratios of 1:1, 1:5, 1:10, 1:20 and 1:40 for 24h and the co-culture conditioned medium was collected which was used to stimulate A549 cells. Treatment with conditioned medium obtained from co-culture of A549 and THP-1 cells in 1:10 ratio was more potent than other ratios in enhancing the proliferation of A549 cells (Figure 3.1). The conditioned medium obtained from homotypic culture of A549 or THP-1 cells or unconditioned medium (control) was however, less effective. The results are thus indicative of possible release of mitogenic factors from either or both the cell types when co-cultured.

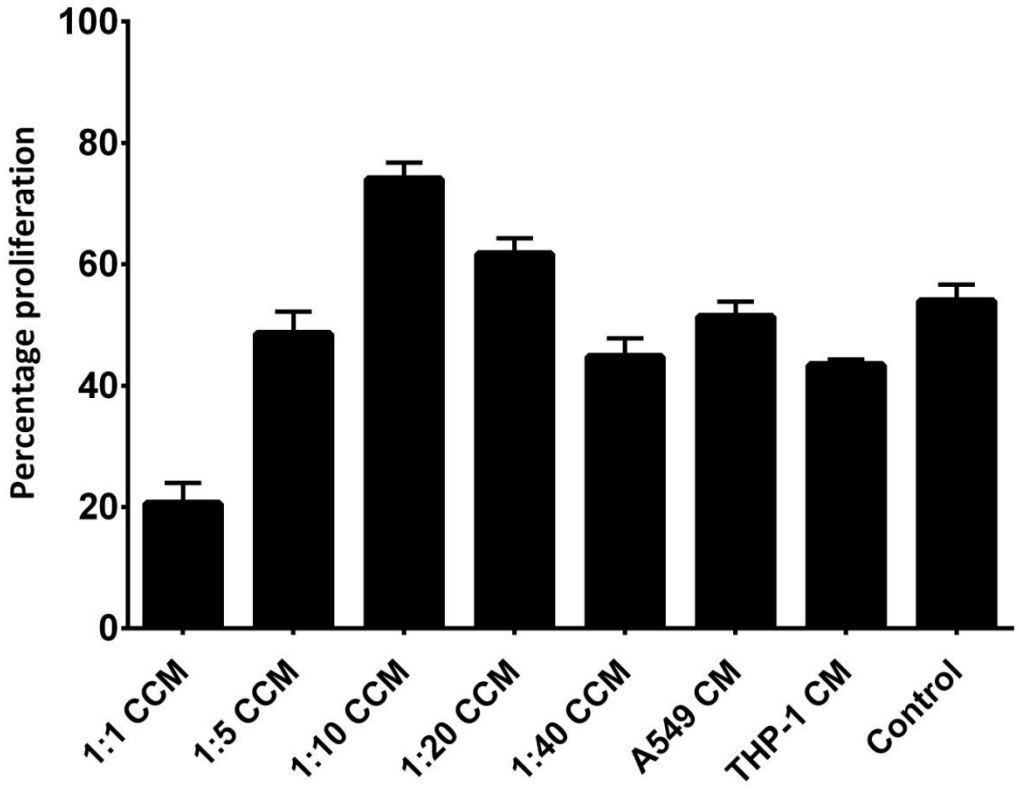


Figure 3.1 Treatment with co-culture conditioned medium increases the proliferation of A549 cells. A549 cells were grown in A549:THP-1 co-culture conditioned media (CCM) or homotypic conditioned media (CM) as described in materials and methods. After 24h, rate of cell proliferation was determined by MTT assay. Data represented as average percentage proliferation ±SD of results obtained from at least three independent experiments.

3.2 Conditioned Medium obtained from co-culture of A549 and THP-1 enhances the migration of A549 cells

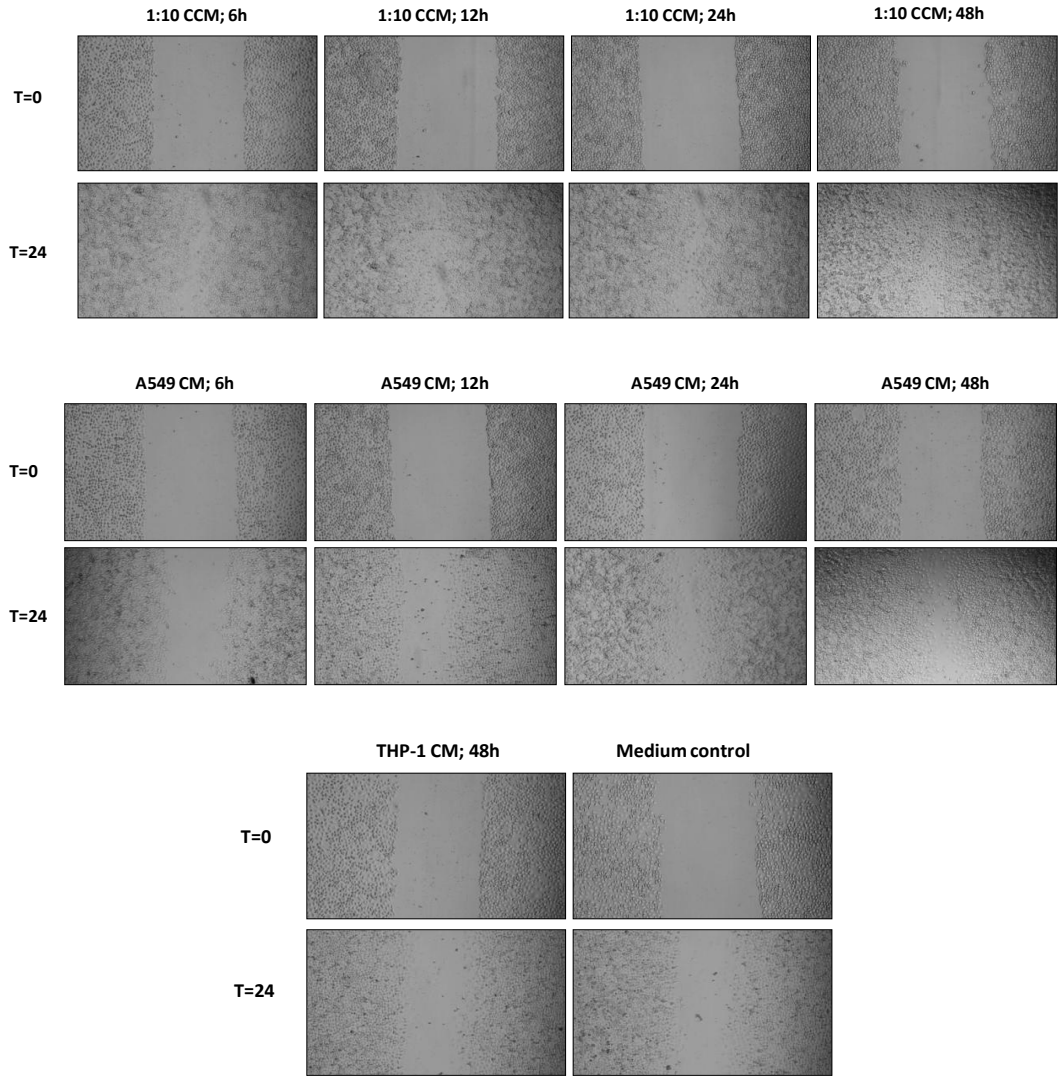
Since, the co-culture conditioned medium accentuated the proliferation of A549 cells, we tried to evaluate the effect of co-culture conditioned medium on the metastatic behavior of A549 cells. A549 and THP-1 cells were co-cultured in the ratio of 1:10 (most effective co-culture ratio as evaluated by MTT assay) for different time points to collect the conditioned medium. To investigate whether the co-culture conditioned medium promotes the cell migration, A549 cells were grown to sub-confluency (70-80%), wounded and then cultured in co-culture conditioned medium (CCM) or homotypic conditioned medium (CM) obtained from A549 cells or THP-1 cells and unconditioned medium (control). After 24h of exposure to conditioned medium, A549 cells treated with 48h co-culture conditioned medium showed almost complete obliteration of the wounded area, although 24h, 12h and 6h co-culture conditioned medium proved to be equally effective (Figure 3.2). However a noteworthy observation was the closure of wound by treatment with homotypic conditioned medium obtained from A549 cells, with 48h conditioned medium being most effective followed by 24h, 12h and 6h. This effect of the homotypic conditioned medium was however, less pronounced than the co-culture medium. Cells treated with homotypic conditioned medium obtained from THP-1 cells or unconditioned medium (medium control) showed marginal healing. These findings thus suggest that the reciprocal interactions between A549 and THP-1 cells result in the secretion of factors from either or both the cell types that in turn promote the migration of A549 cells. It is also explicit that A549 cells themselves secrete certain factors which in an autocrine manner enhance their migration.

3.3 Conditioned Medium obtained from co-culture of A549 and THP-1 increases the invasiveness of A549 cells

We next investigated the effect of co-culture conditioned medium on the invasive behavior of A549 cells. In the similar fashion as above A549 and THP-1 cells were co-cultured in the ratio of 1:10 for different time points to collect the conditioned medium. To determine whether co-culture conditioned medium could promote invasiveness of A549 cells, an *in vitro* invasion assay was performed.

A549 cells in co-culture or homotypic conditioned medium were seeded in the upper chamber of matrigel coated transwell insert with chemoattractant in the lower chamber. It was observed that A549 cells treated with 48h co-culture conditioned medium showed maximal invasion across matrigel coated membrane of the insert when compared with 24h, 12h and 6h co-culture conditioned medium, thus pointing to the secretion of soluble mediators from either or both the cell types during their reciprocal interactions in co-culture (Figure 3.3). A similar but less marked effect was seen when cells were seeded in homotypic conditioned medium obtained from A549 cells, with 48h culture medium being most effective followed by 24h, 12h and 6h. Treatment with homotypic conditioned medium obtained from THP-1 cells showed no obvious effects.

(A)


1:10 CCM; 6h
1:10 CCM; 12h
1:10 CCM; 24h
1:10 CCM; 48h
T=0
T=24
A549 CM; 6h
A549 CM; 12h
A549 CM; 24h
A549 CM; 48h
T=0
T=24
THP-1 CM; 48h
Medium control
T=0
T=24

(B)

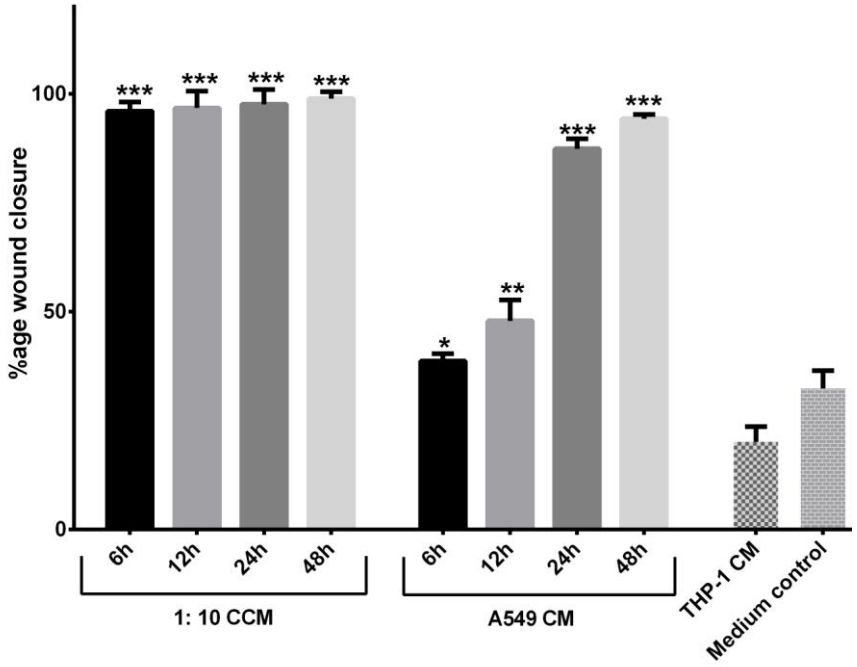
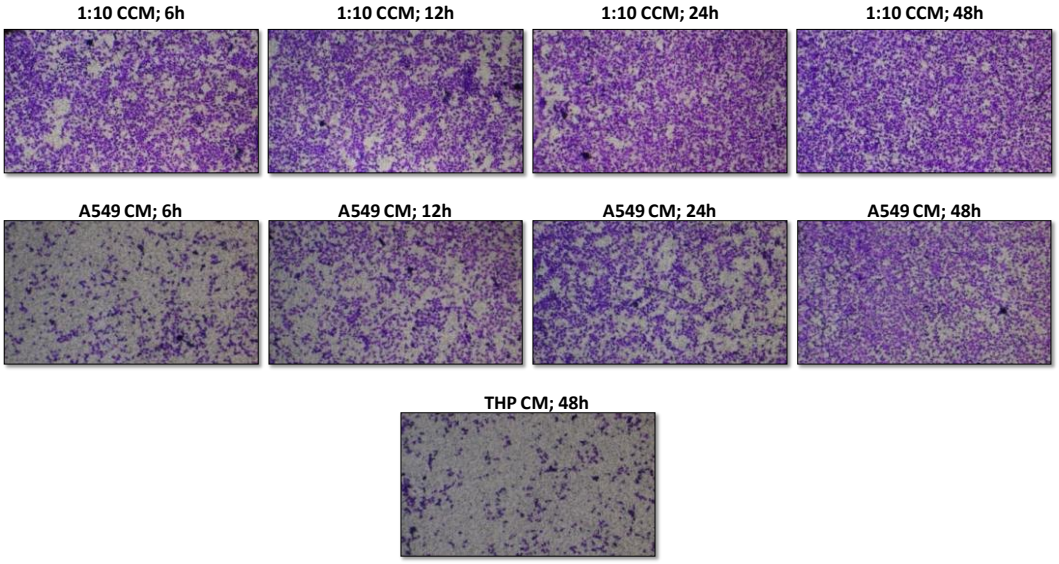


Figure 3.2 Co-culture conditioned medium enhances the migration of A549 cells. A549 cells were grown to sub-confluency in 30mm culture dishes and scratched with sterile tip; incubated in conditioned medium obtained from various co-culture or homotypic culture conditions. **(A)** Scratched areas were photographed at zero hour (T=0) and then subsequently later at 24h (T=24). **(B)** The scratched areas were quantified in three random fields in each case, and data were calculated from at least three independent experiments and represented as mean ±SD. $**p < 0.01$; $***p < 0.001$ compared to control.

(A)



(B)

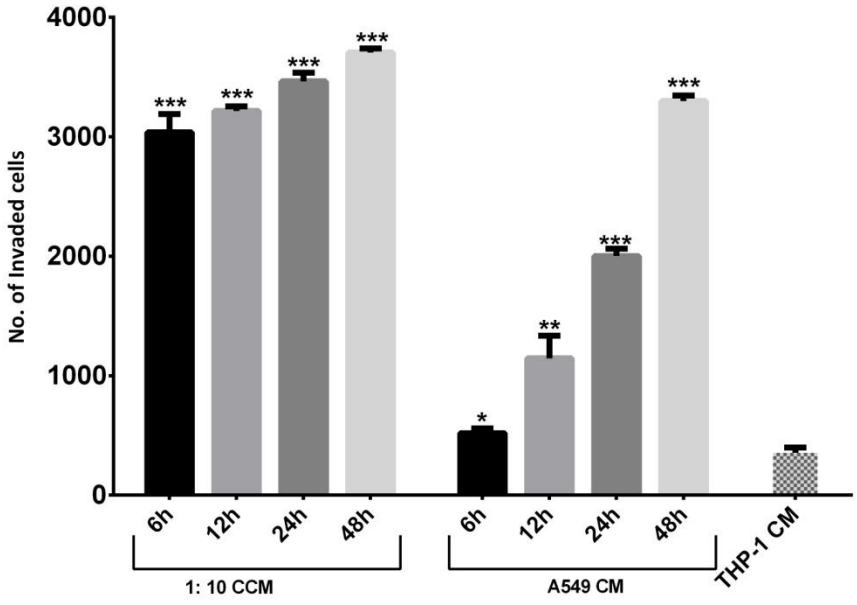


Figure 3.3 Co-culture conditioned medium promotes the invasiveness of A549 cells. A549 cells were seeded in matrigel coated transwell chambers containing conditioned medium obtained from various co-culture or homotypic culture conditions and allowed to migrate. **(A)** After 24h, the migrated cells were fixed, stained, and photographed **(B)** Quantification of invasive cells. Data represented as mean ±SD of results obtained from at least three independent experiments. ***p < 0.01; ***p < 0.001* compared to control.

3.4 Cytokine regulation in Co-cultured cells

Cytokines secreted by activated tumor stromal cells modulate tumor growth and enhance the invasiveness of tumor cells by activation of oncogenic signalling pathways in tumor cells including activation of NF-κB by TNF-α, STAT-3 by IL-6 and SMAD by TGF-β. The increased motility and invasiveness of the tumor cells at the invasive front have been postulated to be propelled by the inflammatory milieu present in the tumor microenvironment. Therefore we assessed the release of pro-inflammatory as well as anti-inflammatory tumor promoting cytokines in our co-culture setup.

3.4.1 *Co-culture of A549 and THP-1 induces the release of TNF-α from THP-1 cells.*

TNF-α is a prototypical pro-inflammatory cytokine that has been implicated in the early stages of carcinogenesis. In order to establish the role of lung carcinoma cells in mediating the release of TNF-α from monocytes, human lung carcinoma cells, A549 were co-cultured with human monocytes, THP-1 in varying ratios of 1:1, 1:5, 1:10, 1:20 and 1:40, respectively, for various time points including 6h, 12h, 24h and 48 h. After co-culture, conditioned medium was harvested and levels of TNF-α were measured by enzyme-linked immunoassay. All the tested co-cultures showed significant production of TNF-α, however, the most potent TNF-α release was observed at co-culture ratios of 1:10 (Figure. 3.4 A). The TNF-α levels increase with time and the response peaks at 48h time point. Neither A549 nor THP-1 cultures alone produced any TNF-α. Similar kinetics of TNF-α were also observed when THP-1 cells were incubated in the conditioned medium obtained from A549 cells (Figure 3.4 B) These results, thus indicate that a stimulus, not only cell associated but also released from A549 cells activates the pro-inflammatory cascade in THP-1 cells.

3.4.2 *Co-culture of A549 and THP-1 induces the release of IL-6 from THP-1 cells*

IL-6 is another pro-inflammatory cytokine, promoting tumor establishment and progression. When A549 and THP-1 cells were co-cultured, a significant release of IL-6 was observed, across all the tested ratios and time points (Figure 3.5 A). The kinetics of IL-6 release were similar to that of TNF-α, with a strong response observed at 48h time point. An optimal IL-6 concentration was detected when the two cell types were co-cultured in the ratio of 1:10, an observation akin to TNF-α response from THP-1 cells. It was also observed that neither A549 nor THP-1 cells alone produce any IL-6. Under stimulation with conditioned medium obtained from A549 cells, similar kinetics of IL-6 response were observed with 48h time point being optimal (Figure 3.5 B).

3.4.3 *Co-culture of A549 and THP-1 induces the release of TGF-β from THP-1 cells but with different kinetics*

TGF-β is an anti-inflammatory cytokine with a complex and paradoxical role in cancer pathogenesis varying with cell type and the stage of tumorigenesis. When immunoassayed for TGF-β production, among all the co-culture ratios (data not shown), only 1:10 ratio of A549 and THP-1 cells exhibited TGF-β response. Following the kinetics of TGF-β under these conditions, it was observed that TGF-β is detectable at 12h time point, unlike TNF-α and IL-6 which are expressed as early as 6h post co-culture (Figure 3.6). The levels increase thereafter and the response peaks at 48h time point. Intriguingly, THP-1 cells do not release any TGF-β when treated with conditioned medium obtained from A549 cells. Neither A549 nor THP-1 cells alone were observed to produce any TGF-β. Absence of TGF-β at early time points and its expression at later time points supports the well-documented role of TGF-β as a modulator of invasion and metastasis at the advanced stages of tumorigenesis.

3.4.4 *Co-culture of A549 and THP-1 enhances the release of MMP-9 from THP-1 cells*

Matrix metalloproteinases (MMPs) plays a pivotal role in tumor growth and the multistep processes of invasion and metastasis, including proteolytic degradation of ECM, alteration of the cell-cell and cell-ECM interactions, migration and

angiogenesis. The co-culture ratio of 1:10 was found to induce the maximal release of MMP-9 from THP-1 cells (Figure 3.7). A549 cells themselves do not secrete any detectable MMP-9, however THP-1 cells exhibit marked release of MMP-9 which gets enhanced during co-culture. As shown in Figure 3.7, gelatinase with molecular weight consistent with an identity of MMP-9 was the main gelatinase expressed in the co-culture conditioned medium.

The attributes of increased proliferation, motility and invasiveness of A549 cells imparted by co-culture conditioned medium may thus be correlated to the production of TNF-α, IL-6, TGF-β and MMP-9 when A549 and THP-1 cells are co-cultured.

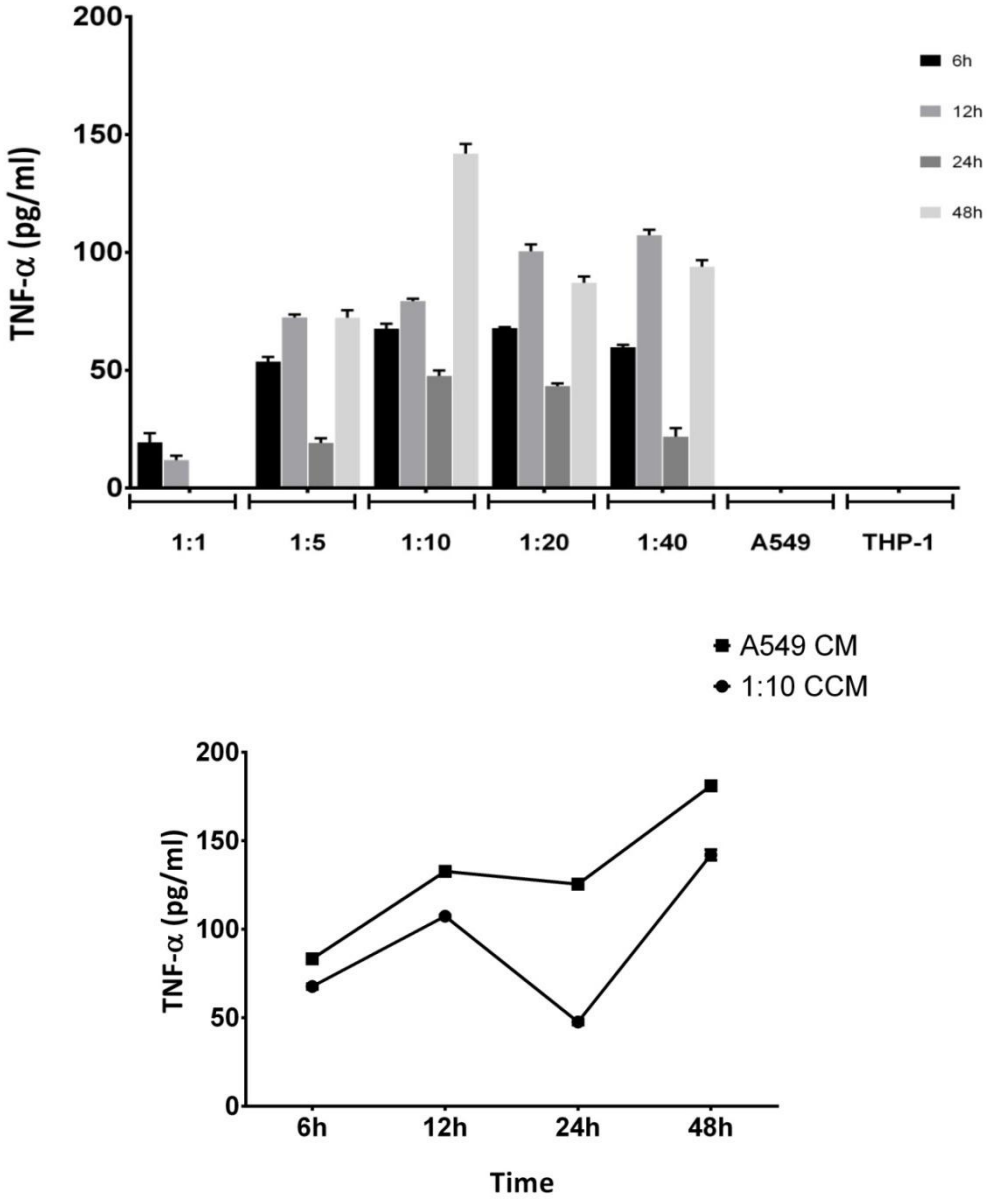


Figure 3.4 Co-culture of A549 and THP-1 cells induces the release of TNF-α from THP-1 cells. **(A)** A549 cells were co-cultured with THP-1 cells in the selected ratios of 1:1, 1:5, 1:10, 1:20 and 1:40 for various time points and the co-culture conditioned medium was immunoassayed for TNF-α. **(B)** Kinetics of TNF-α release from THP-1 cells after stimulation with 48h A549 conditioned medium (A549 CM) compared to its direct co-culture with A549 (1:10 CCM). Data represented as mean ±SD of results obtained from at least three independent experiments.

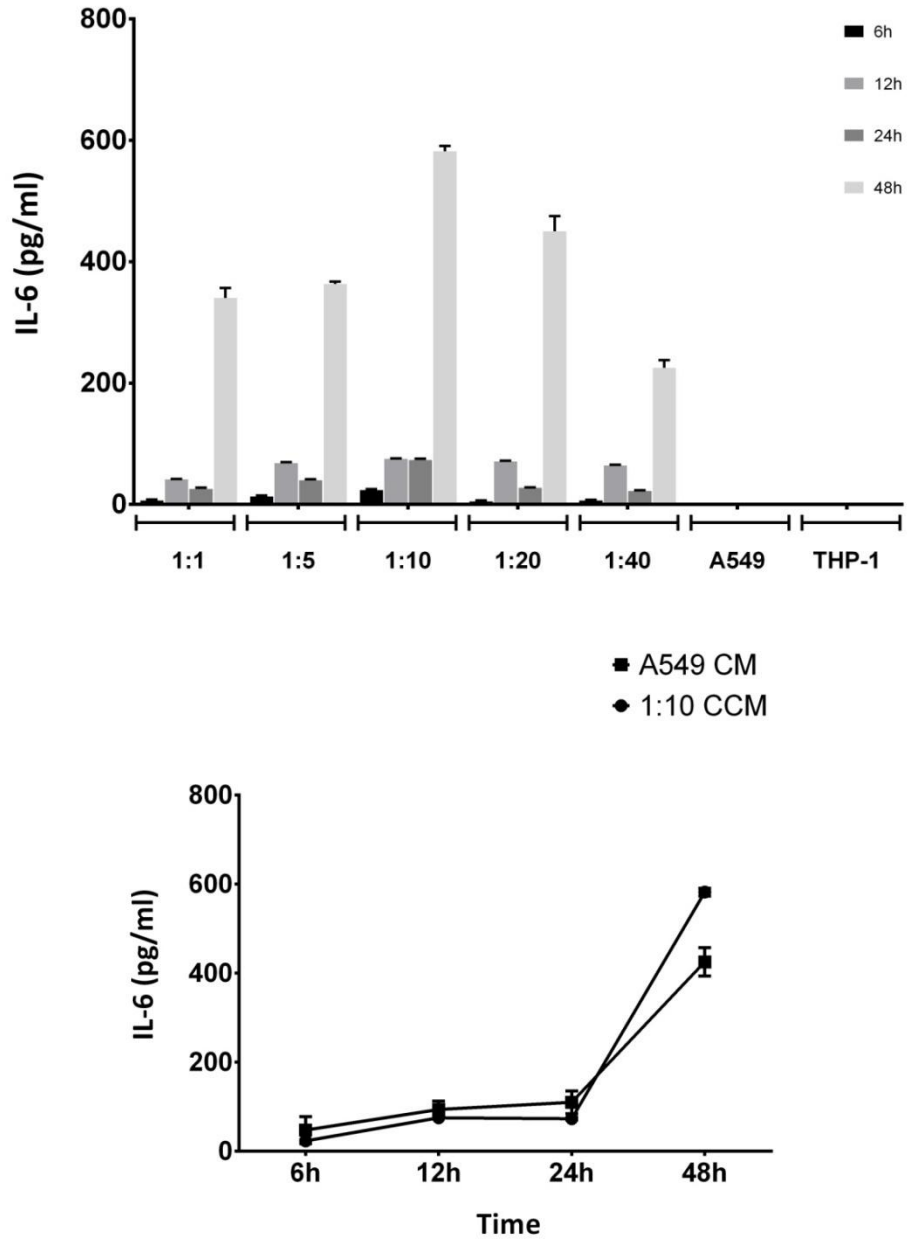


Figure 3.5 Co-culture of A549 and THP-1 cells induces the release of IL-6 from THP-1 cells. **(A)** A549 cells were co-cultured with THP-1 cell in the selected ratios of 1:1, 1:5, 1:10, 1:20 and 1:40 for various time points and the co-culture conditioned medium was immunoassayed for IL-6. **(B)** Kinetics of IL-6 release from THP-1 cells after stimulation with 48h A549 conditioned medium (A549 CM) compared to its direct co-culture with A549 (1:10 CCM). Data represented as mean ±SD of results obtained from at least three independent experiments.

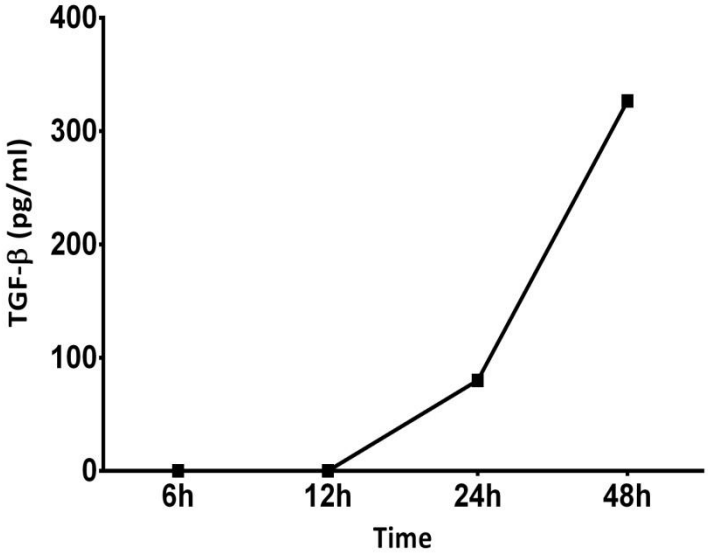


Figure 3.6 Co-culture of A549 and THP-1 cells induces the release of TGF-β from THP-1 cells. A549 cells were co-cultured with THP-1 cells in the ratios of 1:10 for various time points and the co-culture conditioned medium was immunoassayed for TGF-β. Data represented as mean ±SD of results obtained from at least three independent experiments.

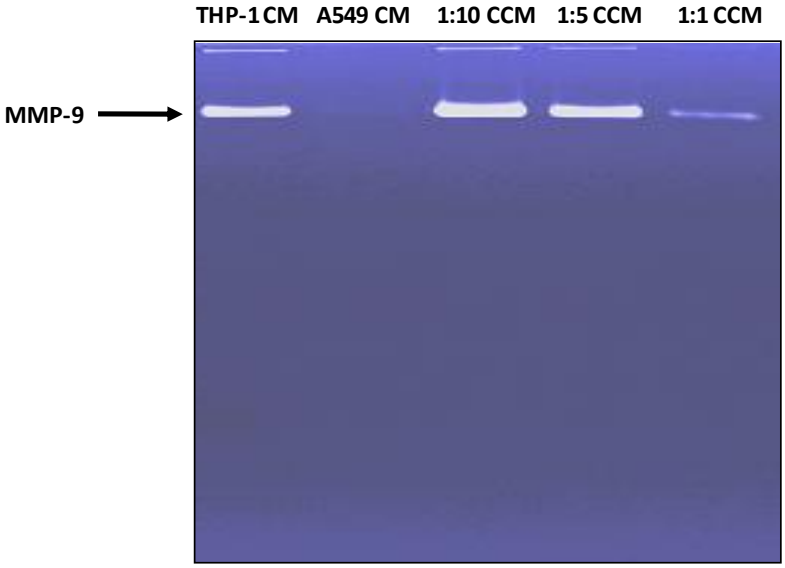


Figure 3.7 Co-culture of A549 and THP-1 upregulates the expression of MMP-9 from THP-1 cells. A549 and THP-1 was co-cultured in the indicated ratios for 24h and the co-culture conditioned medium was subjected to gelatin zymography as described in materials and methods.

3.5 Identification of secretory stimulus from A549 cells by an immunosecretomics approach

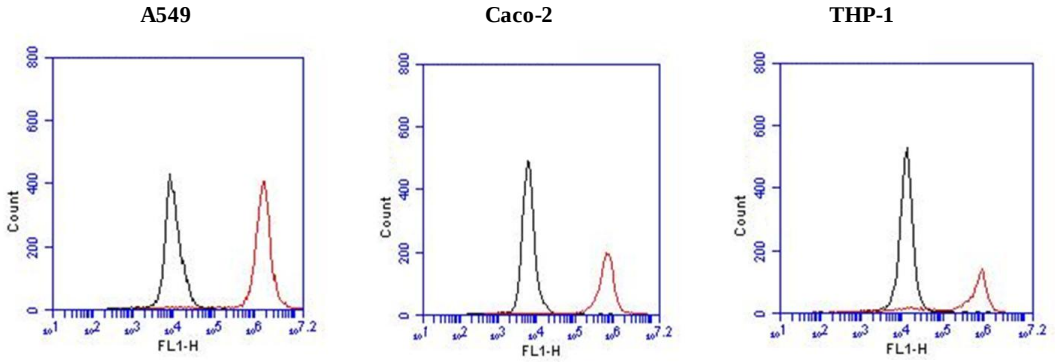
The cancer secretome includes the extracellular matrix components and all the proteins that are released from a given type of a cancer cell such as adhesion molecules, growth factors, cytokines, shed receptors etc. Most of these proteins act in an autocrine or paracrine fashion, resulting in the acquisition of a favorable milieu for the progression of tumors. Immunoscreening of cancer cell secretome may serve as a valuable tool for the identification of protein(s) secreted by cancer cells. The approach is based on the immune recognition system to scan for the cell surface or secretory proteins. Therefore an advantage of this strategy is that antibodies are immediately generated against the secretory proteins or proteins present at the cell surface, which may serve as valuable reagents to identify the secretory proteome. In order to characterize the immunologically relevant secretory proteins of A549 cells, a xenograft model was used and the strategy involved immunization of BALB/c mice with A549 cells. Anti-sera collected from immunized animals was used as a tool to probe the secretory proteins of A549 cells.

3.5.1 *Antisera shows Predominant reactivity against A549 cells*

The specificity of anti-sera against the A549 cells was first analyzed by flow cytometry. A549 cells in culture were detached and stained with anti-sera or pre-immune sera. As shown in (Figure 3.8 A), anti-sera showed higher reactivity against A549 cells as compared to other cancer cells such Caco-2 and THP-1.

The specificity of anti-sera was further verified against the secretory proteins of A549 cells by ELISA. As shown in the figure, anti-sera showed a predominant reactivity against the secretory proteins of A549 cells as compared to other cancer cell lines (Figure 3.8 B). The results thus indicate that the anti-sera contained a preponderant repertoire of antibodies specific to the secretory proteins of A549 cells.

(A)



(B)

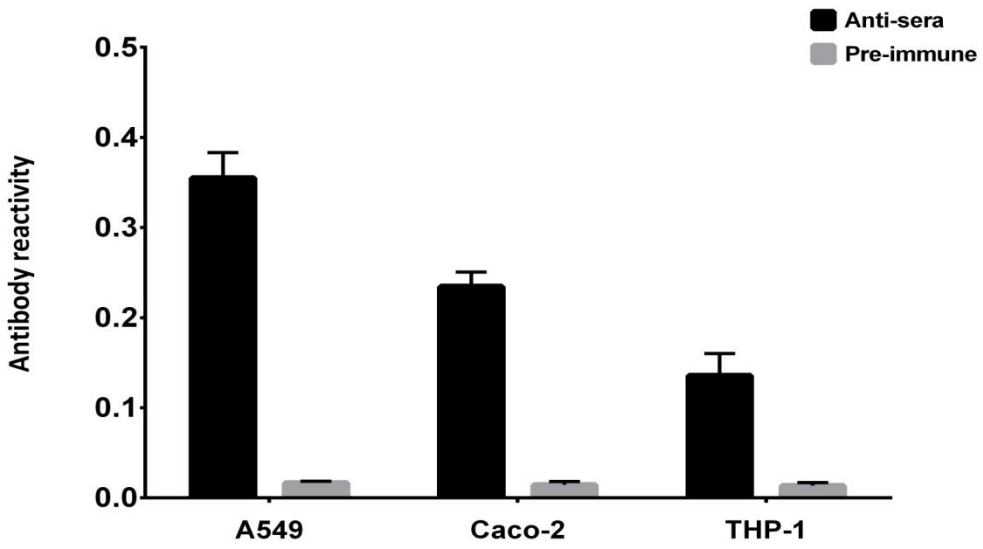


Figure 3.8 Anti-sera shows specific reactivity against A549 cells. (A) Flow cytometric analysis for binding of pre-immune sera (black) and anti-sera (red) to A549, Caco-2, and THP-1 cells respectively. **(B)** Reactivity of the anti-sera against the secretory proteins. Conditioned medium containing secretory proteins of A549, Caco-2 and THP-1 cells was coated on the 96-well ELISA plate and probed with anti-sera or pre-immune sera. Data represented as mean ±SD of results obtained from at least three independent experiments

3.5.2 *Detection of immunogenic secretory proteins of A549 cell line*

Immunoblotting was used to detect the immunologically active secretory proteins of A549 cell line. A549 and other cancer cell were grown in DMEM supplemented with 1% FBS or various media supplements for optimal protein secretion and minimal interference from serum proteins. The conditioned medium (A549 cells grown in DMEM supplemented with 1% FBS) was harvested after 48h, subjected to SDS-PAGE and immunoblotting with anti-sera (Figure 3.9 A). A protein corresponding to ~250 kDa was detected in the conditioned medium of both A549 lung carcinoma and Caco-2 colon carcinoma cell lines. However two other proteins corresponding to ~55kDa and ~50kDa respectively were detected only in the conditioned medium of A549. Albumin (~60 kDa) was detected in the controls as well as the conditioned medium of all the cell lines tested. The conditioned medium of THP-1, a monocytic leukemia cell line did not show any specific reactivity. The pre-immune sera as expected tested negative in terms of reactivity against secretory proteins (Figure 3.9 B).

In order to ensure that the above detected proteins were not artifacts obtained due to contamination of samples with orthologous proteins from serum, A549 cells were grown in various low protein supplements to characterize the secretory proteins that showed reactivity against the anti-sera. Intriguingly only ~250 kDa protein was detected specifically when A549 cells were grown in these low protein supplements for secretome analysis (Figure 3.10 A and Figure 3.10 B). Among all the tested media supplements, LPM proved to be suitable for conditioning as the presence of protein contaminants was minimal and the cell death was negligible (comparable to that of DMEM supplemented with 1% FBS) as evaluated by LDH activity assay (Figure 3.10 C). LPM was therefore selected and used to grow cells for identification of the ~250 kDa protein. Both A549 and Caco-2 secreted ~250 kDa protein in LPM as revealed by western blot analysis (Figure 3.11 A).

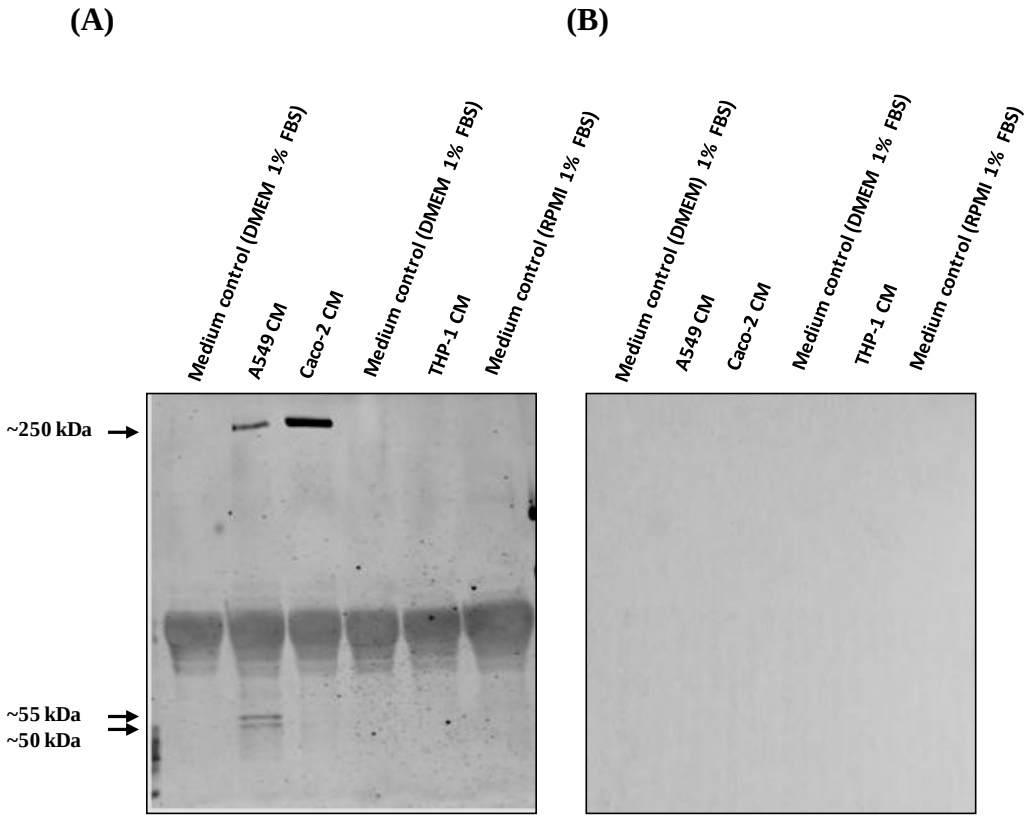


Figure 3.9 Immunoblot analysis of secretory proteins in the conditioned medium of A549, Caco-2 and THP-1 cell lines. Cells were grown in DMEM supplemented with 1% FBS and the conditioned medium (CM) was collected after 48 hours, separated on SDS-PAGE along with controls and transferred on membrane which was probed with **(A)** anti-sera and **(B)** pre-immune sera.

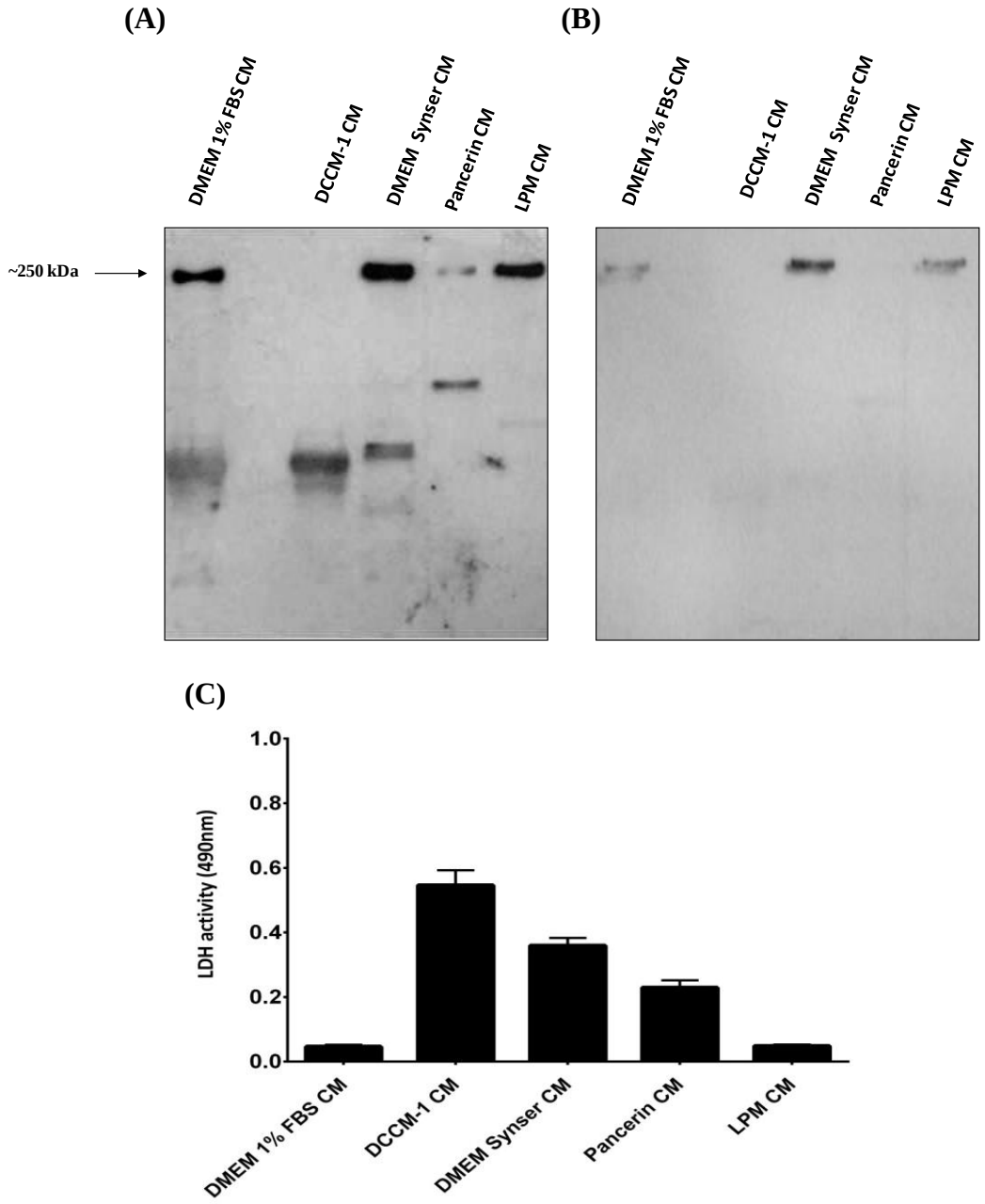


Figure 3.10 Detection of authentic secretory proteins of A549 cell line. Cells were grown in DMEM supplemented with 1% FBS, DMEM supplemented with synser, DCCM-1, Pancerin or LPM. **(A)** Conditioned medium was harvested after 48h, subjected to SDS-PAGE and immunoblotting with anti-sera. **(B)** Immunoblot showing strong and specific reactivity of ~250 Kda protein with anti-sera when membrane was exposed to X-ray film for a few seconds. **(C)** LDH activities in various media formulations used for conditioning, normalized by cell number.

3.5.3 *Identification of ~250 kDa secretory protein as Fibronectin by Mass Spectrometry (LC/MS/MS)*

Immunoblot analysis revealed that the anti-sera could recognize ~250 kDa protein in the conditioned medium of A549 and Caco-2 cell lines (Figure 3.11 A). Therefore the conditioned medium was collected from A549 cell line and subjected to SDS-PAGE (Figure 3.11 B). The aimed protein was excised from silver stained gel, subjected to in-gel digestion and the resulting peptides were analyzed by mass spectrometry (Table 3.1). Generated data was searched for the identity using MASCOT 2.4 as search engine on Proteome discoverer 1.3. The data was searched against both Uniprot Swiss-Prot database (non redundant database with reviewed proteins) and Uniprot TrEMBL database (database with un-reviewed proteins). Finally the protein was identified as Fibronectin by correlating the spectra with the entries in the Swiss-Prot (536789 sequences; 190518892 residues) using Mascot search engine. (Table S1 and Table S2).

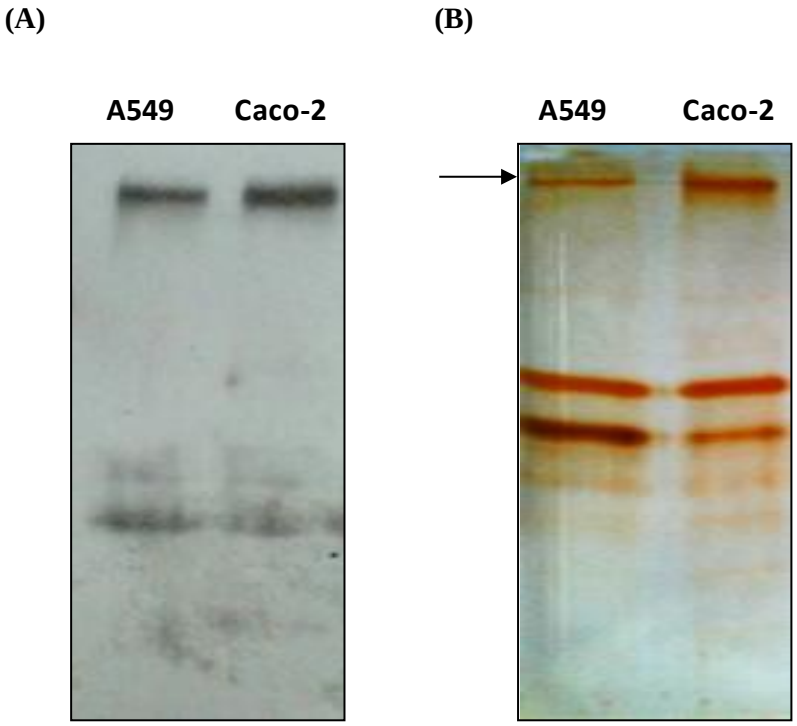


Figure 3.11 Identification of ~250 kDa band. (A) Immunoblot showing ~250 kDa protein in the conditioned medium of A549 and Caco-2 cell lines. **(B)** Silver stained SDS-PAGE profile of the LPM conditioned medium of A549 and Caco-2 cells. Band indicated by arrow was excised and analyzed by LC/MS/MS.

Table 3.1 Characteristic features of Fibronectin identified by LC/MS/MS analysis.

Accession (swiss prot)	Description	Score	Coverage	Unique peptides	Peptides	PSMs	Aminoacids	M.wt (kDa)	Cal. PI
P02751	Fibronectin	6942	42.83	69	69	191	2386	262.5	5.71

3.6 Expression of Fibronectin in the conditioned medium of A549 and other cell lines

The results obtained by LC/MS/MS analysis were further validated by evaluating the expression of Fibronectin in the conditioned medium of A549 and other cell lines by immunoblotting using commercial polyclonal anti-Fibronectin antibody. As shown in the (Figure 3.12), Fibronectin was only detected in the conditioned medium of A549 cell line, an adenocarcinoma cells line established from human lungs. The finding confirmed the results obtained by LC/MS/MS analysis. In contrast, the conditioned medium obtained from the cancer cell lines of different origins tested negative for the expression of Fibronectin.

3.7 Fibronectin release from A549 cells increases with Time

The homotypic conditioned medium obtained A549 cells imparted marked migratory abilities and invasiveness to the A549 cells in an autocrine manner (Figure 3.2 and Figure 3.3), with 48h conditioned medium being most effective followed by 24h, 12h and 6h. To determine whether the presence of Fibronectin has any role in such a phenomenon, expression of Fibronectin was evaluated in the conditioned medium of A549 cells in a time dependent manner. It was observed that the Fibronectin secretion increases with time, with maximum release at 48h (Figure 3.13). Such kinetics sustained till 60 hours after which cells became unhealthy (data not shown). These findings thus demonstrate the role of secretory Fibronectin in mediating the migration and metastasis of A549 cells in an autocrine manner.

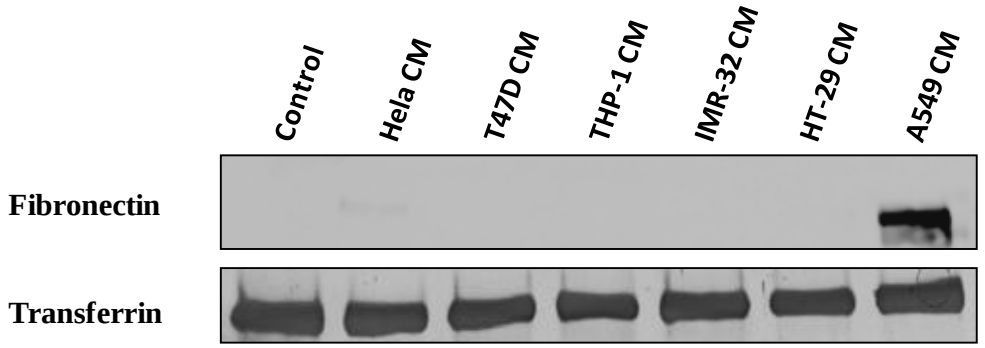


Figure 3.12 Immunoblot showing expression of Fibronectin in the conditioned medium obtained from various cell lines. Conditioned medium was harvested after 24h and evaluated for expression of Fibronectin by immunoblotting using commercial polyclonal anti-Fibronectin antibody.

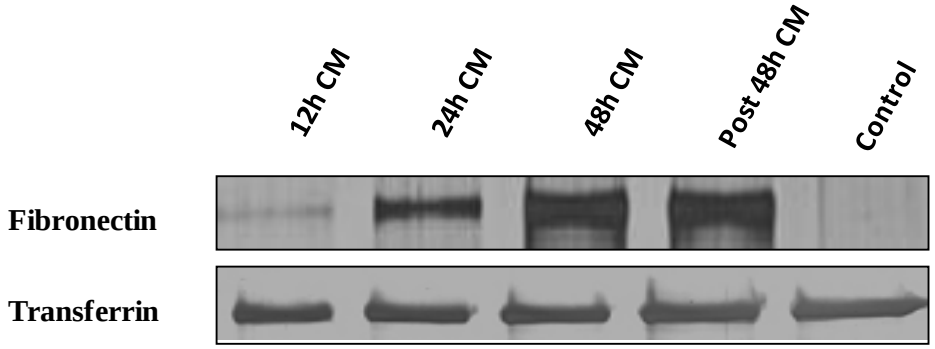


Figure 3.13 Immunoblot showing time dependent expression of Fibronectin in the conditioned medium of A549 cells. Conditioned medium was harvested after indicated time points and evaluated for expression of Fibronectin by immunoblotting.

3.8 Co-culture of A549 and THP-1 increases the secretion of Fibronectin from A549 cells

In order to evaluate the role of secretory Fibronectin in co-culture mediated release of pro-metastatic cytokines from THP-1 cells and co-culture conditioned medium conferred survival, migration and invasiveness to A549 cells, expression of Fibronectin was evaluated in the co-culture conditioned medium in a time dependent manner. It was observed that maximal release of Fibronectin occurred when A549 and THP-1 cells were co-cultured in the ratio of 1:10. Furthermore, the expression of Fibronectin increased from 12 hour to 48 hours, with 48 hour time point being optimal (Figure 3.14).

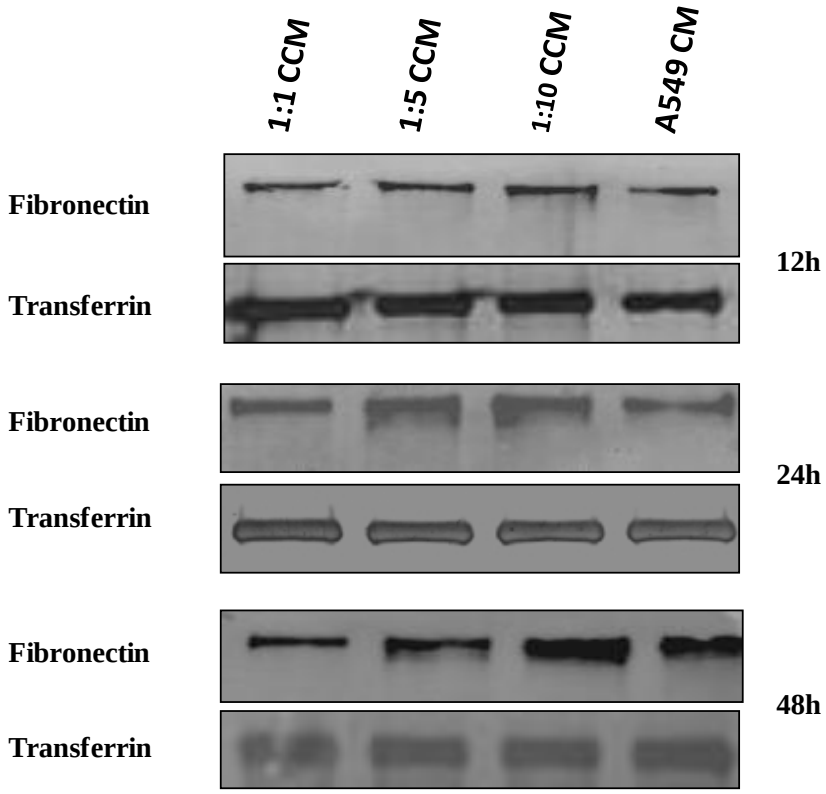


Figure 3.14 Immunoblot showing time dependent expression of Fibronectin in the conditioned medium when A549 and THP-1 cell lines are co-cultured. A549 and THP-1 cell lines were co-cultured in the ratio of 1:1, 1:15 and 1:10 respectively for indicated time points, conditioned medium was harvested and evaluated for expression of Fibronectin by immunoblotting.

3.9 Expression analysis of EDA and EDB exons in Fibronectin

Fibronectin is a high molecular weight adhesive glycoprotein that consists of three different types of repeating units designated type I, type II and type III (Hynes, 1990) (Figure 1.7). The Fibronectin gene encodes 15 type III repeats which are constitutively expressed and 2 repeats that are alternatively spliced designated the extra domain A (EDA) and extra domain B (EDB) (Kornblihtt *et al*., 1996). EDA and EDB are encoded by single exons and their inclusion or exclusion from Fibronectin mRNA is determined by a highly regulated alternative splicing mechanism. The Fibronectin isoforms lacking both EDA and EDB domains are the most abundant forms present in the normal tissues. However under certain conditions EDA and/or EDB may be included in the Fibronectin isoforms which may in turn impart pathological features associated with a disease phenotype. Fibronectin containing EDA has been found to be more potent than Fibronectin lacking EDA in promoting cell spreading and cell migration irrespective of presence or absence of EDB (Manabe *et al*., 1997).

To investigate the presence of EDA and EDB exons in the A549 encoded Fibronectin mRNA, Reverse-Transcription PCR was performed. RNA was isolated from A549 (Figure 3.15) and other cell lines and reverse transcribed to cDNA which was attempted to be amplified using EDA or EDB exon specific primers. The results indicated that A549 cell line tested positive for both the EDA and EDB exons (Figure 3.16), however the other cell lines which did not secrete Fibronectin in the culture medium only expressed EDB exon or did not express either of the exons (Figure 3.17). The DNA sequence analysis of amplified products of both the exons confirmed their identity as EDA and EDB respectively (Data S1 and Data S2). These observations were further validated by evaluating the expression of EDA at protein level by immunoblotting (Figure 3.18).

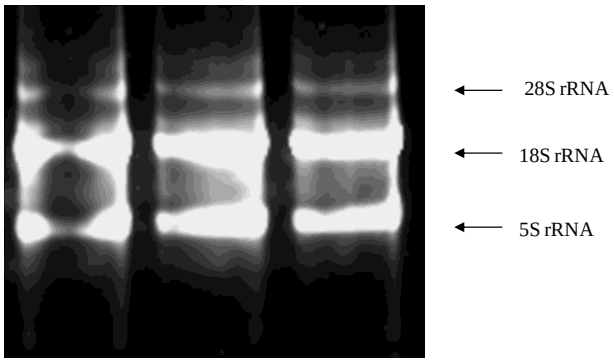


Figure 3.15 Agarose gel showing the integrity of isolated RNA.

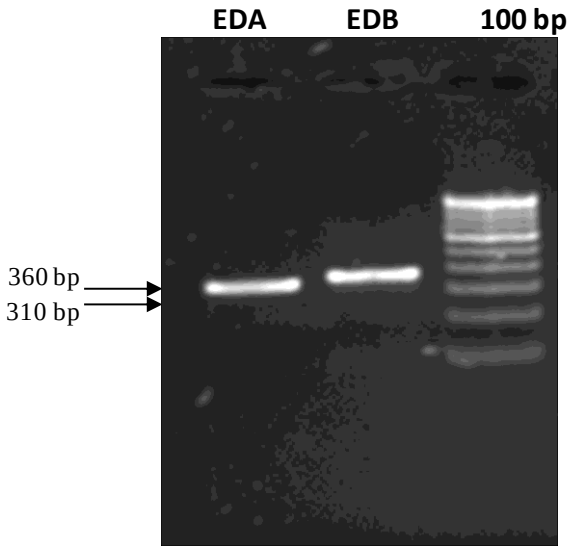


Figure 3.16 Agarose gel showing amplification of 310 bp and 360 bp fragments representing EDA and EDB exons of Fibronectin in A549 cell line.

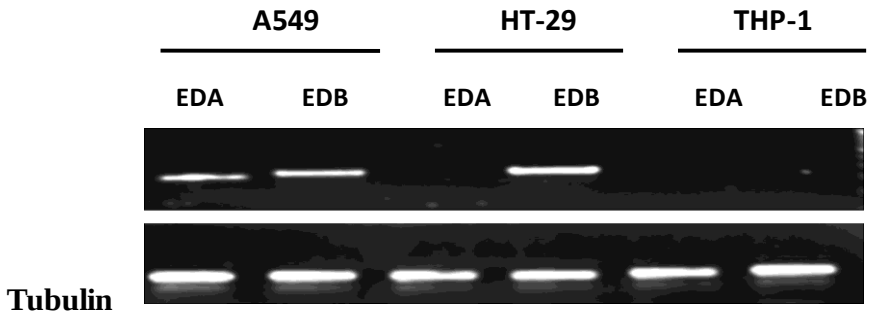


Figure 3.17 Semi-quantitative reverse transcription PCR showing differential expression of EDA and EDB exons in A549, HT-29 and THP-1 cell lines.

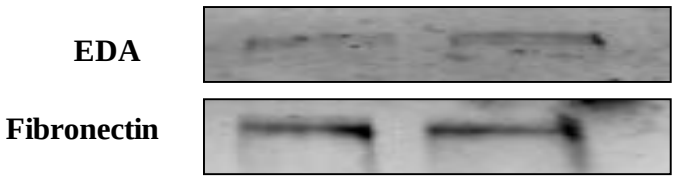


Figure 3.18 Immunoblot showing expression of EDA in Fibronectin secreted by A549 cells.

3.10 Cloning and Expression of EDA exon in bacterial system

It was now evident that A549 cells, as a result of tumorigenesis-triggered aberrant post transcriptional mechanism specifically express EDA which may therefore act as a signal to release Fibronectin into the extracellular microenvironment. EDA was found to be expressed in the Fibronectin secreted by A549 cells (Figure 3.18), thus making it imperative to elucidate its role as a functional entity of Fibronectin. Numerous functions have been ascribed to EDA including cell adhesion, wound healing, matrix assembly, dimer formation, cell differentiation etc. Therefore cloning of EDA was carried out with the aim to express and purify the resulting protein, which was later used in various *in vitro* functional assays.

3.10.1 *Reverse Transcription PCR and Amplification of EDA exon*

RNA was isolated from A549 and other cell lines using trizol reagent. The extracted RNA was reverse transcribed to cDNA using oligo dT primers. In order to facilitate the cloning of EDA exon, cDNA obtained was amplified using primers specific to EDA exon. Forward and reverse primers were designed to carry an ECORI and BamHI sites respectively at the 5` end to ensure directional cloning in pGEX-4T2 vector. As shown, fragment corresponding to 310 bp representing EDA exon was succesfully amplified by PCR (Figure 3.19 A).

3.10.2 *Isolation of Plasmid, restriction digestion, Ligation and screening*

The plasmid DNA (pGEX-4T2) was isolated from DH5-α E.coli cells and its quality was assessed on Agarose gel (Figure 3.19 B). Subsequently the isolated plasmid and purified amplified EDA product were digested separately with ECORI and BamHI (Figure 3.19 C). The digested products were ligated using T4 DNA ligase. The resulting ligation mixture was transformed into chemically competent DH5-α cells. The transformed colonies were screened by double digestion, whereby the isolated plasmid was digested with ECORI and BamHI which resulted in the release of EDA insert from the recombinant vector (Figure 3.19 D). The PCR amplification of EDA using recombinant vector as the template further validated the successful cloning (Figure 3.19 E).

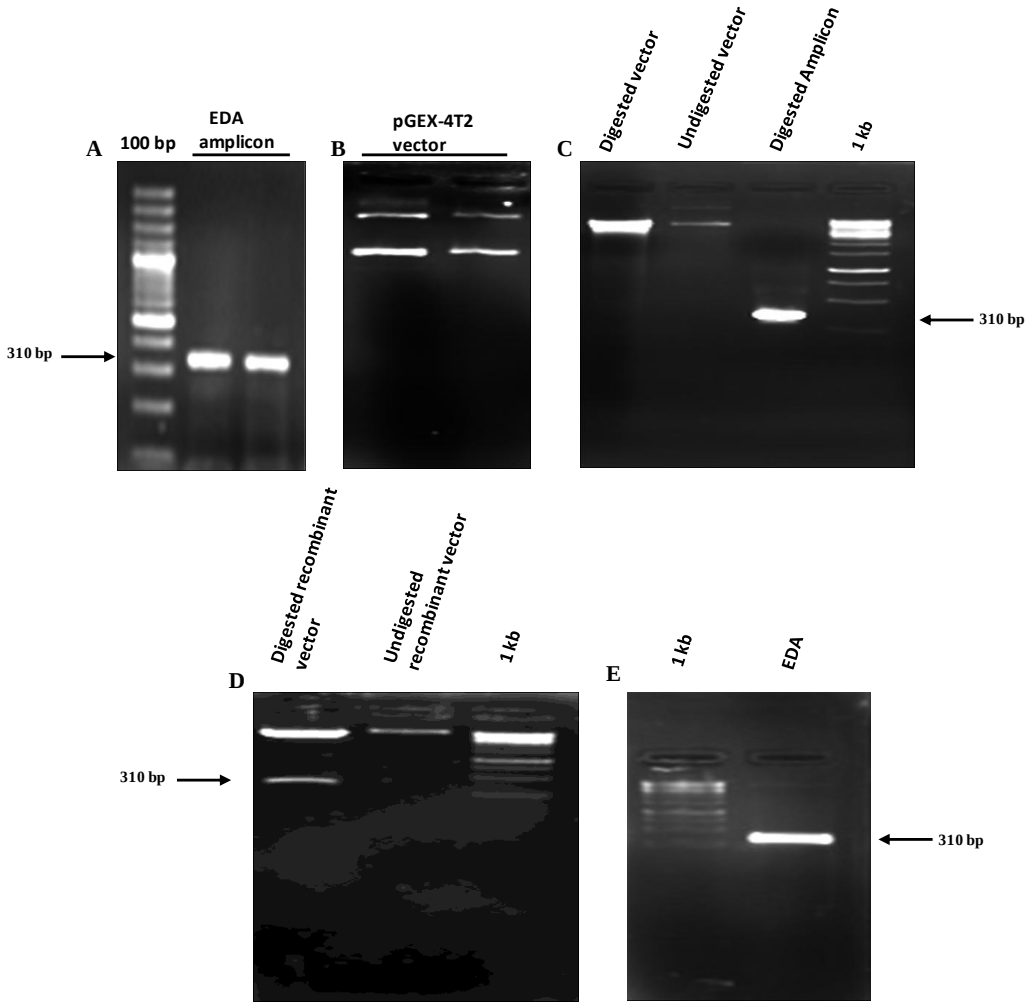


Figure 3.19 Cloning of EDA (A) 1.2% agarose gel showing PCR amplification of EDA exon using forward and reverse primers containing ECORI and BamHI sites respectively **(B)** 1% Agarose gel showing isolation of plasmid **(C)** 1% Agarose gel showing restriction digestion of PGEX-4T2 vector and EDA insert **(D)** 1% Agarose gel showing restriction digestion of recombinant pGEX-4T2 vector **(E)** 1.2 % agarose gel showing PCR amplification of EDA using recombinant pGEX-4T2 vector as template.

3.10.3 *Expression and purification of EDA*

After successful cloning of EDA, the recombinant plasmid was isolated from DH5-α cells and transformed into chemically competent BL21-DE3 (pLysS) cells for expression. Expression of cloned EDA as GST fusion protein was induced using IPTG and analyzed by SDS-PAGE (Figure 3.20 A). SDS-PAGE confirmed the production of 37 kDa fusion protein which was subsequently purified using GSH-Agarose beads (Figure 3.20 B). Purified protein was made free of endotoxins by endotoxin removal columns for further use in *in vitro* studies.

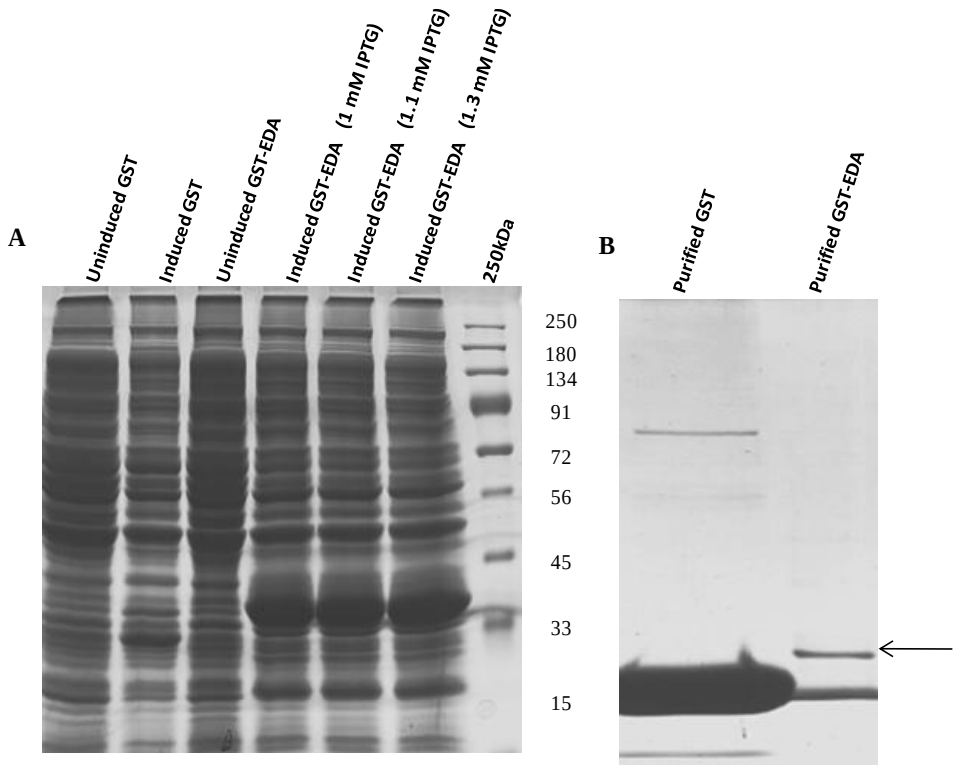


Figure 3.20 Expression and purification of GST-EDA protein (A) 12% SDS-PAGE showing induction of 37 kDa GST-EDA fusion protein **(B)** 10% SDS-PAGE showing purification of GST and GST-EDA fusion protein *(arrow indicates purified GST-EDA protein).*

3.11 Validating the role of secretory Fibronectin in Lung Tumor Microenvironment

3.11.1 *Fibronectin secreted from A549 binds to THP-1 cells*

To determine whether the Fibronectin secreted from A549 cells binds to THP-1, THP-1 cells were cultured for 24h in conditioned medium containing Fibronectin (obtained from A549 cells) or the conditioned medium depleted of Fibronectin (Figure 3.21). THP-1 cells under both the treatments differentiated as was reflected by their adherence to the surface of cell culture plates. However only THP-1 cells cultured in conditioned medium containing Fibronectin showed maximal binding of Fibronectin to their surfaces as determined by in-cell western analysis (Figure 3.22). In contrast THP-1 cells grown in conditioned medium depleted of Fibronectin showed marginal binding to THP-1 cells. These data thus confirm the binding of secreted Fibronectin to THP-1 cells which in turn may mediate downstream activation of various signalling pathways in these cells.

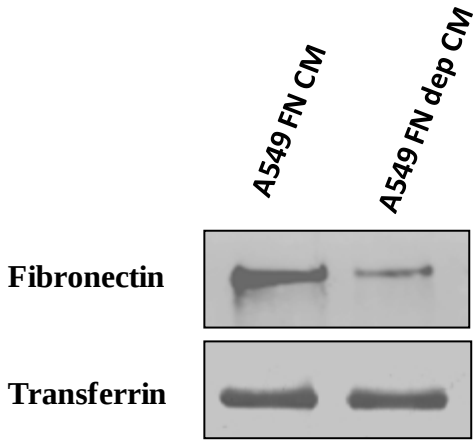


Figure 3.21 Immunoblot showing expression of Fibronectin in conditioned medium of A549 cells containing Fibronectin (A549 FN CM) and conditioned medium depleted of Fibronectin (A549 FN dep CM). Depletion of Fibronectin was carried out by immunoprecipitation as described in materials and methods.

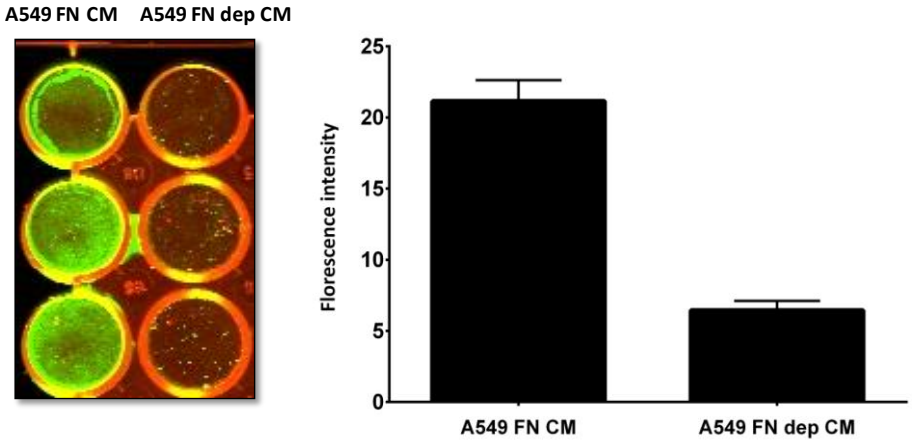


Figure 3.22 In-cell western assay showing binding of Fibronectin to the surface of THP-1 cells. (A) THP-1 cells were incubated in the conditioned medium of A549 cells containing Fibronectin (A549 FN CM) or conditioned medium depleted of Fibronectin (A549 FN dep CM). Cells were then treated with anti-Fibronectin antibody and imaged using LI-COR infrared imager. **(B)** Average florescent intensity of each well was calculated in arbitrary units using LI-COR-ODYSSEY software.

3.11.2 *Depletion of Fibronectin from the conditioned medium of A549 ablates the pro-inflammatory response from THP-1 cells*

Having established that the co-culture of A549 and THP-1 cells mediates the release of pro-inflammatory cytokines from THP-1 as well as the Fibronectin secreted from A549 cells binds to THP-1 when co-cultured, we set out to investigate (i) the effect of this binding on downstream pro-inflammatory response from THP-1 cells and (ii) the indispensability of EDA to such response. Culture of THP-1 cells in the conditioned medium obtained from A549 for 24h led to the release of TNF-α and IL-6. This response was however drastically reduced when THP-1 cells were cultured in Fibronectin depleted conditioned medium (Figure 3.23 A and Figure 3.23 B). An interesting observation was the marginal release of such cytokines from THP-1 cells when cultured in the conditioned medium obtained from cancer cell lines which do not release any Fibronectin such as HT-29, thus pointing to the role of Fibronectin in mediating pro-inflammatory response from THP-1 cells.

Furthermore we also examined whether the recombinant EDA is able to stimulate THP-1 cells to produce any pro-inflammatory cytokines. After culture in the medium supplemented with recombinant EDA (10 μg/ml) for 24h, THP-1 cells produced appreciable levels of TNF-α and IL-6 (Figure 3.23 A and Figure 3.23 B), which was comparable to the direct stimulation with Fibronectin containing conditioned medium. GST (used as control, since EDA was expressed as a GST tagged fusion protein) was not able to produce any response from THP-1 cells. Taken together these findings demonstrate that A549 cell line secreted Fibronectin elicits the pro-inflammatory response from THP-1 cells and EDA acts as a functional elicitor of such a response.

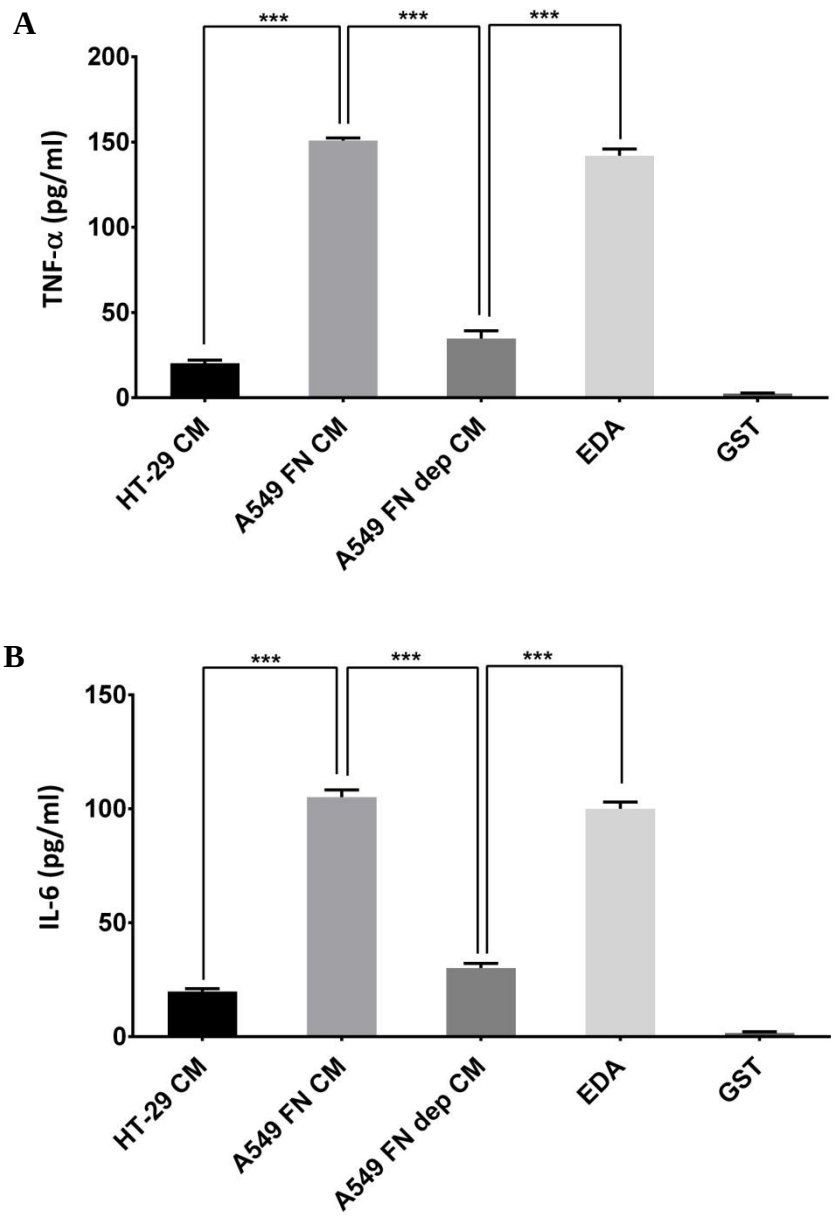


Figure 3.23. Fibronectin mediates the pro-inflammatory response from THP-1 cells. THP-1 cells were incubated in the conditioned medium of A549 cells containing Fibronectin (A549 FN CM) or depleted of Fibronectin (A549 FN dep CM), conditioned medium of HT-29 (HT-29 CM) and medium supplemented with 10 μg/ml recombinant EDA (EDA) or GST. The levels of **(A)** TNF-α and **(B)** IL-6 were immunoassayed after 24h of incubation. Data represented as mean ±SD of results obtained from at least three independent experiments. ****p < 0.001* compared to control.

3.11.3 *Expression of NF-κB P65 subunit in cytoplasmic and nuclear fractions of THP-1 cells*

NF-κB signalling cascade is considered indispensable to an inflammatory response. Once activated NF-κB P65 subunit homodimerises or heterodimerises with other subunits and translocates to nucleus leading to transcription of pro-inflammmatory and pro-survival genes. To gain an insight into the cellular mechanisms underlying the Fibronectin mediated pro-inflammatory responses, we evaluated the expression of NF-κB P65 subunit in the cytoplasmic and nuclear fractions of THP-1 cells under various conditions. Incubation of THP-1 cells in Fibronectin containing conditioned medium or medium supplemented with recombinant EDA (10 μg/ml) for 3h increased the expression of P65 in the nuclear fraction of THP-1 cells with corresponding decrease in the cytoplasmic fraction (Figure 3.24). These results were consistent with the expression pattern of P65 when THP-1 cells were treated with LPS (100 ng/ml)**.** As expected, culture of THP-1 cells in Fibronectin depleted conditioned medium or conditioned medium lacking Fibronectin led to the localization of P65 predominantly in the cytoplasm of THP-1 cells.

3.11.4 *Fibronectin mediates the pro-inflammatory responses via TLR-4*

The response of THP-1 cells exposed to Fibronectin containing conditioned medium or recombinant EDA was similar to that observed when treated with lipopolysaccharide (Figure 3.24). Therefore it is very likely that Fibronectin may be recognized by the same receptor on the THP-1 cells, which prompted us to examine the ability of conditioned medium containing Fibronectin or recombinant EDA to activate TLR-4, a signal receptor activated by LPS. To unravel the receptor on THP-1 cells to which Fibronectin or EDA binds, THP-1 cells were blocked with 5μg/ml of either anti-TLR-4 or anti-TLR-2 antibodies for 60 min before stimulation with conditioned medium or recombinant EDA for 24h. TLR-4 blocking on THP-1 cells subverted the pro-inflammatory response elicited by conditioned medium or recombinant EDA (Figure 3.25 A and Figure 3.25 A). However the blockade with anti-TLR-2 antibody showed no obvious effects on the expression of TNF-α and IL-6 from THP-1. These findings thus suggest that the A549 cell line secreted Fibronectin mediates the pro-inflammatory response through its EDA which engages TLR-4 on monocytes.

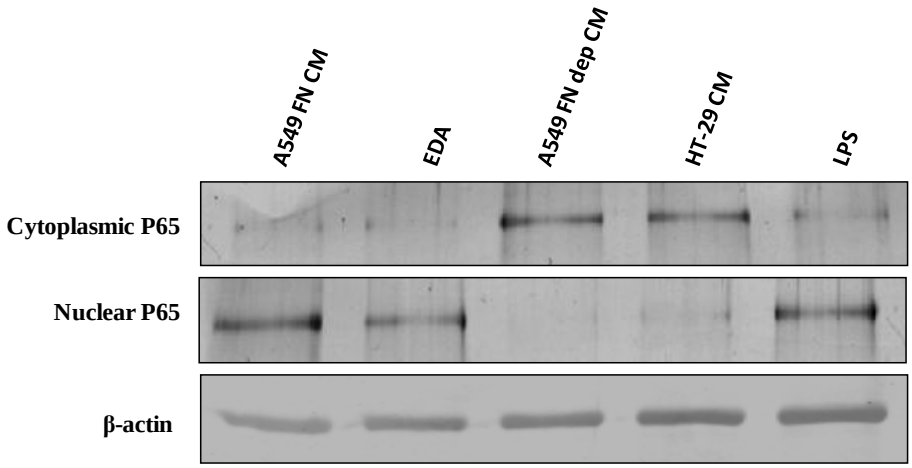


Figure 3.24 Immunoblot showing expression of NF-κB P65 subunit in the cytoplasmic and nuclear fractions of THP-1 cells. THP-1 cells were incubated in the conditioned medium of A549 cells containing Fibronectin (A549 FN CM) or depleted of Fibronectin (A549 FN dep CM), conditioned medium of HT-29 cells (HT-29 CM) and medium supplemented with 10 μg/ml recombinant EDA (EDA) or 100 ng/ml LPS for 3h and the expression of NF-κB in the cytoplasmic and nuclear fractions was evaluated by immunoblotting.

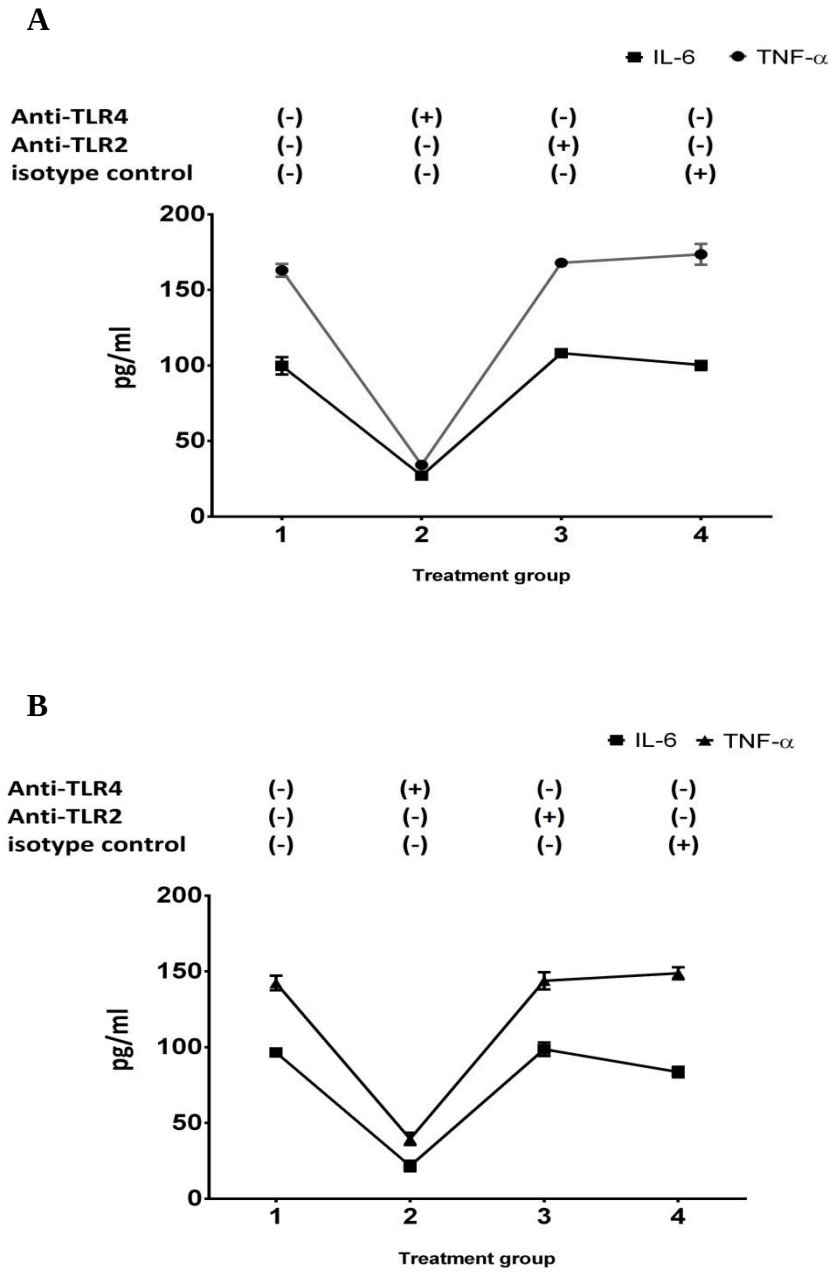


Figure 3.25 Fibronectin mediates the pro-inflammatory response via TLR-4. THP-1 cells were blocked with 5μg/ml of either anti-TLR-4 or anti-TLR-2 antibodies for 60 min before before incubation in **(A)** conditioned medium of A549 cells containing Fibronectin or **(B)** medium supplemented with 10 μg/ml recombinant EDA. The levels of TNF-α and IL-6 were immunoassayed after 24h of incubation. Data represented as mean ±SD of results obtained from at least three independent experiments.

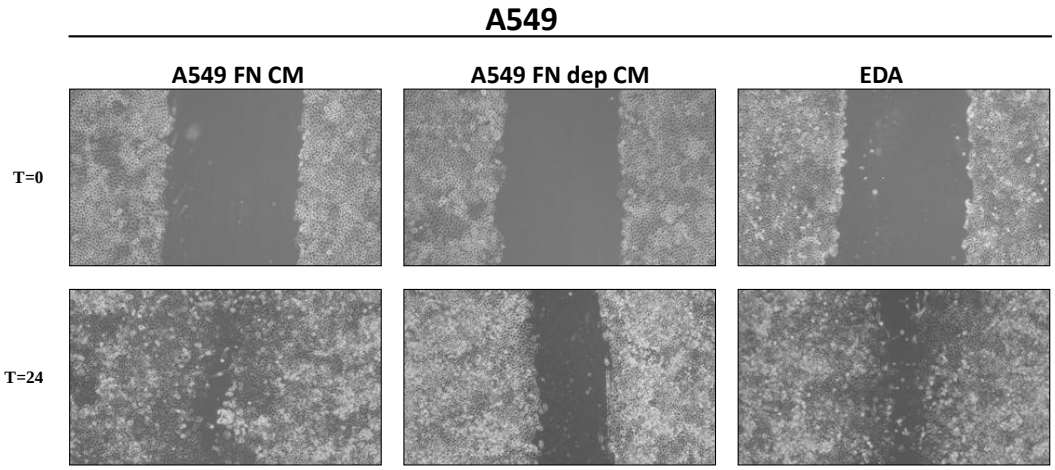
3.11.5 *Fibronectin secreted from A549 cells acts in an autocrine manner to increase their migration*

Having demonstrated that the culture of A549 cells in co-culture conditioned medium (obtained from co-culture of A549 and THP-1) or homotypic conditioned medium (obtained from A549 cells) enhances their migration and that both the types of conditioned media contain Fibronectin, we next sought to assess the involvement of Fibronectin in this phenomenon. A549 cells were grown to sub-confluency, wounded and then cultured in (i) conditioned medium containing Fibronectin (ii) conditioned medium depleted of Fibronectin and (iii) medium supplemented with recombinant EDA. It was observed that after 24 hours, A549 cells treated with Fibronectin containing conditioned medium showed almost complete healing of the wounded area, however the migration of these cells was significantly ablated in Fibronectin depleted conditioned medium (Figure 3.26). An interesting and noteworthy observation was the closure of wound when A549 cells were grown in medium supplemented with recombinant EDA. These findings could also be extended to cancer cell lines of different origin such HT-29 and T47D, although T47D was less responsive.

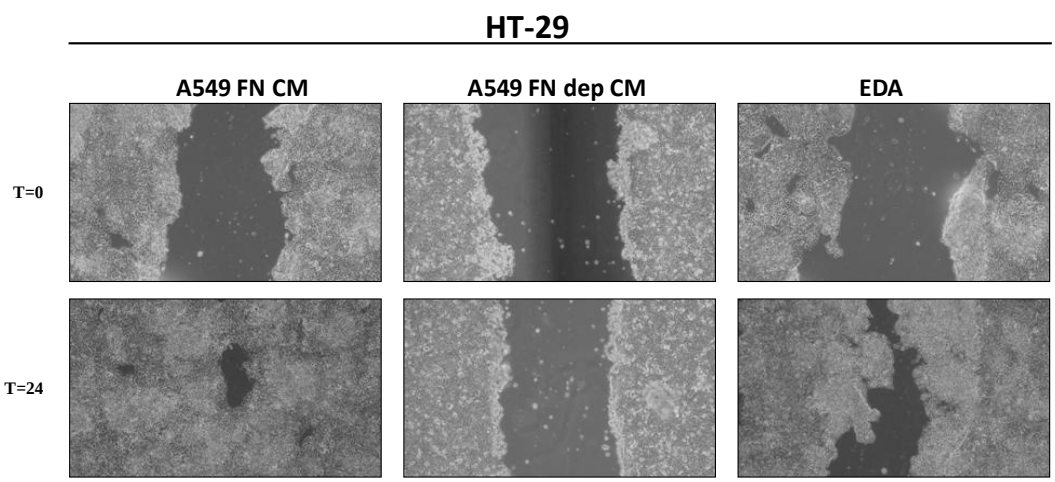
3.11.6 *Fibronectin secreted from A549 cells acts in an autocrine manner to increase their Invasiveness*

To substantiate the involvement of Fibronectin in co-culture conditioned medium or homotypic conditioned medium induced invasion, A549 cells were cultured in transwell chambers in presence of (i) conditioned medium containing Fibronectin (ii) conditioned medium depleted of Fibronectin and (iii) medium supplemented with recombinant EDA. After 24 hours, conditioned medium containing Fibronectin was found to increase the cell invasion significantly when compared with the conditioned medium depleted of Fibronectin. As observed during wound healing assay, recombinant EDA proved to be equally efficient in promoting invasion across the matrigel coated membrane of transwell inserts (Figure 3.27). The results showed consistency when extended to other cancer cell lines.

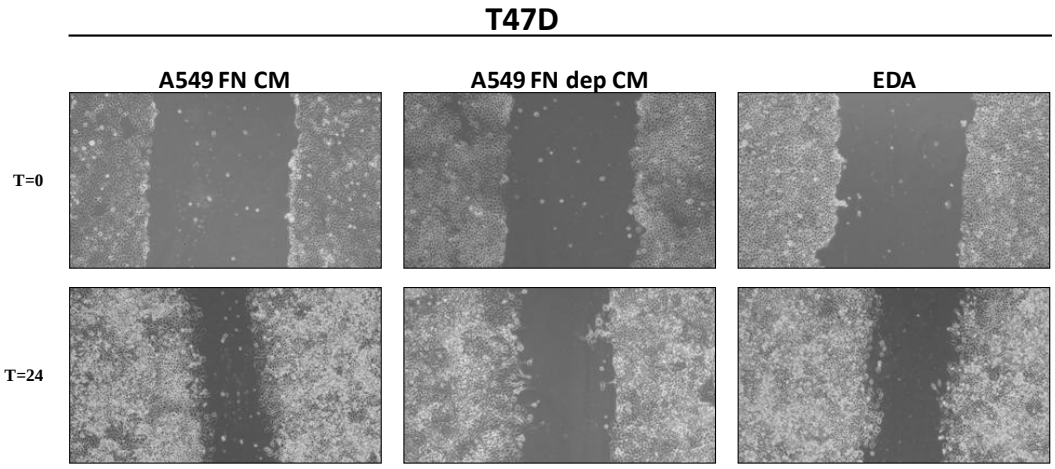
(A)


A549
A549 FN CM
A549 FN dep CM
EDA
T=0
T=24

(B)


HT-29
A549 FN CM
A549 FN dep CM
EDA
T=0
T=24

(C)



(D)

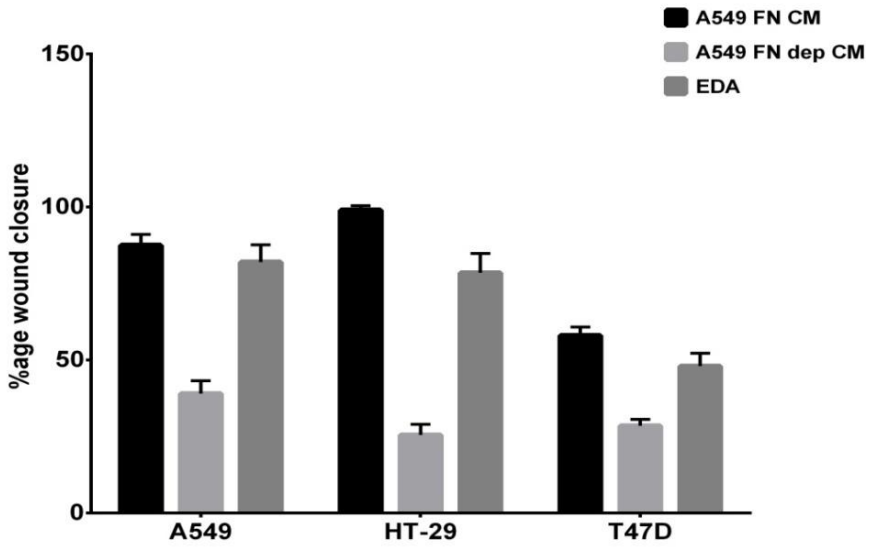
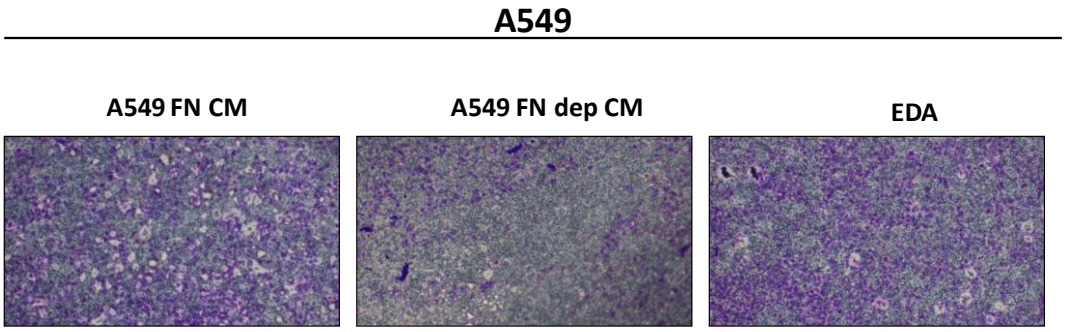
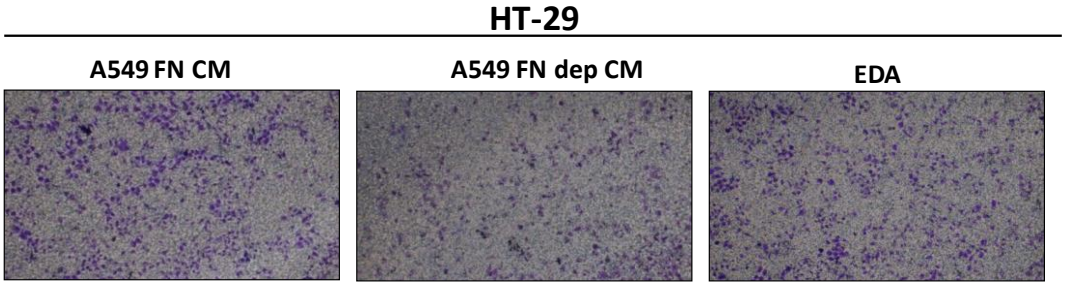


Figure 3.26 Fibronectin secreted from A549 cells promotes their migration. Cells **(A)** A549 **(B)** HT-29 and **(C)** T47D were grown to confluence in 30mm culture dishes and scratched with sterile tip; incubated in conditioned medium of A549 containing Fibronectin (A549 FN CM) or depleted of Fibronectin (A549 FN dep CM) and medium supplemented with 10 μg/ml recombinant EDA (EDA). Scratched areas were photographed at zero hour (T0) and then subsequently later at 24h (T24). **(D)** The scratched areas were quantified in three random fields in each case, and data were calculated from at least three independent experiments and represented as mean ±SD.

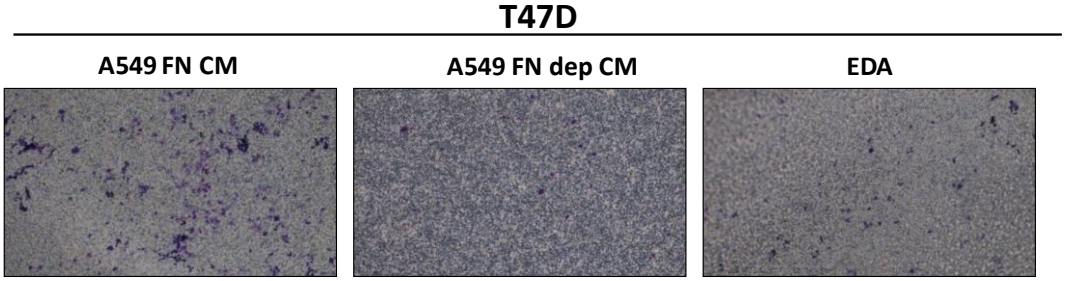
(A)


A549
A549 FN CM
A549 FN dep CM
EDA

(B)


HT-29
A549 FN CM
A549 FN dep CM
EDA

(C)


T47D
A549 FN CM
A549 FN dep CM
EDA

(D)

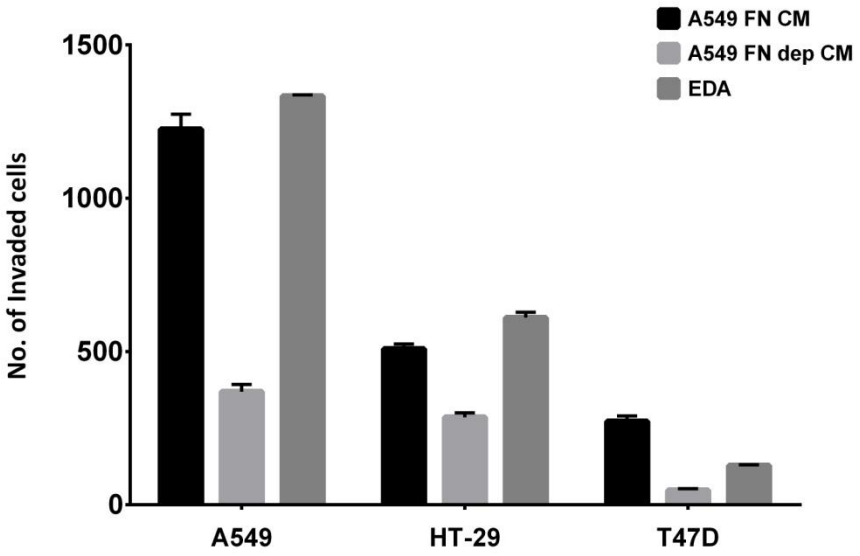
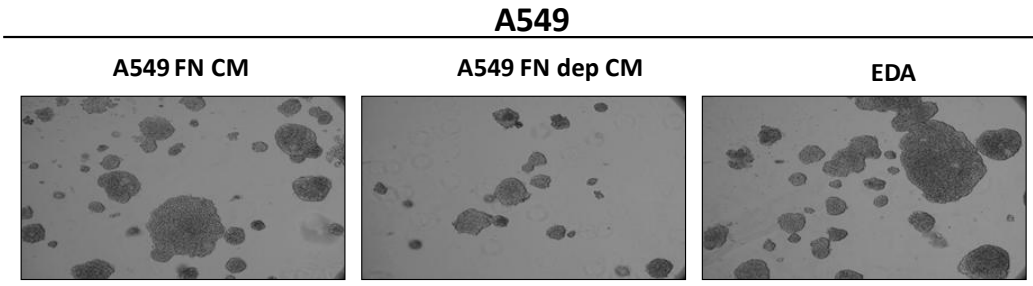


Figure 3.27 Fibronectin secreted from A549 cells promotes their invasiveness. Cells **(A)** A549 **(B)** HT-29 and **(C)** T47D were seeded in collagen coated transwell chambers containing conditioned medium of A549 with Fibronectin (A549 FN CM) or depleted of Fibronectin (A549 FN dep CM) and medium supplemented with 10 μg/ml recombinant EDA (EDA). After 24h, the migrated cells were fixed, stained, and photographed **(D)** Quantification of invasive cells. Data represented as mean ±SD of results obtained from at least three independent experiments.

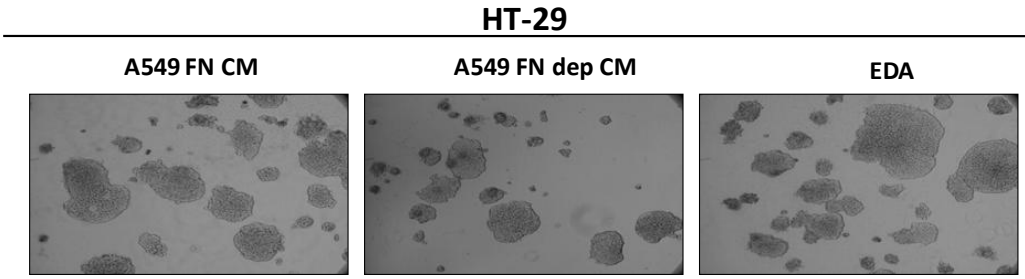
3.11.7 *Fibronectin secreted from A549 cells acts in an autocrine manner to enhance their tumorigenicity*

Anchorage-independent growth, the ability of a cell to proliferate without attachment to, or spreading onto, a substratum, is one of the hallmarks of transformation and the most accurate *in vitro* indication of tumorigenicity. To assess the tumorigenic potential imparted by secretory Fibronectin or its associated EDA, soft agar colony formation assay was performed wherein A549 cells were grown under attachment independent conditions in presence of (i) conditioned medium containing Fibronectin (ii) conditioned medium depleted of Fibronectin and (iii) medium supplemented with recombinant EDA for 14 days. It was observed that, A549 cells treated with Fibronectin containing conditioned medium formed maximum number of colonies, however the colony efficiency of these cells was significantly decreased in Fibronectin depleted conditioned medium (Figure 3.28). In consonance with the migration and invasion assays, recombinant EDA was equally efficient in promoting attachment independent growth of A549 cells. These results could also be extended to other cancer cell lines.

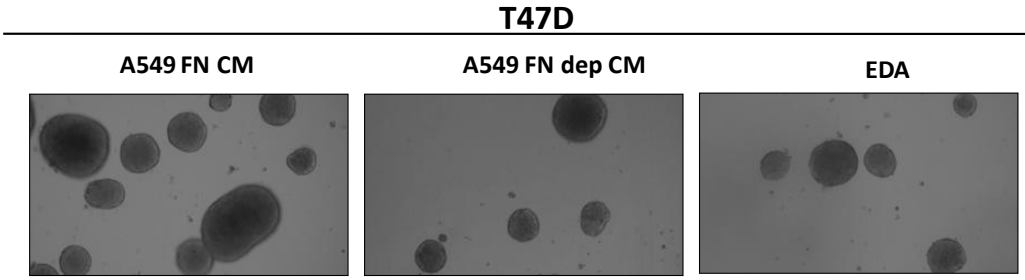
(A)


A549
A549 FN CM
A549 FN dep CM
EDA

(B)


HT-29
A549 FN CM
A549 FN dep CM
EDA

(C)


T47D
A549 FN CM
A549 FN dep CM
EDA

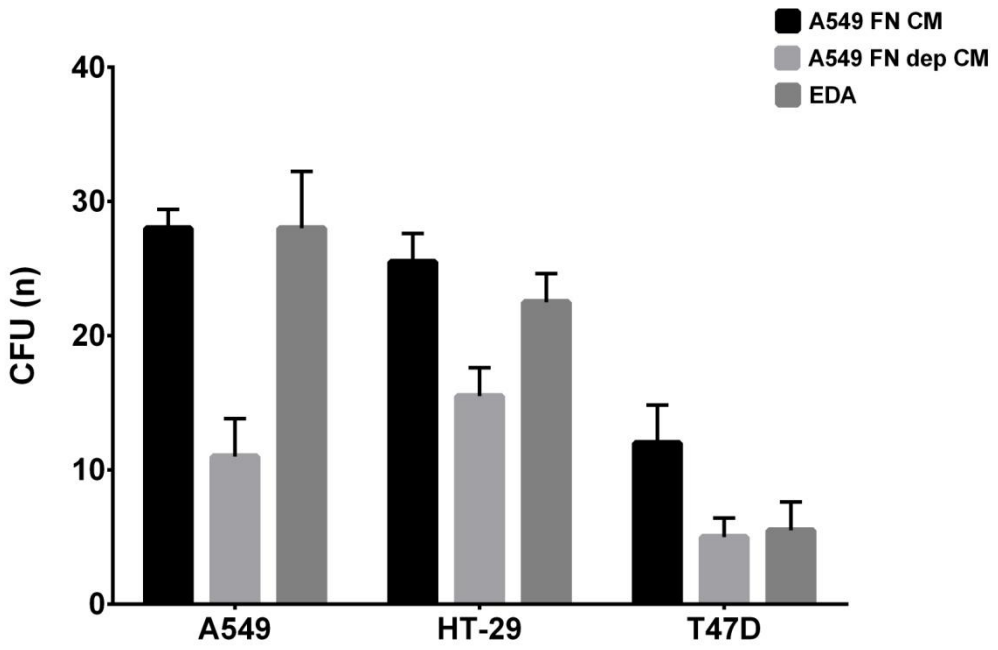


Figure 3.28 Fibronectin secreted from A549 cells promotes their anchorage independent growth. Cells **(A)** A549 **(B)** HT-29 and **(C)** T47D were seeded in the top layer containing 0.4% agar and cultured for 14 days in conditioned medium of A549 containing Fibronectin (A549 FN CM) or depleted of Fibronectin (A549 FN dep CM) and medium supplemented with 10 μg/ml recombinant EDA (EDA). Colonies were photographed and counted per four fields under a microscope. **(D)** The number of colonies was counted and the data was interpreted as CFU**.** Data represented as mean ±SD of results obtained from at least three independent experiments.

3.11.8 *Fibronectin secreted from A549 cells induces their Epithelial-Mesenchymal transition*

Cells undergoing Epithelial-Mesenchymal Transition (EMT) lose epithelial characteristics and exhibit changes in cell adhesion, migration, polarity and gene expression. In order to validate the *in vitro* findings of Fibronectin or EDA induced migration and invasion of A549 cells, we sought to evaluate the shift in expression levels of the signature markers of EMT by immunoblotting. To this end, A549 cells were grown in conditioned medium containing Fibronectin (obtained from A549 cells) or depleted of Fibronectin, conditioned medium obtained from cancer cell lines which do not release any Fibronectin (HT-29) as well as the medium supplemented with recombinant EDA or GST (used as control, since EDA was expressed as a GST tagged fusion protein) for 24 hours. A critical feature of EMT is the downregulation of epithelial markers like E-cadherin and expectedly the treatment of A549 cells with Fibronectin containing conditioned medium or EDA supplemented medium led to the comprehensive decrease in expression of E-cadherin with concomitant upregulation of mesenchymal markers, N-cadherin, and vimentin (Figure 3.29). Furthermore the expression of the EMT-associated transcription regulators snail and zeb-1, both of which repress E-cadherin transcription were found to increase when A549 cells were cultured in Fibronectin containing conditioned medium or EDA supplemented medium, contrary to their growth in conditioned medium lacking or depleted of Fibronectin.

These results were also confirmed by the immunofluorescence staining of A549 cells (Figure 3.30). Together these findings strongly suggest that Fibronectin or its associated EDA released by A549 cells acts in an autocrine manner to promote their EMT phenotype which in turn mediates the migration, invasiveness and anchorage independent growth of these cells. It is also clear that EDA acts as functional entity of such a response.

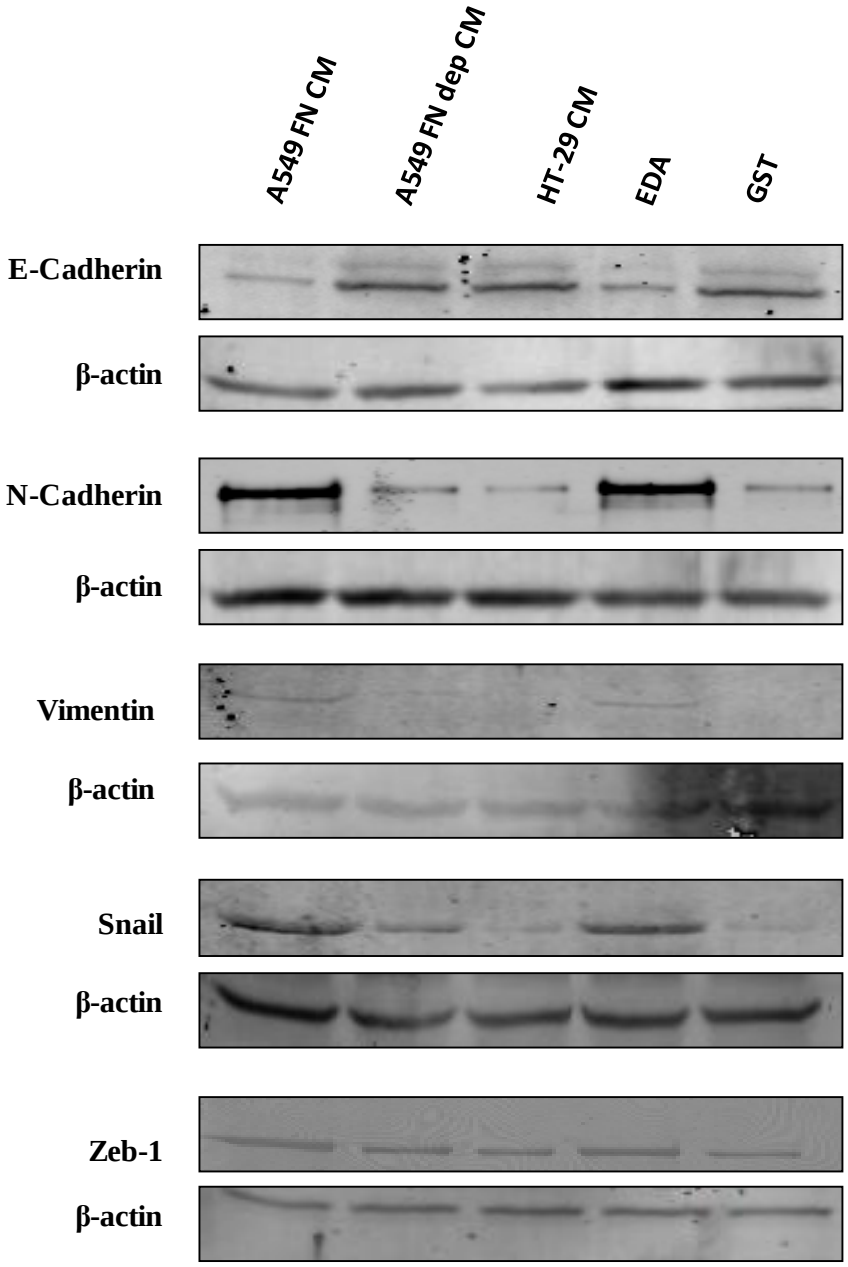
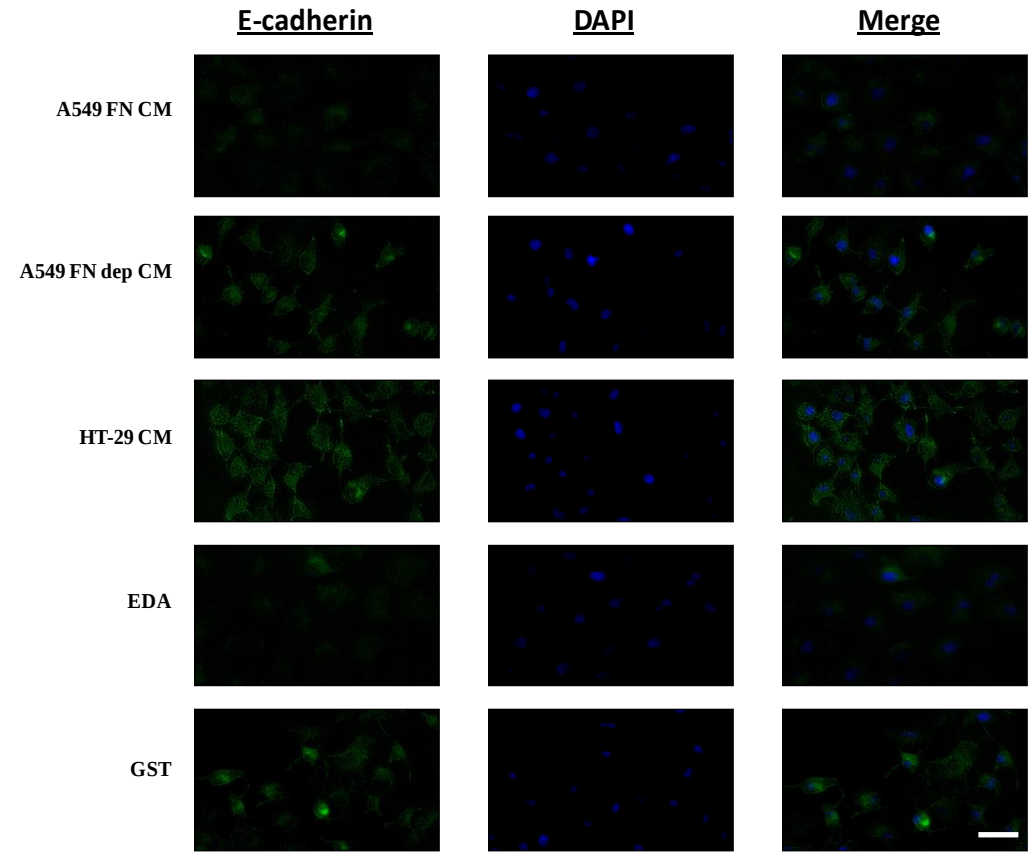
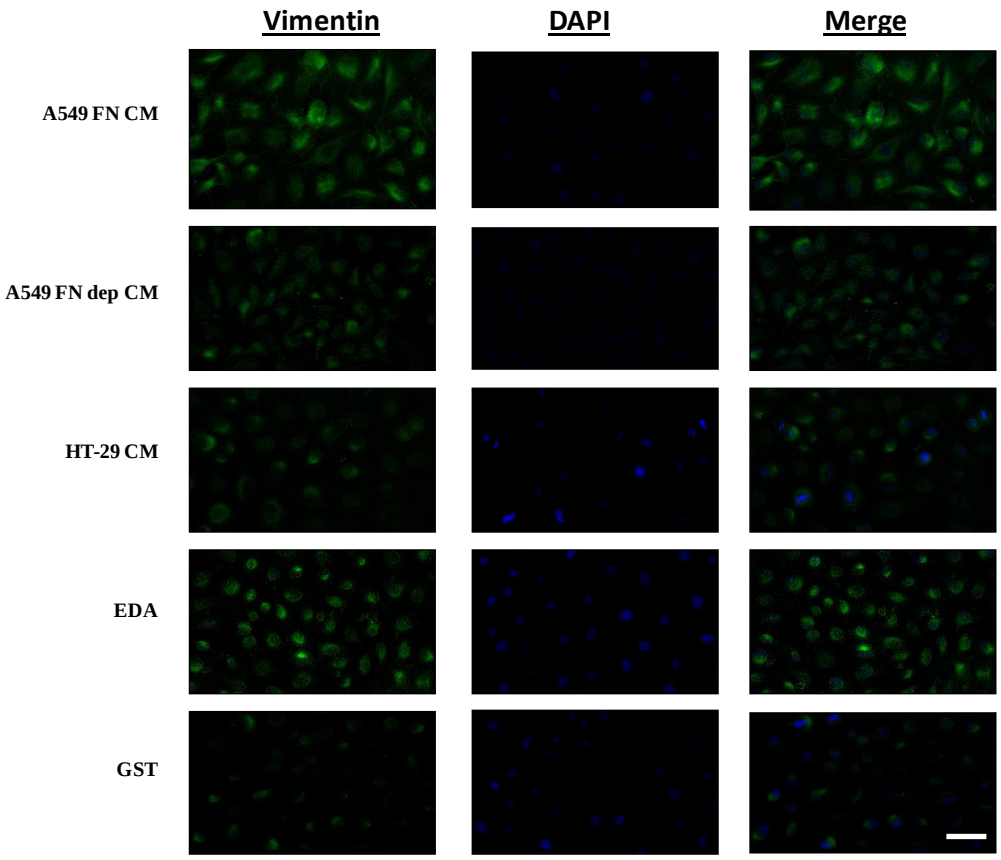


Figure 3.29 Immunoblot showing expression of E-cadherin, N-cadherin, Vimentin, Snail and Zeb-1 in A549 cells. A549 cells were grown to confluence in six well tissue culture plates and treated with conditioned medium of A549 containing Fibronectin (A549 CM) or depleted of Fibronectin (A549 FN dep CM), conditioned medium of HT-29 (HT-CM) and medium supplemented with 10 µg/ml recombinant EDA (EDA) or GST for 24h, followed by lysis and immunoblotting.

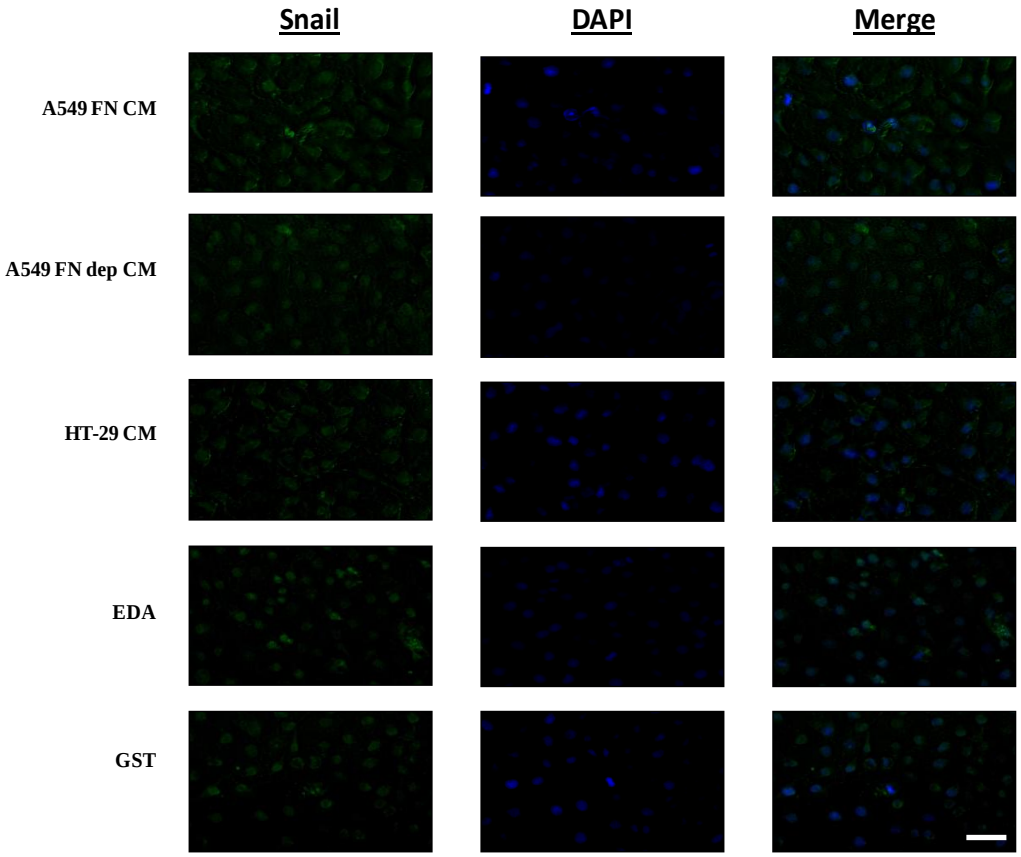
(A)



(B)



(C)



(D)

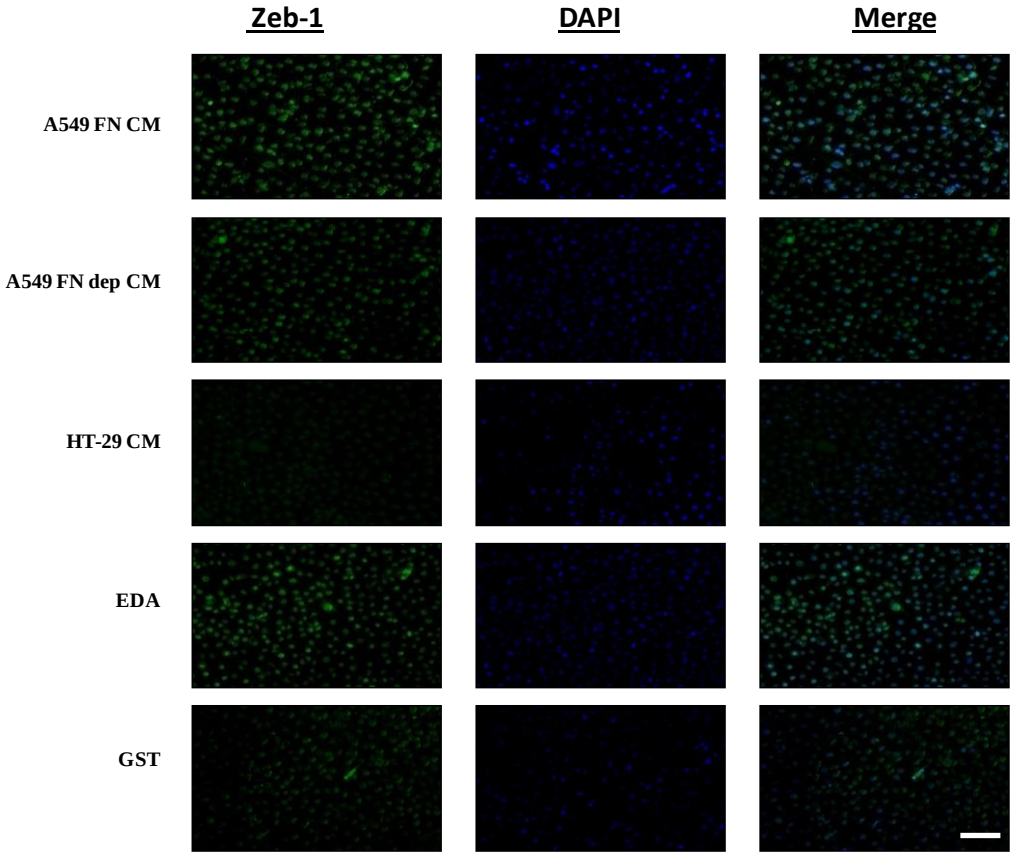


Figure 3.30 Immunoflorescence staining of A549 cells showing expression of (A) E-cadherin (B) Vimentin (C) Snail and (D) Zeb-1. A549 cells were grown to confluence in twenty-four well tissue culture plates and treated with conditioned medium of A549 containing Fibronectin (A549 FN CM) or depleted of Fibronectin (A549 FN dep CM) and medium supplemented with 10 µg/ml recombinant EDA (EDA) or GST for 24h, followed by staining with relevant primary antibody and alexa fluor labeled secondary antibody. Scale bar, 100 µm

The propensity for tumors to progress and metastasize reflects not only the oncogenic mutations in the cancer cells but also dynamic and reciprocal interactions involving non-malignant stromal cells in the tumor microenvironment. Stromal cells that infiltrate a developing tumor include the immune cells, fibroblasts, adipocytes, endothelial cells, and perivascular cells, all of which may orchestrate events critical to tumor formation, progression and evolution towards metastasis. Amongst the stromal cells, macrophages constitute the predominant recruited cells which support tumor growth and facilitate the metastatic dissemination (Bingle *et al*., 2002; Qian and Pollard 2010). There are considerable clinical studies which demonstrate a strong correlation between a high population of TAMs and poor prognosis or survival in lung, breast, ovarian, and cervical cancers (Komohara *et al*., 2014; Zhang *et al*., 2012). Compelling evidences suggest that several pro-inflammatory factors produced by macrophages, promote tumor growth, angiogenesis, invasion, and metastasis. However, the molecular mechanisms by which tumor cells interact with macrophages and re-educate them for a phenotype that is beneficial for tumor progression and metastasis remains poorly defined. These interactions can be related to the cancer cell secreted factors that directly activate myeloid cells particularly macrophages. Concerted action of the secretory factors from both cell types may involve paracrine or autocrine signaling loops and culminate in tumor cell survival, proliferation and, metastasis. In this study, we demonstrated that the interaction between lung carcinoma cells and monocytes/macrophages is a two-way process and involve cancer cell derived soluble factors that are capable of modulating the monocytes to generate an inflammatory microenvironment congenial for metastatic growth *in vitro*.

To evaluate the mechanisms by which lung cancer cells and monocytes regulate their biological properties in a reciprocal manner, we established an *in vitro* model mimicking the tumor microenvironment scenario by co-culturing human lung carcinoma cells, A549 and human monocytes, THP-1 in varying ratios ordering as 1:1, 1:5, 1:10, 1:20 and 1:40, respectively. The conditioned medium from the co-culture of A549 and THP-1 cells in 1:10 ratio was observed to be most potent in enhancing the proliferation of A549 cells. Such an observation is comparable to the mitogenic effect of factors secreted into the tumor microenvironment *in vivo*

(Zhong *et al*., 2008). In consonance with these results, treatment of A549 cells with co-culture conditioned medium also enhanced their migration and invasiveness. However a noteworthy observation was acquisition of increased metastatic capacity with homotypic conditioned medium obtained from A549 cultures. This intriguing phenomenon may be explained by two aspects. One is that the reciprocal interactions between A549 and THP-1 cells results in the secretion of oncogenic factors from either or both the cell types that in turn promote the metastatic capacity of A549 cells and the another is A549 cells themselves secrete such factors which mediate the similar effects in an autocrine manner.

To investigate whether the above observed effects involve activation and subsequent secretion of factors from THP-1 cells post co-culture, all the co-cultures were tested for the production of tumor promoting cytokines. All the tested co-cultures showed significant production of TNF-α and IL-6, however, the most effective pro-inflammatory response (high TNF-α and high IL-6) was observed at a particular co-culture ratios of 1:10. A high TNF-α and IL-6 microenvironment points to the possible dependence of above observed co-culture imparted metastatic phenotype on inflammation. *In vivo*, TNF-α along with IL-6 has been demonstrated to mediate tumor promotion in hepatocellular carcinoma (Park *et al*., 2010). In various human malignancies, high levels of circulating TNF-α and IL-6 have been observed to act as risk factors and correlate with disease prognosis. Indeed gender biased production of IL-6 accounts for much higher hepatocellular carcinoma load in males as compared to females (Naugler *et al*., 2007). In contrast to the production of TNF-α and IL-6 in various co-culture conditions, TGF-β was observed to be produced at later time points post co-culture. TGF-β is known to activate SMAD transcription factors and MAPKs which in turn control the expression of various other metastatic regulators (Yang and Weinberg, 2008). Furthermore, elevated levels of TGF-β have often been found to be associated with poor prognosis. Thus co-culture conditioned medium enhanced cell migration and proliferative properties of the A549 cells is in accordance with the known tumor promoting abilities of TNF-α, IL-6 and TGF-β. Expression kinetics of these cytokines also supports their well documented role in

inducing the expression of MMP-9 observed under co-culture conditions (Yu *et al*., 2007).

During tumorigenesis, immune responses may be triggered by the binding of aberrantly expressed molecules from tumor cells to the receptors on immune cells. In order to identify the A549 cell line derived secretory stimulus that may have presumptuously mediated the activation and simultaneous production of tumor promoting cytokines from THP-1 cells, an *in vivo* immunoscreening approach was used. Secretory protein fractions obtained from A549 cells under different conditions, aimed to avoid artifacts resulting due to contamination with orthologous proteins from serum, were probed with the anti-sera obtained from BALB/c mice immunized with A549 cells. Using this strategy, Fibronectin was successfully identified as immunogenic secretory protein in A549 cell line. Although Fibronectin was also detected in the conditioned medium of Caco-2, a colon adenocarcinoma cell line but other cancer cell lines used in this study did not show any release of Fibronectin. This specific release of Fibronectin in the extracellular medium by A549 and Caco-2 cells could be related to the type of tumors, from which these cell lines are established. However since HT-29, another colon adenocarcinoma cell line tested negative for Fibronectin release, it could be argued that this specific expression may also be associated with the stage and grade of tumor.

We investigated the expression of Fibronectin in the homotypic cultures of A549 and post co-culture with THP-1 cells. A time course analysis of Fibronectin protein expression in the conditioned medium of A549 cells showed a time dependent increase in Fibronectin release. The observation that the homotypic conditioned medium accentuates the migratory abilities and invasiveness of A549 cells in a time dependent manner could be attributed to this expression pattern of Fibronectin and its concomitant autocrine action. When A549 and THP-1 cells were co-cultured, intriguingly the expression of Fibronectin showed upregulation, thus pointing to the tumor supportive role of monocytes/macrophages present in the vicinity of the tumor cells. After establishing the important role played by lung cancer cell derived Fibronectin, we sought to differentiate this Fibronectin from plasma Fibronectin. Different isoforms of Fibronectin are generated by the alternative splicing of combination of three exons including extra domain A

(EDA), extra domain B (EDB) and connecting segment III. The soluble plasma Fibronectin produced by hepatocytes lacks both the EDA and EDB segments while as cellular Fibronectin produced by epithelial cells may contain one or both the segments (Pankov and Yamada, 2002). Interestingly, inclusion of these exons is elevated during embryonic development and decreases substantially after birth and with ageing (Chauhan *et al*., 2004). However under certain conditions like tissue fibrosis, tissue repair and angiogenesis, the embryonic splicing pattern may be temporarily re-established. Although the functions of EDA and EDB have not been fully unraveled, their ablation is however, lethal and leads to embryonic death. Fibronectin containing EDA has been found to be more potent than Fibronectin lacking EDA in promoting cell spreading and cell migration irrespective of presence or absence of EDB (Manabe *et al*., 1997). The EDGIHEL sequence within the EDA makes it an independent functional entity of Fibronectin protein and facilitates its binding to various receptors.

In this study we demonstrated that the A549 cells express Fibronectin isoforms containing both the EDA and EDB exons. However the other cell lines which did not secrete Fibronectin in the culture medium expressed either EDB alone or did not express them altogether. The fact that the A549 cells specifically express EDA emphasizes the role played by EDA in the secretion of Fibronectin. The ability of EDA to modulate the lung tumor microenvironment was further tested by its cloning and expression in bacterial system. The expressed protein was subsequently used in all the *in vitro* functional assays and its activity was compared with the total secretory Fibronectin, with the aim to ascribe the role played by secretory Fibronectin in modulation of lung tumor microenvironment to the presence of EDA.

We explored the possibility of Fibronectin acting as a ligand and binding to the receptors on THP-1 cells, thereupon triggering an inflammatory cascade. A study by, Kim *et al* (Kim *et al*., 2009) investigated the role of cancer cell secreted factors that directly activate myeloid cells particularly macrophages in a murine model. Conditioned medium from Lewis Lung carcinoma cells was found to induce secretion of IL-6 and TNF-α than conditioned medium from non-metastatic cells. The study identified extracellular matrix proteoglycan versican as a macrophage activator and this activation was found to occur through Toll-like

receptor family members TLR-2 and TLR-6. Taking clues from this study and having already confirmed the presence of Fibronectin in the conditioned medium of A549 cells with preliminary proofs of it acting as a regulator of migration and metastasis, we set out to investigate the paracrine activity of the Fibronectin secreted by A549 cells. Indeed depletion experiments confirmed the binding of secreted Fibronectin to the THP-1 cells. This observation was further substantiated by the finding that culture of THP-1 cells in the conditioned medium depleted of Fibronectin or conditioned medium of non-Fibronectin secreting cell lines led to the ablation of pro-inflammatory response from THP-1 cells. Furthermore, THP-1 cells when stimulated with recombinant EDA produced considerable levels of pro-inflammatory cytokines, comparable to their culture in the conditioned medium containing Fibronectin. These findings were further evidenced by the appropriate localization of NF-κB P65 subunit in the cytoplasmic or nuclear fractions under various experimental conditions. The possibility of involvement of a receptor, upstream of NF-κB mediated above observed responses led to the evaluation of Toll like receptors as candidates. Toll like receptors recognize both microbial-pathogen associated molecular patterns and non-microbial endogenous ligands (Piccinini and Midwood, 2010). Of the known TLR's in mammalian system, TLR-2 and TLR-4 have known endogenous ligands. It was observed that pre-treatment of THP-1 cells with anti-TLR-4 antibody led to the attenuation of pro-inflammatory response when stimulated with conditioned medium containing Fibronectin or recombinant EDA. Collectively these results demonstrate that tumor cell secreted Fibronectin elicits the TLR-4 dependent pro-inflammatory response from monocytes and EDA acts as a functional elicitor of such a response. The data explains the ability of cancer cells to depose the host innate immune system to generate an inflammatory microenvironment congenial for metastatic growth.

Finally we provided several lines of evidence demonstrating that A549 cell line secreted Fibronectin potentiates their migration, invasion and anchorage independent growth in an autocrine manner by activating the EMT program. It was observed that A549 cells when grown in Fibronectin depleted conditioned medium show lesser migration, invasion and colony formation, contrary to their growth in Fibronectin containing conditioned medium or medium supplemented

with recombinant EDA. This was further verified by the downregulation of epithelial markers like E-cadherin with concomitant upregulation of mesenchymal markers, N-cadherin and vimentin as well as EMT-associated transcription regulators snail and zeb-1 when cells were grown in Fibronectin containing conditioned medium or recombinant EDA supplemented medium compared to their growth in conditioned medium lacking or depleted of Fibronectin.

In summary this study identifies the tumor cell derived EDA-containing Fibronectin as a novel oncogenic secretory factor in lung tumor microenvironment potentiating the migration and invasion of tumor cells. We demonstrate that the lung carcinoma cell secreted EDA-containing Fibronectin mediates an interplay between tumor cells and immune cells by engaging TLR-4 on monocytes, thus generating an inflammatory microenvironment congenial for metastatic growth. On the other hand EDA-containing Fibronectin through its autocrine action accentuates the metastatic capacity of lung carcinoma cells by activating the EMT program. On the basis of these observations, we propose a model of communication between lung carcinoma cells and monocytes (Figure 4.1).

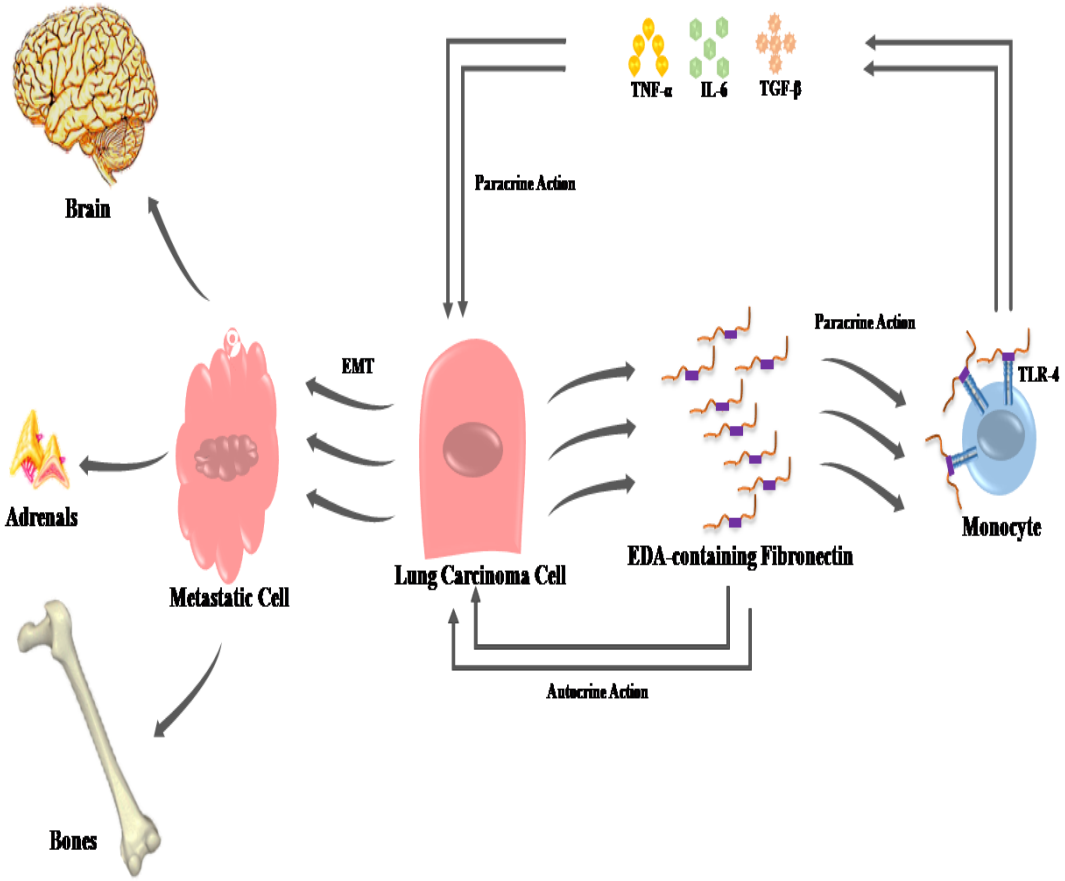


Figure 4.1 Proposed model of communication between Lung carcinoma cells and monocytes.

The conclusions from the present study can be summarized as follows:

- Treatment of lung carcinoma cells with the conditioned medium obtained from co-culture of lung carcinoma and monocytes enhanced their proliferation, migration and invasiveness. However the conditioned medium obtained from the homotypic culture of lung carcinoma cells was found to equally promote their migration and invasion.
- Lung carcinoma cells trigger the release of Pro-inflammatory cytokines viz TNF-α and IL-6 from monocytes which may in turn fuel their progression and metastasis.
- Lung carcinoma cell induced expression of TGF-β from monocytes at later time points supports the well-documented role of TGF-β as a modulator of invasion and metastasis at the advanced stages of tumorigenesis.
- Expression of TNF-α and IL-6 from monocytes by direct co-culture with lung carcinoma cells or their conditioned medium points to a stimulus which is not only tumor cell associated but also secretory in nature.
- Fibronectin was identified as potentially immunogenic secretory protein by an *in vivo* immunoscreening approach.
- Secretion of Fibronectin was found to increase in a time dependent manner in the homotypic cultures of lung carcinoma cells which can be correlated to the homotypic conditioned medium facilitated migration and invasion of lung carcinoma cells.
- Secretion of Fibronectin was enhanced in the co-cultures of lung carcinoma and monocytes.
- Lung carcinoma cells express both EDA and EDB domains, however the cancer cell lines which did not secrete Fibronectin expressed either EDB only or did not express them altogether, thus pointing to the role played by EDA in the secretion of Fibronectin and its associated pathogenesis.
- Lung carcinoma cell derived EDA-containing Fibronectin elicits NF-κB mediated TLR-4 dependent pro-inflammatory response from monocytes.
- Lung carcinoma cell derived EDA-containing Fibronectin potentiates their migration, invasion and anchorage independent growth in an autocrine manner by activating the EMT program.

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Table S1. Identification of Fibronectin by searching the mass spectrometry generated data against Swiss-Prot using proteome discoverer 1.3

Accession	Description	Score	Coverage	Unique Peptides	PSMs	AAs	MW [kDa]	calc. pI
P02751	Fibronectin OS=Homo sapiens GN=FN1 PE=1 SV=4 [FINC_HUMAN]	6942.20	42.83	69	191	2386	262.5	5.71
P00761	Trypsin OS=Sus scrofa PE=1 SV=1 - [TRYP_PIG]	1032.60	39.39	6	40	231	24.4	7.18
P02787	Serotransferrin OS=Homo sapiens GN=TF PE=1 SV=3 - [TRFE_HUMAN]	738.81	32.81	21	23	698	77.0	7.12
O15230	Laminin subunit alpha-5 OS=Homo sapiens GN=LAMA5 PE=1 SV=8 - [LAMA5_HUMAN]	552.82	5.79	15	16	3695	399.5	7.02
P04264	Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6 [K2C1_HUMAN]	387.74	19.25	0	11	644	66.0	8.12
A5A6M6	Keratin, type II cytoskeletal 1 OS=Pan troglodytes GN=KRT1 PE=2 SV=1 [K2C1_PANTR]	387.74	19.47	0	11	637	65.4	7.81
P11047	Laminin subunit gamma-1 OS=Homo sapiens GN=LAMC1 PE=1 SV=3 [LAMC1_HUMAN]	350.20	6.03	7	7	1609	177.5	5.12
P13645	Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10 PE=1 SV=6 [K1C10_HUMAN]	329.83	14.38	6	6	584	58.8	5.21
O75369	Filamin-B OS=Homo sapiens GN=FLNB PE=1 SV=2 [FLNB_HUMAN]	306.39	4.88	10	10	2602	278.0	5.73

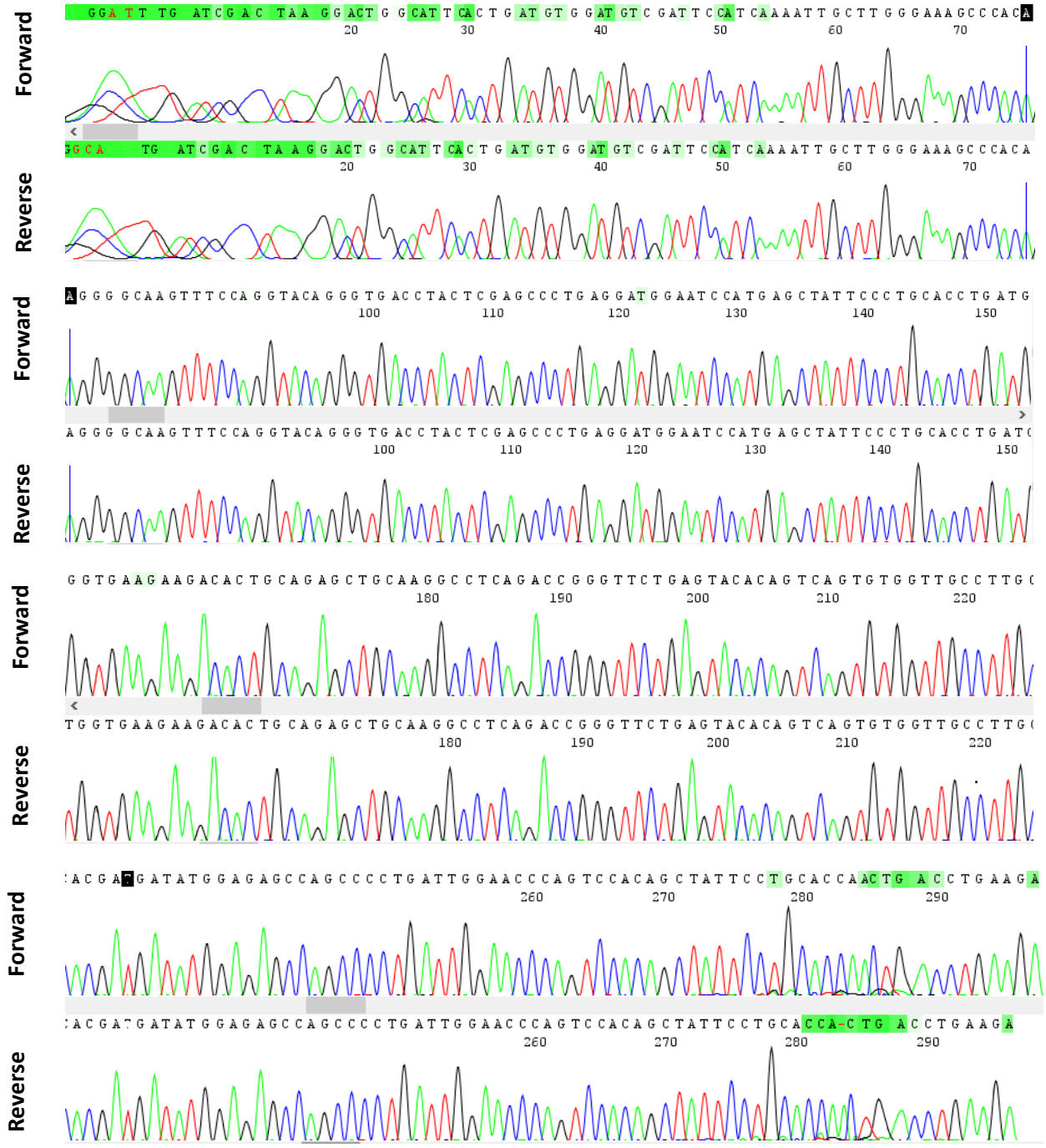
P35908	Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens GN=KRT2 PE=1 SV=2 [K22E_HUMAN]	225.37	12.36	5	7	639	65.4	8.00
P02769	Serum albumin OS=Bos taurus GN=ALB PE=1 SV=4 [ALBU_BOVIN]	220.97	15.49	8	8	607	69.2	6.18
P07942	Laminin subunit beta-1 OS=Homo sapiens GN=LAMB1 PE=1 SV=2 [LAMB1_HUMAN]	154.41	2.86	3	3	1786	197.9	4.94
P49788	Retinoic acid receptor responder protein 1 OS=Homo sapiens GN=RARRES1 PE=2 SV=2 [TIG1_HUMAN]	98.04	7.48	2	2	294	33.3	8.51
P98160	Basement membrane-specific heparan sulfate proteoglycan core protein OS=Homo sapiens GN=HSPG2 PE=1 SV=4 [PGBM_HUMAN]	94.42	0.87	3	3	4391	468.5	6.51
P02790	Hemopexin OS=Homo sapiens GN=HPX PE=1 SV=2 [HEMO_HUMAN]	74.32	4.76	0	2	462	51.6	7.02
Q5R543	Hemopexin OS=Pongo abelii GN=HPX PE=2 SV=1 - [HEMO_PONAB]	74.32	4.76	0	2	462	51.6	6.92
Q90474	Heat shock protein HSP 90-alpha 1 OS=Danio rerio GN=hsp90a.1 PE=1 SV=3 [H90A1_DANRE]	59.03	4.00	0	2	725	83.3	5.08
Q76LV2	Heat shock protein HSP 90-alpha OS=Bos taurus GN=HSP90AA1 PE=2 SV=3 [HS90A_BOVIN]	59.03	3.96	0	2	733	84.7	5.01

Table S2. Identification of Fibronectin by searching the mass spectrometry generated data against TrEMBL using proteome discoverer 1.3

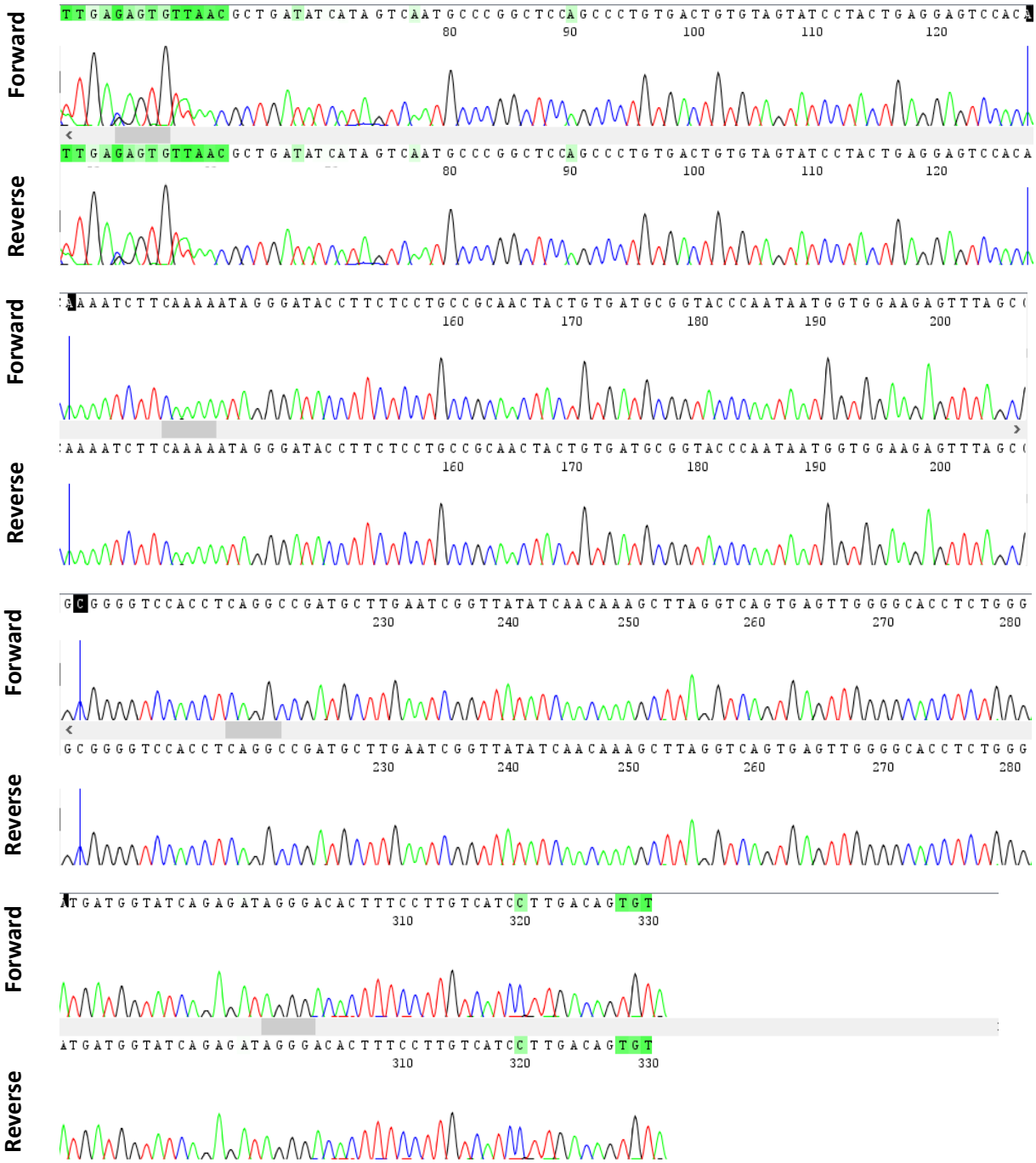
Accession	Description	Score	Coverage	Unique Peptides	PSMs	AAs	MW [kDa]	calc. pI
E9PE77	Anastellin OS=Homo sapiens GN=FN1 PE=4 SV=1 [E9PE77_HUMAN]	3034.17	35.15	41	100	2330	256.3	5.80
G1KRT6	Uncharacterized protein OS=Anolis carolinensis GN=FN1 PE=4 SV=1 G1KRT6_ANOCA]	372.98	4.67	1	18	2482	273.8	5.68
Q06AH7	Transferrin OS=Homo sapiens GN=TF PE=2 SV=1 [Q06AH7_HUMAN]	350.33	17.77	0	10	698	76.9	7.21
Q53H26	Transferrin variant (Fragment) OS=Homo sapiens PE=2 SV=1 - [Q53H26_HUMAN]	350.33	17.77	0	10	698	77.0	7.03
G3R4X1	Uncharacterized protein OS=Gorilla gorilla gorilla GN=TF PE=4 SV=1 [G3R4X1_GORGO]	350.33	17.77	0	10	698	77.0	7.21
C5IWV5	Trypsinogen OS=Sus scrofa PE=2 SV=1 - [C5IWV5_PIG]	237.33	18.70	0	12	246	25.9	7.18
F1SRS2	Uncharacterized protein OS=Sus scrofa GN=Ssc.41697 PE=3 SV=1 - [F1SRS2_PIG]	237.33	18.70	0	12	246	25.9	7.18
G3QDX0	Uncharacterized protein OS=Gorilla gorilla gorilla GN=KRT10 PE=3 SV=1 - [G3QDX0_GORGO]	226.82	15.31	0	5	490	51.8	5.07
H2QCX3	Uncharacterized protein OS=Pan troglodytes GN=ENSG00000186395 PE=3 SV=1 - [H2QCX3_PANTR]	226.82	13.00	0	5	577	58.2	5.16
G1RIT4	Uncharacterized protein OS=Nomascus leucogenys GN=KRT9 PE=3 SV=1 - [G1RIT4_NOMLE]	207.56	12.70	0	4	622	61.9	5.19
H2QCZ6	Uncharacterized protein OS=Pan troglodytes GN=ENSG00000171403 PE=3 SV=1 - [H2QCZ6_PANTR]	207.56	12.68	0	4	623	62.1	5.24

H6VRG1	Keratin 1 OS=Homo sapiens GN=KRT1 PE=3 SV=1 - [H6VRG1_HUMAN]	200.99	13.64	0	6	645	66.1	8.12
H6VRG3	Keratin 1 OS=Homo sapiens GN=KRT1 PE=3 SV=1 - [H6VRG3_HUMAN]	200.99	13.66	0	6	644	66.1	7.80
G3QLP7	Uncharacterized protein OS=Gorilla gorilla gorilla GN=KRT1 PE=3 SV=1 - [G3QLP7_GORGO]	200.99	13.71	0	6	642	65.9	8.12
H2R1Z0	Uncharacterized protein OS=Pan troglodytes GN=LOC100615215 PE=3 SV=1 - [H2R1Z0_PANTR]	200.99	13.95	0	6	631	65.4	8.54
H2RBT7	Uncharacterized protein OS=Pan troglodytes GN=LOC100615215 PE=4 SV=1 - [H2RBT7_PANTR]	200.99	13.81	0	6	637	65.2	9.33
F6WVB2	Uncharacterized protein OS=Macaca mulatta GN=LOC715265 PE=4 SV=1 - [F6WVB2_MACMU]	177.62	4.48	0	5	1607	177.5	5.15
F7CQA4	Uncharacterized protein OS=Equus caballus GN=LAMC1 PE=4 SV=1 - [F7CQA4_HORSE]	177.62	4.62	0	5	1559	172.4	5.07
F7CV04	Uncharacterized protein (Fragment) OS=Equus caballus GN=LAMC1 PE=4 SV=1 - [F7CV04_HORSE]	177.62	4.53	0	5	1591	175.9	5.08
G3QIX4	Uncharacterized protein OS=Gorilla gorilla gorilla GN=LAMC1 PE=4 SV=1 - [G3QIX4_GORGO]	177.62	4.47	0	5	1609	177.4	5.14
G3S795	Uncharacterized protein OS=Gorilla gorilla gorilla GN=LAMC1 PE=4 SV=1 - [G3S795_GORGO]	177.62	4.47	0	5	1611	177.6	5.14

Data S1. Chromatograms of Forward and Reverse Sequenced EDA of A549 cells



Data S2. Chromatograms of Forward and Reverse Sequenced EDB of A549 cells



Related Publications:

- A Amin, TA Mokhdomi, S Bukhari, SH Wani, AH Wafai, GN Lone, A Qadri, RA Qadri (2015) Tectorigenin ablates the inflammation-induced epithelial–mesenchymal transition in a co-culture model of human lung carcinoma. Pharmacological Reports 67 (2), 382-387.
- A Amin, S Bukhari, TA Mokhdomi, N Anjum, AH Wafai, Z Wani, S Manzoor, GN Lone, RA Qadri (2015) Comparative proteomics and global genome-wide expression data implicate role of ARMC8 in lung cancer. APJCP 16 (9), 3691-3696.
- A Amin, TA Mokhdomi, S Bukhari, RA Qadri (2015) Omics in Lung Cancer: From Clinical Associations to Technological Advances. Sara Book Publication, India (ISSN: 9781630426330).

Other Publications:

- A Amin, SH Wani, TA Mokhdomi, S Bukhari, AH Wafai, JI Mir, RA Qadri (2013) Investigating the pharmacological potential of Iris kashmiriana in limiting growth of epithelial tumors. Pharmacognosy Journal 5 (4), 170-175.
- S Bukhari, TA Mokhdomi, NA Chikan, A Amin, H Qazi, SH Wani, AH Wafai, RA Qadri (2015) Affinity proteomics led identification of vimentin as a potential biomarker in colon cancers: insights from serological screening and computational modeling. Molecular BioSystems 11 (1), 159-169.
- NA Chikan, S Bukhari, N Shabir, A Amin, S Shafi, RA Qadri, TN Patel (2015) Atomic Insight into the Altered O6-Methylguanine-DNA Methyltransferase Protein Architecture in Gastric Cancer. PloS one 10 (5), e0127741-e0127741.
- TA Mokhdomi, S Bukhari, SH Wani, A Amin, AH Wafai, JI Mir, NA Chowdri, RA Qadri (2014) Diagnostic relevance of primary investigations in early referral and management of colorectal cancer patients. American Journal of Clinical Cancer Research 2 (1), 14-20.