Studies on regulation of TGF-β and markers of epithelial to mesenchymal transition in breast cancer cells

Thesis Submitted to
Jawaharlal Nehru University
For the Award of the Degree of

DOCTOR OF PHILOSOPHY

Rahul Dev Malage

School of Life Sciences
Jawaharlal Nehru University
New Delhi 110 067
INDIA
2008

School Of Life Sciences
Jawaharlal Nehru University
New Delhi 110067

Certificate

*The research work embodied in this thesis entitled "**Studies on regulation of TGF-β and markers of epithelial to mesenchymal transition in breast cancer cells**" has been carried out at the School of Life Sciences, Jawaharlal Nehru University, New Delhi. This work is original and has not been submitted so far, in part or in full for the award of any other degree or diploma of any other university.*

Dec, 2008

Prof. Pramod Kumar Yadava
(Supervisor)

Rahul Dev Malage
(Scholar)

Prof. R. K. Kale
(Dean)

Dedicated to .....

My beloved Mother and Father!

ACKNOWLEDGEMENT

The journey of PhD would not have been accomplished without blessings of those who compassionately stood by.....helping hands of those that generously rose for.....in all ups and downs!

I am fortunate being showered with the blessings and compassion of my esteemed supervisor and mentor Prof. Pramod Kumar Yadava. He offered me the liberty and opportunity to pursue the ideas in PhD under his able guidance. He always stood by me in all circumstances. His crystallized views and critical suggestions could shape this work. His persistent encouragement and intellectual freedom given to me would always be the source of creation in the exploration of further studies.

I am indebted to all my teachers who showed me the path of knowledge, tolerance and quest for curiosity.

My thanks goes to my lab mates, SLS community members, friends, companions and all my well-wishers who were the fellow-travelers of my research.

I am very grateful to the Dean, SLS, Prof. R.K. Kale and previous Dean, SLS, Prof. Pramod Kumar Yadava for providing me equipped facility in CIF and lively environment in SLS. Events of Biospark and celebrations of Spandan were great refreshment and a get-together.

My gratitude goes to Dr.Vandana from IARI for her generous help of IEF-strips. I remember her gentle and kind attitude. I also extend this gratitude to Mr. Hardip from ICGEB for spending his valuable time in discussion and demonstration of 2D gel casting unit.

I would be very obliged to acknowledge CSIR for providing me scholarship to accomplish the task of PhD Programme.

I am very much thankful to the family members of SLS from CIF: Khan Sir, Alexander Sir, S.P. Sharma Sir, S.K. Mishra Ji, Aslam Ji, Meena Ji for their love and timely co-operation to me while working in CIF. I am grateful to SLS, administrative office family members, Ganguli Ji, Mam Raj Ji, Ramesh Ji, Pehaluddin Ji, Shambu Ji, Satish Ji, Butola Ji, Mange Ram Ji, Vinod Ji, Ram Kripal Ji for their help and support. I extend my hearty thanks to Meenu Maam and Shiny Maam for their caring attitude and offering me all time help.

I cannot forget the company of my lab mates who are the most cherished part during my PhD programme. I am very lucky to have them such as Dr. Pradip, Dr. Ravindra, Dr. Anju, Suresh, Jitendra, Rajendra, Vijay, Anshuman, Chanchal, Jyoti, Ashima, Prashant, Akhil and Hussain. I remember early days with Dr. Pradip, Ravi, Anju, Jitu, Raj and all members of that time who were always refurbished with full of joy and sense of humor and ready to bear my short-poems that I shared with them during tea time in canteen. I am glad to offer thank to Suresh for his valuable suggestions, fruitful discussions and updates on literature regarding telomerase and cancer biology. Moreover the assistance of Chottelal ji to sort out the problems during work would always be regarded the best co-ordination.

I would be grateful to all my batch-mates: Kamal, Vikas and his spouse Puja, Manoj from SES, Prasad and his spouse Angana, Sudhanshu and his spouse Sanghmitra, Sanjay, Manoj Kumar, Vijay, Ganesh, Jitendra, Anil, Zubeda, Bimala, Dola and Krishna Prakash (KP). They were always there to help me out in any moment anywhere. I treasure those memorable moments of stage show I performed with Sudhanshu, Dibyendu and Manoj in cultural program of Spandan.

I am very delighted to convey my best regards to my friend Kishan Ji (KP) who was the fellow-traveler of my struggle and hardship during my studies. I found him like a pillar of support in all good and bad times. Also, I would like to acknowledge my other friends like Audesh and Anil for their unconditional support and care.

My sincere thanks goes to my friends from SBT and SES who were supportive and cooperative during research. They are Dr. Subhash, Dr. Neeraj; I remember their generous help for 10L Mili-Q water during crisis in SLS that could help me to perform 2D experiments. I also can not forget the all time assistance offered by Dr. Vivek, Anup and Anuj to me, especially, in fluorescence imaging and photomicrography.

I express thankfulness to my friend Mr. Rajiv (SIT-Bioinformatics) for his incredible help in finding binding motifs of transcription factors in various promoters of the genes. I would love to acknowledge Vivek Ji (SIT-Bioinformatics) for his valuable discussions on bioinformatics and its applications in wet-lab research. His unconditional support and caring attitude would be the strength of great friendship.

I am nostalgic about those nights I spent during experiments and writing the thesis. In such a moment the company of Mr. Ravi (SLS) and Suranjit has been a motivating factor at a time of stimulating discussions on science, career and multi-aspects of life during having a tea at 24x7 dhaba in the nights of chilled winter.

I thankfully cherish the thoughtful suggestions and sincere attitude of Mr. Mahendra Ji, Satpal , Sanju, Vijay, Rakesh, Hanumanta, OP Ji, Sachin saab, Arshad saab and Shyam.

I have no words to express my profound sense of gratefulness for my CIF friends, Saurabh, Ashish, Jugendra Ji, Pankaj, Pratik and Vikrant Ji for their devoted-help and persistent support in 2D, MALDI and flowcytometry experiments. I admire Ashish for his support he gave me to initiate the 2D experiment. I also extend the gratitude to my friend Jugendra Ji who assisted me in MALDI analysis and MALDI-protocol. I have hearty regards and love for my younger friends Pankaj and Pratik for their support in flowcytometry experiments and analysis. I appreciate Vikrant Ji for his valuable suggestions in schematics and thesis.

I have deep respect and appreciation for Dr. Nita. I admire her spirit of work, jovial nature and vibrant smile. I feel fortunate for having encouragement and cheerful support while making the thesis more vivacious with full of rainbow.

I would like to acknowledge with great feelings of kind regards to my vehicle Maruti-800 (DL-2CK-3316). I attribute all those days and nights of work during chilled winter for five consecutive years and all my happiness to my car as it could make the travel safe especially in nights, bring several moments of joy and keep me happy in all times.

I would love to acknowledge my friend Mr. Farid. I admire his critically evaluated suggestions in thesis. I have an enduring back up of care and concern from my beloved friend Mr. Ashutosh.

Eternal flow of inspiration I always have from my dear friend Subhradip saab and his spouse Ruby. Their care and love for me would not be expressed in words. I could perceive the 'art of science' through the outstanding insight of him. I could perceive the 'science of art' through the wonderful philosophy of him. I cherish the liberty of learning in those days and nights we spent together with full of science and philosophy in his lab at AIIMS. An incomparable support and concern from both Miya and Biwi during PhD would always be the kindled sentiment in heart.

Here...., my fingers came to a standstill while writing....I breathe deep, shut my eyes and delved into the very beginning of the journey, I started from Kolhapur. No wonder! My heart was full of gratitude and love for my affectionate friends of native place, Appasaab, Prakash (Paka seth), Sanju (Master), Sunil saab, Amit saab, Sachin & Sachin, Dipak, Rafik saab, Kishor saab, Ranjit, Harun, Abhiraj, Vivek, Sunil, Hemant saab, Anand, Suhas Ji, Firoz, Rahul Awate & Rahul (Idali), Prashant, Sudarshan, Mahesh & Mahesh, Shivaji, Shivraj, Raju, Binay saab, Sanjay. Their timeless love, affection, encouragement and push-up to set the acceleration onto the journey of PhD and future endeavors would always be there.

I have deep gratitude for Sachin saab, Amit saab and Paka seth for their fullest support, love and care during PhD. Their visit in JNU could bring moments of utmost happiness to me.

Last but not the least, feelings of affection with deep respect and gratitude are beyond the expression in words for my family. My late mother and father would always be the everlasting source of strength and tolerance. My thesis is a humble and heartfelt tribute and worship to them. I have deep gratitude for my Shaku Mausi and Kaka for their parental love and care.

My family members are pillars of my strength and trust. I have motherly love and care from my elder sister Maya and my beloved brother-in-law Ramesh Ji from whom I always gain wisdom and spirit of happiness. I adore my elder brother Milind Raj and Madhu Vahini and younger brother Prasad for their love, all-over support and care during PhD. My love and affection goes to my niece Reshma, Samruddhi and Akanksha.

Without the support and encouragement of my family members the thesis would not have been accomplished.

An experience of SLS is full of joy and sorrow. It was a great time being there. I owe each and every one for giving me the opportunity to express my sincere thanks to them.

Rahul Dev Malage

CONTENTS

ABBREVIATIONS

ALK:	Activin receptor like kinase
AML-1:	Acute myelogenous leukemia 1 protein
AML-2:	Acute myelogenous leukemia 2 protein
AP-1:	Activation protein-1
APS:	Ammonium per sulphate
ATF:	Activating transcription factor
ATP:	Adenosine 5'-triphosphate
bHLH:	basic helix-loop-helix
BMP:	Bone morphogenetic protein
BRIT:	Board of radiation and isotope technology
BSA:	Bovine serum albumin
bZIP:	basic region-leucine zipper
CBFA1:	Core binding factor 1
Cbl-b:	Cas-Br-M ecotropic retroviral transforming sequence b
CBP:	CREB binding protein
cDNA:	complementary deoxyribose nucleic acid
CDS:	Coding DNA sequence
Ci:	Curie
cpm:	counts per minute
CREB:	cAMP responsive element binding protein
c-Ski:	cellular oncogene of Sloan-Kettering viruses group
CXCR4:	Chemokine, CXC motif, receptor 4
DAPI:	4'-6-Diamidino-2-phenylindole
DEPC:	Diethyl pyrocarbonate
DMEM:	Dulbeco's modified eagle's medium
DNA:	Deoxyribonucleic acid
DTT:	Dithiothreitol
ECM:	Extra-cellular matrix
EDTA:	Ethylenediamine tetraacetic acid
EMSA:	Electrophoretic mobility shift assay
EMT:	Epithelial to mesenchymal transition

Erk:	Extra-cellular signal regulated kinase
Evi-1:	Ecotropic viral integration site1 protein
FCS:	Fetal calf serum
FITC:	Fluorescein isothiocyanate
FOXC2:	Forkhead box protein C2
Gdf3:	Growth differentiation factor 3
HAB:	Human AB serum
HDAC3:	Histone deacetylase 3
HEPES:	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HER 2:	Human epithelial growth factor receptor 2
HES1:	Hairy and enhancer of split 1(*Drosophila*)
HEY1:	Hairy/Enhancer-of-split related with YRPW motif 1
HRP:	Horseradish peroxidase
hrs:	hours
ID1:	Inhibitor of DNA binding 1, dominant negative helix-loop-helix protein
IEF:	Isoelectrofocussing
IFN-γ:	Interferon -gamma
IL1:	Interleukin 1
JNK:	Jun N-terminal kinase
KCl:	Potassium chloride
Kd:	kilo Dalton
L:	Litre
LEF-1:	Lymphoid enhancer binding factor 1
LTBPs:	Latent TGFβ-binding proteins
M:	Molar
MALDI-TOF:	Matrix assisted laser desorption /ionization- Time of flight
MAP:	Mitogen-activatied protein
MAPK:	Mitogen-activated protein kinase
mCi:	milli Curie
Mek:	MAPK/ERK kinase
MET:	Mesenchymal-epithelial transition
MH:	Mad homology
MKK3:	Mitogen-activated protein kinase kinase 3

ml:	millilitre
mM:	millimolar
MMPs :	Matrix metalloproteases
MSG1:	Melanocyte-specific gene 1
MUC1:	Mucin1
NCCS:	National centre for cell science, Pune
NF-κB:	Nuclear factor kappa-B
ng:	nanogram
NMuMG:	Normal mouse mammary gland epithelial cells
PAGE:	Polyacrylamide gel electrophoresis
PAK1:	p21 protein (Cdc42/Rac)-activated kinase 1
PAR6:	Partitioning defective-6 protein
PBS:	Phosphate-buffered saline
PBST:	Phosphate-buffered saline with Tween 20
PDGF:	Platelet-derived growth factor
PGK1:	Phosphoglycerate kinase 1
PI3K:	Phosphatydil inositol-3 kinase
PKC:	Protein kinase C
PMF:	Peptides mass fingerprinting
pmole:	picomole
PMSF:	Phenylmethylsulphonyl flouride
PVDF:	Polyvinylidene fluoride
Rho:	(Ras homology) subfamily of Ras-like small GTPases
RNA:	Ribonucleic acid
Roc1:	Regulator of cullins 1
rpm:	revolutions per minute
R-Smad:	Receptor-activated Smad
RT-PCR:	Reverse transcription-Polymerase chain reaction
SBE:	Smad-binding element
SDS:	Sodium dodecyl sulphate
SDS-PAGE:	Sodium dodecyl sulphate -Polyacrylamide gel electrophoresis
SIP1:	Smad interacting protein-1
Skp1:	S-phase kinase-associated protein 1

Smad:	Composite name from **Sma** (*Caenorhabditis elegans*) and **Mad** (Mothers against decapentaplegic) (*Drosophila melanogaster*)
SMURF1:	SMAD specific E3 ubiquitin protein ligase 1
SNIP1:	Smad nuclear- interacting protein1
SPARC:	Secreted protein, acidic and rich in cysteine (extracellular matrix protein)
TEMED:	Tetramethylenediamine
TFE3:	Transcription factor E3
TGF-β:	Transforming growth factor- beta
TGFβRI:	TGF beta type 1 receptor
TGFβRII:	TGF beta type 2 receptor
TGIF:	Transforming growth factor beta-induced factor
TLE3:	Transducin like enhancer of split 3(*Drosophila*)
TNF-α:	Tumor necrosis factor-alpha
TrCP1:	Thyroid hormone receptor coactivating protein1
Tris:	[tris (hydroxymethyl) aminomethane]
U:	Unit
Vol.:	Volume
w/v:	weight/volume ratio
Wnt:	Wingless type, int-1 (mouse) and wingless (*Drosophila*)
ZEB1:	Zinc finger E-box-binding homeobox 1
ZO-1:	Zonula occludens 1
µ:	Micro
µg:	Microgram
µl:	Microlitre
^{0}C:	Degree Celsius

Introduction

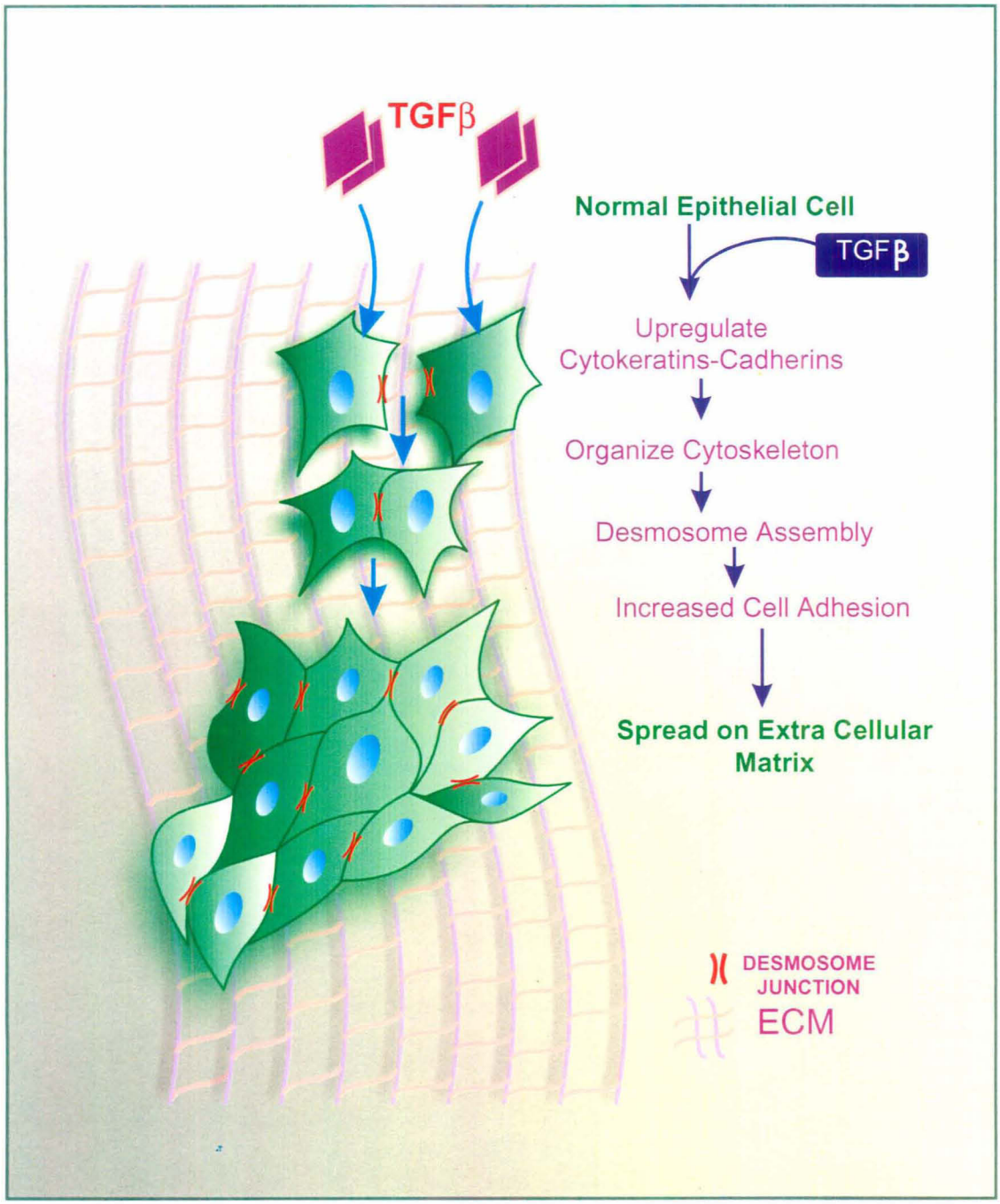


Plate I:TGF-β induces normal epithelial cell to organize with intact cell junctions e.g. Desmosome junctions and polar orientation on extra-cellular matrix (ECM).

1 INTRODUCTION

Cancer is a consequence of a series of events including dedifferentiation, uncontrolled cell division, change of cell adhesion properties and change in invasiveness. A major contribution to cancer progression is downregulation of cell adhesion molecules, which allows change in cell shape during metastasis (Takeichi, 1991, 1993). Essentially the delocalization and downregulation of E-cadherin is a pivotal event in epithelial to mesenchymal transition (EMT). E-cadherin expression limits the ability of the epithelial cell to move and migrate. Its downregulation correlates with dedifferentiation and metastasis (Battle et al., 2000; Cano et al., 2000). EMT occurs during critical phase of embryonic development in many animal species. Cells undergo EMT during gastrulation, neural crest cell migration and malignant progression of certain epithelial tumors (Savagner, 2001). EMT can occur in response to signaling from growth factors or oncogenes and is marked by transformation of polarized noninvasive epithelial cells into nonpolar, invasive mesenchymal cells that break through the basement membrane and invade the interstitial extra-cellular matrix (ECM). During transitions mesenchymal cells acquire a morphology that is appropriate for migration in an extra-cellular matrix environment and settlement in areas that are involved in organ formation which involves interaction between epithelial and mesenchymal cells. It took a long time for EMT to be recognized as a potential mechanism for carcinoma progression. One of the main reasons for this is that EMT can not be followed along time and space in tumor.

Various carcinoma cell lines (epithelial cell origin) undergo partial or complete EMT in vitro. The mechanism that governs EMT is now being unraveled and many parallels are being found between these processes in embryonic development, in tissue culture and in tumors. Several signaling pathways have been uncovered that are common to EMT in development and tumor progression. The EMT concept, then, provides a new means of identifying genes that are important for the progression of carcinoma towards dedifferentiated and more malignant states and studying the cellular and developmental biology of EMT that may provide insight into the mechanism of tumor progression (Thiery, 2002).

Transforming growth factor-β (TGF-β) induces EMT in epithelial cells and is necessary for acquisition of invasive phenotype in carcinomas (Oft et al., 1998; Akhurst et al., 1999). TGF-β acts as a potent inhibitor of epithelial cell proliferation whereas in contexts with cancer progression, TGF-β promotes tumor cell survival through

autocrine/paracrine interactions within tumor microenvironment. TGF-β triggers context -dependent signaling network to impart different effects on tumor progression at different stages of tumorigenesis. In advanced carcinoma, TGF-β enhances tumor cell proliferation and simultaneously inhibits tumoricidal activity of the immune system, thus accelerating tumor progression (Muraoka et al., 2002). Epithelial to mesenchymal transitions are essential manifestations underlying epithelial cell plasticity during embryogenesis and progression of tumor formation. TGF-β modulates epithelial cell plasticity by inducing EMT in these physiological backgrounds (Zavadil et al., 2001). EMT plays a pivotal role in metastasis of tumors of epithelial origin. In culture of mammalian epithelial cells, TGF-β causes loss of cell-cell junctions and cytoskeletal reorganization that transforms a tightly organized epithelial sheet into population of motile cells. In normal epithelial cell, TGF-β upregulates the expression of epithelial cell -associated cytokeratins, reorganizes the cytoskeleton and induces increased adhesion and spread on extracellular matrix (**Plate I**). Tumor cells essentially require loss of cell-cell adhesion to acquire motility. In cancerous epithelial cell, loss of responsiveness to TGF-β is implicated in downregulation of cytokeratins and E-cadherin. Loss of cytokeratins and E-cadherins lead to desmosome disassembly, decreased adhesion and consequently epithelial to mesenchymal transition (**Plate II**) (Oft et al., 1996; Miettinen et al., 1994; Troyanovsky et al., 1993, 1994).

Loss of cell-cell junctions followed by programmed E-cadherin repression is an important event in development as well as invasion and metastasis. E-cadherin is therefore emerging as one of the caretakers of the epithelial phenotype. There is a direct correlation between lack of E-cadherin production and loss of the epithelial phenotype in vitro. Downregulation of E-cadherin is thought to play a fundamental role during early steps of invasion and metastasis of carcinoma cells. Cadherin-mediated cell adhesion also plays a critical role in early embryonic development where numerous phenotypic changes occur through a mechanism called EMT. The acquisition of a fibroblastic phenotype is accompanied by the loss of the E-cadherin and allows the cell to dissociate from epithelial tissue to migrate freely. This is an essential event during gastrulation movements and neural crest formation but has also been suggested to play a fundamental role during early steps of invasion and metastasis of carcinoma cells. The molecules triggering EMT have been proposed to be involved in tumor progression, invasion and metastasis.

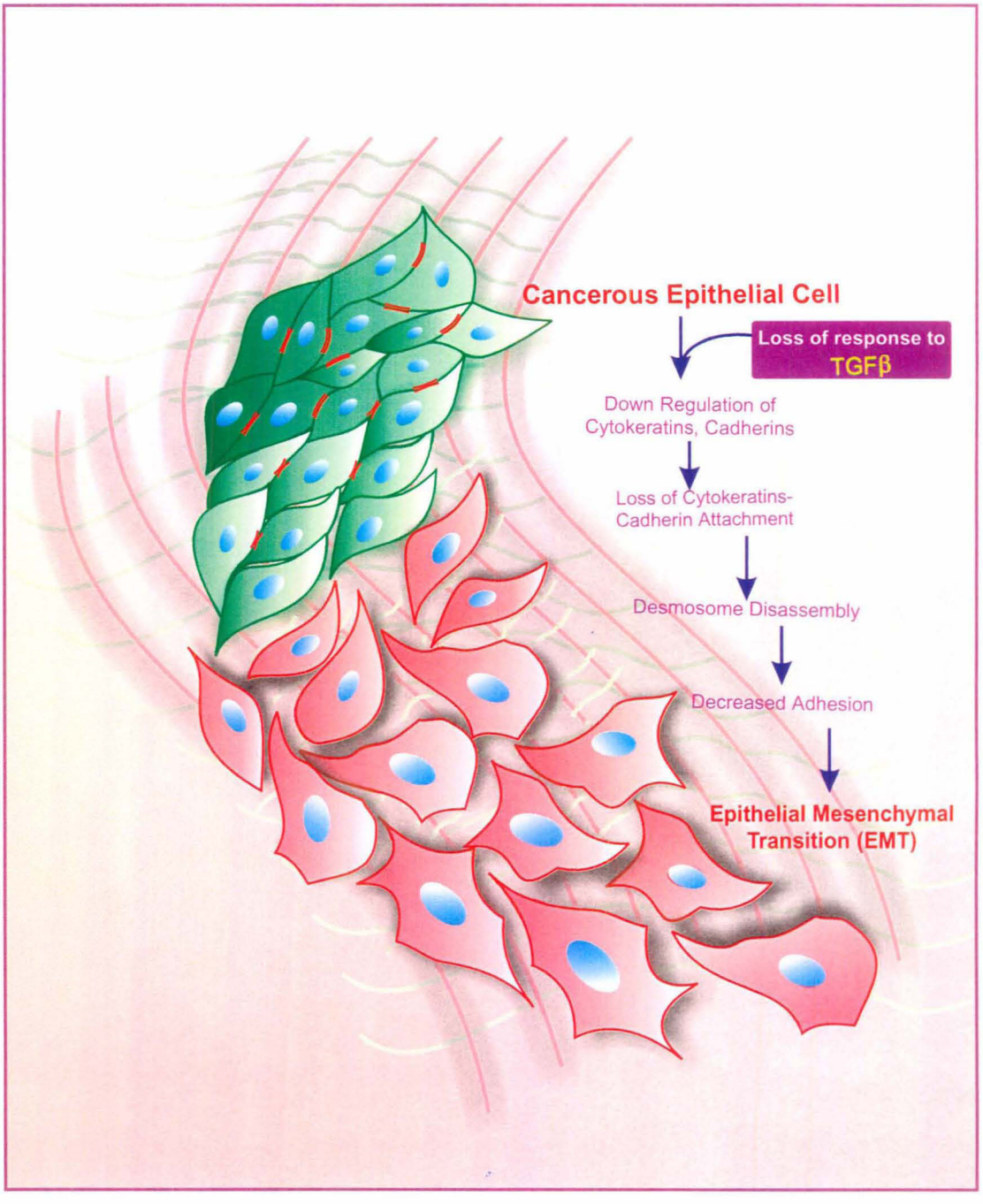


Plate II: Selective loss of TGF-β response encourage cancerous cell to loose junctional assemblies e.g. Desmosome junctions and render into migratory phenotype.

There are multiple mechanisms inactivating the E-cadherin mediated cell adhesion system in cancer, such as gene mutations, promoter hypermethylations, chromatin rearrangement, post-translational truncation or modifications and recently highlighted transcriptional repressors (Rosivatz et al., 2002). Notably, two members of the Snail superfamily of zinc finger protein namely Snail and Slug have shown downregulation of E-cadherin transcription through targeting E-boxes located in 5'proximal sequences of the E-cadherin promoter (Thiery et al., 2002). Snail was found to evoke tumorigenic and invasive properties in epithelial cells on overexpression and proven to be a strong repressor of transcription of the E-cadherin gene (Nieto et al., 2002; Hemavathy et al., 2000). Snail-induced downregulation of E-cadherin expression is necessary for early phases of embryonic development; mice deficient in Snail failed to downregulate E-cadherin levels and to complete gastrulation (Carver et al, 2001). SIP1 (Smad interacting protein-1) is another zinc finger protein postulated as enhancer of invasion as it can repress E-cadherin transcription via promoter binding (Cano et al., 2000). Expression of SIP1 is associated with migratory and invasive mechanisms occurring during embryonic development. High level of SIP1 has been detected during the formation of the neural tube suggesting its key role in the migration of neural crest cells (Eisaki et al., 2000; van Grunsven et al., 2000; Van de Putte et al., 2003). Another molecule known to trigger EMT is Twist a transcription factor containing a helix-loop-helix DNA-binding domain essential for the initiation of N-cadherin expression in *Drosophila*. Twist is possibly involved in the E- to N-cadherin switch during EMT (Rosivatz et al., 2002; Hajra et al., 2002a).

Apart from E-cadherin gene, Snail has additional target genes such as desmoplakin and cytokeratin18, emphasizing its role in tumor progression. Whether Twist may act as indirect repressor of E-cadherin through transcriptional activation of N-cadherin or directly via binding to the E-cadherin promoter and exerting repressor function is not clear. The promoter elements of both human and mouse E-cadherin responsible for the downregulation contain E-boxes. These E-boxes have the core consensus for Snail family proteins. Direct DNA binding assays, as well as yeast one hybrid assays demonstrated that Snail binds to this sequence most efficiently. Transfection experiments also confirm that Snail represses transcription of E-cadherin through these E-boxes (Battle et al., 2000; Cano et al., 2000). Presently, it is not clear how Snail, SIP1 and Twist are regulated themselves. It has been reported that the cytokine TGF-β which is often regulated in human tumors activates various transcription

repressors such as Snail, Slug, SIP1 and Twist. These repressors are known for the repression of various intermediate filaments and cell adhesion molecules such as cytokeratins, desmoplakin and E-cadherin which leads loss of cell-cell junctions initiating EMT. The direct role and contribution of TGF-β to EMT in several types of cancer and its potential association with Snail, Slug, SIP1 and Twist remain unresolved query.

E-box represents core-consensus of CACCTG motif for binding with proteins like Snail. Promoter regions of Snail, Slug and TGF-β also show presence of E-boxes containing CACCTG motif along with other binding motifs likely to be involved in regulation of these molecules through an auto-regulatory control. However regulatory role of E-box containing CACCTG motif in TGF-β1 promoter has not been documented. With this background information available in literature, we hypothesize that Snail, Slug, SIP1 and Twist may be the key regulatory molecules for the expression of TGF-β and auto-regulation of their own through TGF-β-mediated circuits responsible for EMT. This may be one of the TGF-β mediated circuits responsible for the manifestation and progression of EMT.

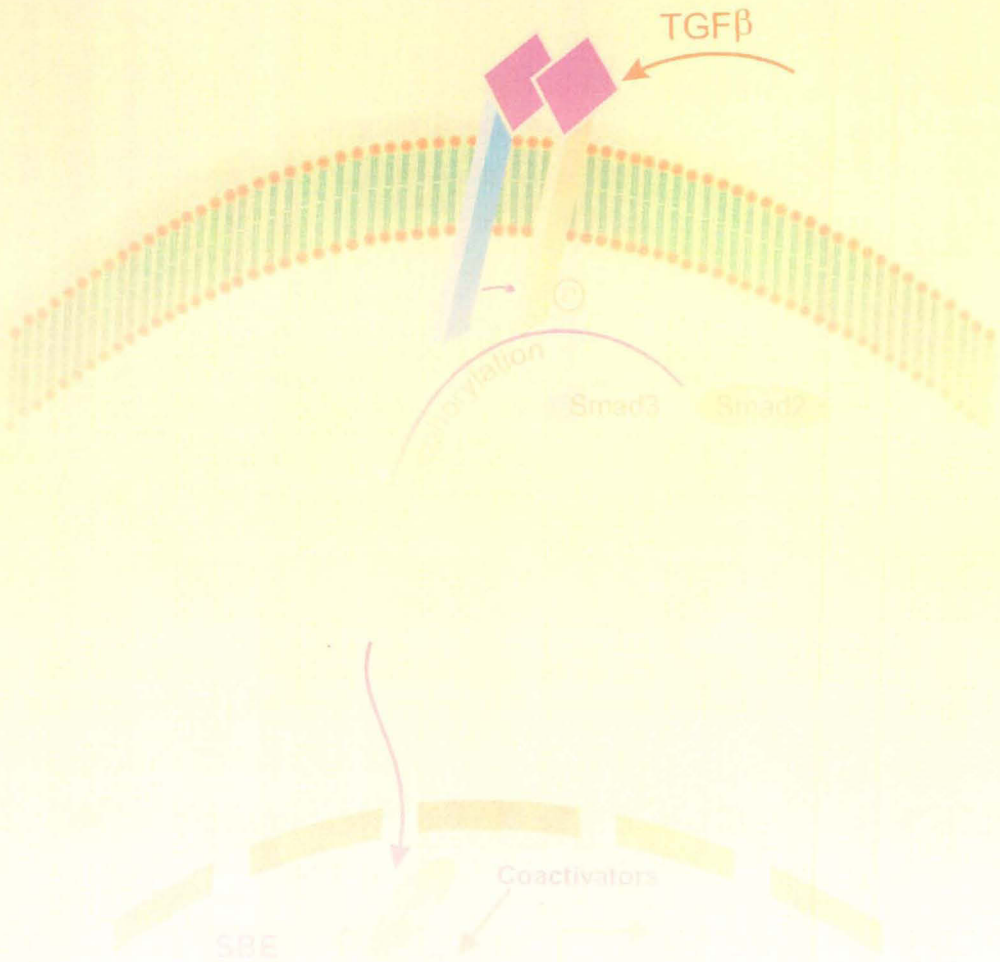
To understand the role of TGF-β1 pathway molecules Snail, Slug, SIP1 and Twist in the onset and progression of the EMT in breast cancer the objectives of the proposed study are:

1. To establish the changes in expression of cytokeratins and vimentin after treatment with TGF-β1 in breast cancer cells (Cell line/Tissue sample).

2. To study the effect of TGF-β1and related regulators (Snail, Slug, SIP1 and Twist) on expression of cytokeratins and vimentin.

3. To study any regulatory interaction of the above molecules with TGF-β1.

4. To construct the possible regulatory circuit around TGF-β1 showing roles of Snail, Slug, SIP1, Twist, cytokeratins, vimentin and E-cadherin.



Review of Literature

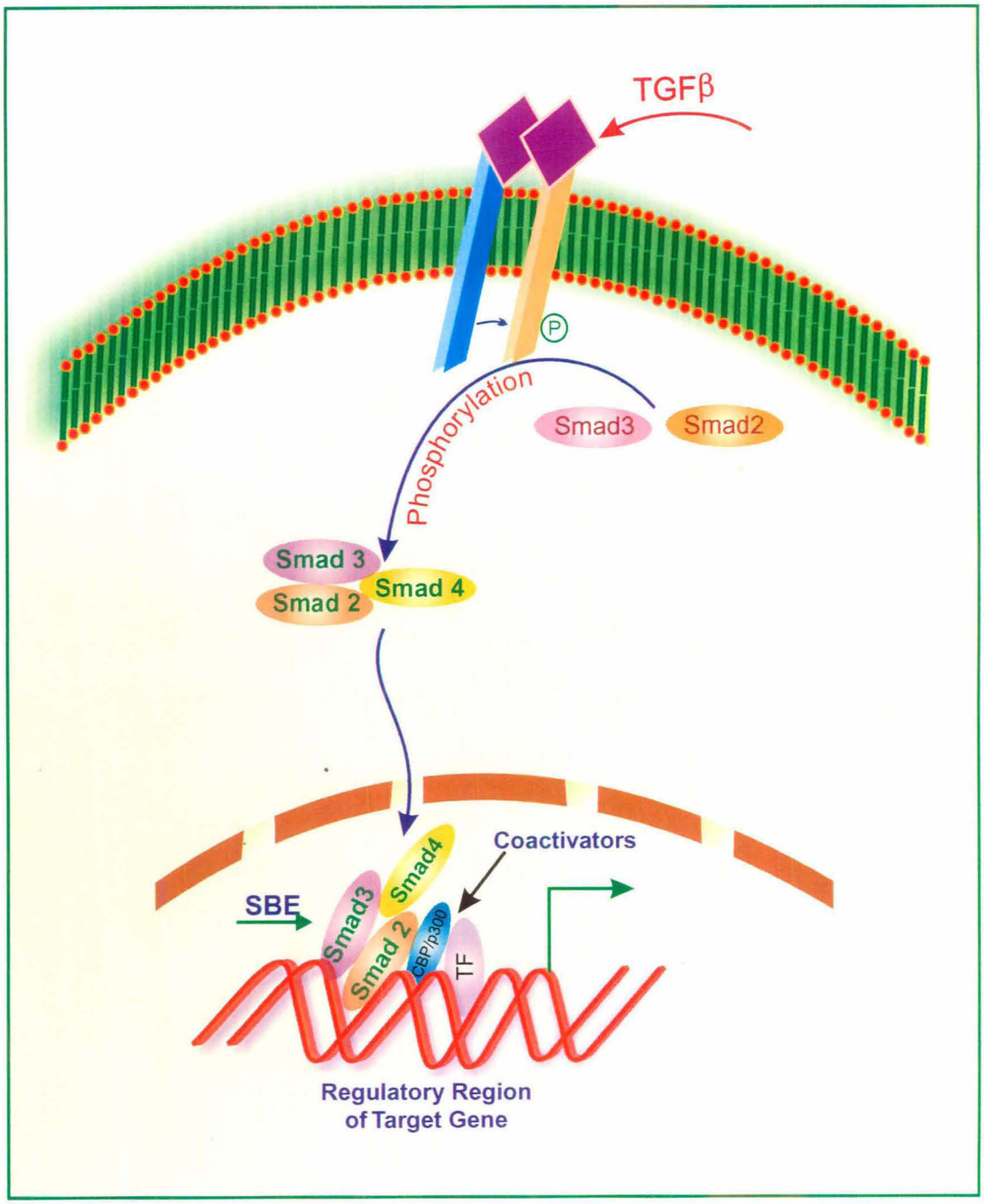


Plate III: Simplified depiction of Smads -mediated TGF-β signaling.

TGF-β activates type 2 receptor -serine- threonine -tyrosine kinase activity which phosphorylates type1 receptor. The phosphorylated type1 receptor facilitates phosphorylation of Smad2 and Smad3. Phosphorylated Smad2,3 forms heterotrimeric complex with Smad4 that translocates into nucleus and interacts with co-activators and transcription factors at Smad binding element(SBE) of target gene.

2 REVIEW OF LITERATURE

2.1 Overview

The epithelial to mesenchymal transition (EMT) can be overviewed as a series of fundamental events in which cell-cell and cell-extracellular matrix (ECM) interactions undergo essential changes to release epithelial cells from the surrounding tissue. It begins with a new transcriptional program that reorganizes cytoskeleton to confer the ability to move through a three-dimensional ECM, to acquire mesenchymal phenotype. EMT is a prerequisite for morphogenesis in development and for onset and progression of metastasis in cancer. Members of transforming growth factor-β family are essential players that lead the interplay of EMT-inducing key transcriptional regulators. Early discoveries in EMT have largely resulted from studies on developmental processes during embryogenesis. Depictions of epithelial and mesenchymal cell types have already been illustrated in the late 19th century with respect to their distinct shape and organization of cell types during embryonic development. Greenburg and Hay (1982) first portrayed the process of epithelial to mesenchymal transition in culture.

Epithelial framework is maintained by cell-cell junctions. These involve tight junctions, cadherin-based adherens junctions, gap junctions, desmosomal junctions connected through the intermediate filament cytoskeleton and cell-ECM interactions mediated by integrins and other molecules. The cell-cell and cell-ECM contacts characterize tissue polarity (Yeaman et al., 1999), which allows different functions for the apical and basal surfaces. On the contrary, mesenchymal cells often exist without cell-cell contacts and characteristic cell polarity. Reorganized structural architecture and extra-cellular matrix environment suitable for mobility is often acquired by `mesenchymal cells (Yokoyama et al., 2003). It is able to produce various MMPs and growth factors, including epidermal growth factor (EGF), hepatocyte growth factor (HGF) and fibroblast growth factor (FGF) families as well as transforming growth factor-β (Radisky et al., 2005).

Onset and progression of EMT is marked by altered cytoskeletal structure and further breakdown of cell-cell junctions and reorganized cell-ECM interactions (Janda et al., 2002; Ozdamar et al., 2005). Activation of key transcription factors is an important event of the EMT (Huber et al., 2004; Nieto, 2002; Peinado et al., 2003). These transcription factors selectively work for the promotion and maintenance of EMT by inducing expression of EMT responsive genes. It creates the feedback loops that help

sustaining the mesenchymal phenotype. Molecular play involved in EMT is characterized as a loss of proteins associated with polarized phenotype of epithelial cells and de novo synthesis of proteins associated with migratory phenotype of mesenchymal cells. Loss of epithelial proteins such as E-cadherin, cytokeratin8, cytokeratin18, desmoplakins, MUC1 and ZO-1 clearly defines epithelial dedifferentiation, whereas gain of expression of mesenchymal intermediate filament protein vimentin defines mesenchymal phenotype in EMT. Various studies propose vimentin expression as a critical marker to distinguish truly manifested EMT from cell scattering, partial EMT or any other form of cytopathy (Eckes et al., 1998; Janda et al., 2002; Grunert et al., 2003). However, vimentin expression is also present in leukocytes, endothelial cells and in some carcinomas without signs of dedifferentiation or EMT (Czernobilsky et al., 1985; Auersperg et al., 1994).

EMT is one of the important fundamental processes involved in embryogenesis (Hay, 2005; Huber et al., 2005; Thiery et al., 2006). It play a critical role at many stages in development, including gastrulaton, where primordial epithelium give rise to mesoderm and delamination of neural crest, where migratory form of cells are produced that migrate to distant site and get incorporated into various embryonic tissues (Nieto, 2001; Shook & Keller, 2003). EMT provides an ability to a cell to separate from neighboring cells and penetrate into and through surrounding tissues and thus facilitate migration of cell through intravasations and extravasations at distant tissues (Thiery, 2002) in progression of tumor metastasis (Kang & Massague, 2004; Yang et al., 2004). It also provides increased resistance to apoptotic agents in tumor progression (Maestro et al., 1999; Vega et al., 2004). EMT reorganizes cellular as well as ECM architecture that overall compromise the integrity of cell-cell and cell-matrix environment and consequently physiological integrity of the tissue. EMT is also observed in chronic inflammation or conditions that promote prolonged tissue disruption that eventually manifest fibrosis and compromise tissue integrity and organ function (Iwano et al., 2002; Kalluri & Neilson, 2003).

On the contrary, the reverse process, known as mesenchymal–epithelial transition (MET) has also been reported to occur during somatogenesis, kidney development, coelomic-cavity formation and in fibrosis. This process is regulated by effectors of EMT, which influence each other (Christ et al., 1995; Funayama et al., 1999; Locascio & Nieto, 2001).

Human embryonic stem (ES) cells represent a useful model system to study EMT events with respect to embryonic development and metastasis in cancer. In human ES cells, E-cadherin functions to stabilize the cortical actin cytoskeletal arrangement. It has been seen that differentiation of human embryonic stem cells accompanied with EMT in monolayer culture is associated with an E to N-cadherin switch, upregulation of E-cadherin repressors Snail, Slug and increased expression of vimentin (Eastham et al., 2007). Similarly, cancer stem cells also have been considered as critical contributors of EMT in context with advanced carcinoma (Prindull et al., 2005). Studies on embryonic stem cells have shown agreement with the capability of cancer stem cell to undergo EMT under in vitro culture conditions (Ullmann et al., 2007). Members of TGF-β family proteins play key roles in the self renewal and maintenance of undifferentiated stem cells. Deregulation of TGF-β signaling may contribute to impaired differentiation and development of cancers.

Overall, several studies support the idea that EMT has a central role in tumor progression. Cancerous cells acquire properties of mesenchymal cells along with altered pattern of gene expression during progression to metastasis. This results into changed adhesive properties and enhanced motility in order to migrate and establish at secondary tumors sites (Sleeman et al., 2000). These events remarkably resemble with the follow ups of EMT during developmental processes and thus show that EMT program has been conserved in development and cancer progression (Thiery, 2002). Strikingly, signaling pathways that regulate developmental EMT are also activated during tumour progression. EMT in development and progression of cancer begins with appearances of several cytokine factors which act as ligands and trigger the signaling into developmental or cancer cell that trigger molecular interplay that precisely work to achieve the objectives of embryogenesis or metastasis.

2.2 The cellular and molecular basis for epithelial to mesenchymal transition

The process of EMT transforms a framework of highly organized tightly connected sheet of epithelial cells into a disorganized motile population of mesenchymal cells. EMT is inevitable event involved in normal development and occurs in a well orchestrated fashion from gastrulation to organogenesis. The reappearance of EMT can occur in adult life under pathological conditions, particularly in fibrosis and epithelial cell plasticity in which it has a major effect on disease progression. EMT has been defined with three

striking changes in cellular and molecular architecture (Boyer & Thiery, 1993; Hay, 1995):

1) Morphological changes from cells displaying characteristic epitheloid pattern with an apical-basal polarity to scattered, spindle-shaped mesenchymal cells with migratory front.

2) Molecular changes accompanying transition from cell-cell junction proteins e.g. E-cadherin, integrins and cytoskeletal proteins (cytokeratin filaments to mesencymal specific vimentin and fibronectin filaments).

3) Functional changes accompanying conversion of stationary cells to motile cells to facilitate migration and intravasation for invasion and metastasis progression.

EMT does not necessarily observe all three changes in spatial fashion. However, it confers ability to a mesenchymal cell to migrate and invade ECM and this is considered a functional hallmark of the EMT program. EMT program is attributed as a necessary prerequisite in embryonic development and in cancer progression (Boyer & Thiery, 1993; Davies, 1996; Yang & Weinberg, 2008).

2.3 TGF-β a key inducer of EMT

TGF-β signaling has a profound role in the onset and progression of EMT during embryonic development. TGF-β signaling regulates expression of genes that play critical roles in the control of cell proliferation, differentiation, cell death and cell migration (Massague et al., 2005). Furthermore, TGF-β receptors also shared with alternative signaling effectors, such as MAPK, PI3K and small GTPases of the Rho family also involved in both gene regulation and cytoplasmic signaling for cell motility, apoptosis and EMT (Moustakas et al., 2005). TGF-β /activin signaling through Smad2 and Smad3 can induce EMT (Valcourt et al., 2005; Piek et al., 1999) whereas, pathways of the BMP through Smad1, Smad5 and Smad8 do not induce marked EMT and are able to inhibit TGF-β induced EMT promoted in normal mammary and lens epithelial cells (Kowanetz et al., 2004; Saika et al., 2006). In vitro studies, along with tumor analyses in mouse models have firmly established that Smad signaling mediates the EMT response to TGF-β family members. Interestingly, comparative analysis of *Smad2* versus *Smad3* liver-specific knockout mice has recently confirmed that TGF-β driven EMT of hepatocytes depends on Smad3 and not on Smad2; in contrast, Smad2 counteracts the EMT response thus acting as a suppressor of hepatocyte dedifferentiation (Ju et al., 2006). Smad

signaling has been shown to be dependent on the common mediator Smad4. In cultured cell models and tissue-specific knockdown of *Smad4* in the mammary gland and the pancreas, RNAi experiment targeting *Smad4* have shown an important role of Smad4 in EMT of epithelial tissue types (Li et al., 2003; Bardeesy et al., 2006; Deckers et al., 2006). Furthermore, recently, Smad4 has been shown to be essential for the transcriptional mechanism that downregulates E-cadherin expression in response to TGF -β (Takano et al., 2007). The mechanism which elicits the EMT in response to Smad signaling involves regulation of several genes implicated in EMT. Large scale gene expression profile in a cell model undergoing EMT in response to TGF-β or in vivo models of carcinoma invasiveness and metastasis, have revealed many potential regulators of EMT (Valcourt et al., 2005; Zavadil et al., 2001; Jechlinger et al., 2003; Kang et al., 2003; Xie et al., 2003; LaGamba et al., 2005).

2.4 Activation of TGF-β

Three isoforms of TGF-β; TGF-β1, TGF-β2 and TGF-β3 are known to be found in mammals. Among these, TGF-β1 is most frequently upregulated in tumor cells (Derynck et al., 1987; Dickson et al., 1987) and has a profound influence on EMT implicated in morphogenesis and tumorigenesis. The TGF-β is an extra-cellular protein secreted as an inactive 'latent' complex, comprising a TGF-β dimer in a non-covalent complex with two prosegments. Several latent TGF-β binding proteins(LTBPs) are linked to prosegments (Miyazono et al., 1993; Munger et al., 1997; Oklu et al., 2000). This latent complex provides an important safeguard against accidental activation and helps in stabilizing the latent TGF-β to the extra-cellular matrix environment where it gets sequestered (Oklu et al., 2000; Taipale et al., 1998). The secretion of TGF-β as a latent complex suggests the regulated activation of TGF-β through the activities of proteases that preferentially degrade the TGF-β prosegments and hence release the highly stable and active TGF-β dimer. Plasmin is known to activate latent TGF-β (Sato et al., 1989; Lyons et al., 1990) at sites of cell migration and invasion (Andreasen et al., 1997). Metalloproteases MMP-9 and MMP-2 can also activate latent TGF-β (Yu & Stamenkovic, 2000) at sites of tumor cell invasion (Stamenkovic et al., 2000; Stetler-Stevenson et al., 2001). Other mechanisms are also found to be involved in TGF-β activation independent of proteases, for example, the extra-cellular matrix protein thrombospondin (Schultz-Cherry et al., 1993; Crawford et al., 1998) and the αvβ6 integrin activate TGF-β through conformational change in TGF-β complex in response to

inflammation (Munger et al., 1999). In different physiological contexts, different mechanisms may regulate TGF-β activation.

2.5 Smads mediated TGF-β signaling

Smads are known to be the only established intracellular effectors of TGF-β signaling. They exist as three subgroups, R-Smads (Smad2 and Smad3), a common Smad (e.g. Smad 4 in vertebrates) and inhibitory Smads (Smad6 and Smad7). The R-Smads have a C-terminal SXS motif which is targeted for direct phosphorylation by the type I receptors at serine residue. C-terminal SXS phosphorylation of the R-Smads leads to the formation of a heterotrimeric complex consisting of two R-Smads and one Smad4. This heterotrimeric complex translocates into the nucleus, where the Smads act as transcription factors (Miyazono et al., 2000; Derynck & Zhang, 2003; Shi & Massague 2003). Smads regulate transcription through formation of a nucleoprotein complex consisting of Smad binding DNA elements, transcription factors and coactivators at the regulatory region of target genes (**Plate III**). R-Smads and Smad4 have a weak intrinsic DNA binding ability that requires less stringent binding sequence or motif than the Smad interacting transcription factors, which possess high binding affinity towards specific DNA sequence. Thus, Smad mediated transcriptional regulation takes place through a Smad binding sequence in proximity with the cognate sequence for the interacting transcription factor. In addition, Smads do interact with coactivators CBP/p300 that allows the Smad complex to enhance the transcription activity of the transcription factors. Thus, Smad complex also acts as a coactivator complex for transcription factors to a wide array of regulation in response to TGF-β signaling. Overall, interactions of Smads with Smad binding DNA sequence in proximity with transcription factor binding element along with co activators suggest the selection of target genes with binding sites for the interacting transcription factors (Derynck & Zhang, 2003; Shi & Massague, 2003; Feng & Derynck, 2005).

2.6 Interactions of Smads with other regulatory proteins

Inhibitory Smads; Smad6 and Smad7 are known to inhibit TGF-β family signaling primarily by interfering with the receptor mediated activation of R-Smads. These inhibitory Smads show competitive interactions with R-Smads for recruitment and phosphorylation by type I receptors (Hayashi et al., 1997; Imamura et al., 1997; Nakao et al., 1997). Several signaling pathways such as Jak/STAT in response to interferon-γ

(Ulloa et al., 1999) and NF-κB signaling in response to inflammatory cytokines such as TNF-α and IL-1 induce expression of Smad6 and Smad7 which composes a decisive point for negative regulation of TGF-β and BMP signaling. Apart from Smad-mediated transcription and repression (Chen et al., 2001; Alliston et al., 2001; Hayes et al., 2001), Smads also interact with many transcription factors together with the DNA binding activity that specifies the promoter binding and transcriptional activation (Massague et al., 2000a; Derynck et al., 1998; Massague et al., 2000b). For example, Smad3 can interact with bZIP transcription factors such as c-Jun (Zhang et al., 1998; Liberati et al.,1999; Qing et al., 2000), ATF-2 (Sano et al., 1999; Hanafusa et al., 1999) or CREB (Zhang & Derynck, 2000), basic helix–loop–helix transcription factors such as TFE3 (Hua et al., 1998, 1999), runt domain transcription factors such as AML-1, AML-2 and CBFA1 (Alliston et al., 2001; Zhang & Derynck, 2000; Hanai et al., 1999; Pardali et al., 2000), nuclear receptors such as the vitamin D3 (Yanagisawa et al., 1999), glucocorticoid (Song et al., 1999), androgen receptors (Hayes et al., 2001; Kang et al., 2001) and STAT-3 (Nakashima et al., 1999). A direct physical interaction of Smads with the transcriptional coactivator CBP/p300 is essential for the transcriptional activation function of Smads (Janknecht et al., 1998; Feng et al., 1998; Shen et al., 1998). Smad3 and Smad4, but not Smad2, have an intrinsic DNA-binding capability (Zhang et al., 1998; Yingling et al., 1997; Zawel et al., 1998; Dennler et al., 1998; Shi et al., 1998) although interactions of Smad complex with transcription factor, defines degree of affinity for a specific promoter sequence. A Smad-binding DNA sequence thus provides a favorable sequence context for Smad close to the high affinity binding sequence for the Smad-interacting transcription factor (Derynck et al., 1998; Yingling et al., 1997; Dennler et al., 1998). This sequence context-dependent binding of a Smad to both the interacting transcription factor and the promoter DNA might explain why TGF-β activates only a subpopulation of the promoters that bind the transcription factor with which a Smad can interact.

2.7 Specificity and crosstalk with Smad signaling

Amplitude of TGF-β mediated transcriptional activation is dependent on recruitment of coactivators and corerpressors in addition to Smad complex and its interaction with CBP/p300. MSG1 has been implicated as another coactivator to amplify the Smad response (Shioda et al., 1998; Yahata et al., 2000). Whereas SNIP1 (Kim et al., 2000), SIP1 (Comijn et al., 2001), SnoN (Stroschein et al., 1999), Evi-1 (Kurokawa et al.,

1998), c-Ski (Luo et al., 1999; Akiyoshi et al., 1999) and TGIF (Wotton et al., 1999) that interact with Smad3 and/or Smad2 have been implicated as a corepressor to inhibit TGF-β responses. Recruitment of corepressors may be the essentially fundamental step followed by EMT in embryogenesis. Tumor cells may exploit similar process in the onset and progression of tumor growth by recruiting the same repressors essential for embryogenesis.

Physical interactions of Smads with various transcription factors as a functional cooperativity, invites crosstalk with other signaling pathways (Piek et al., 1999; Massague et al., 2000c; Zhang et al., 1999; ten Dijke et al., 2000). For example, Smad interaction with TGF-β-responsive AP-1-(c-Jun/c-Fos) or CREB/ATF-binding sites activates mitogen-activated protein (MAP) kinase pathways (Zhang et al., 1998; Sano et al., 1999; Hanafusa et al., 1999; Hocevar et al., 1999; Wong et al., 1999; Yue et al., 2000; Engel et al., 1999). Notably, transcriptional cooperation of Smads with the AP-1 complex at intact AP-1-binding promoter sequence is often required for the expression of extra-cellular matrix proteins and proteases (Chang et al., 1995; Chung et al., 1996), suggesting TGF-β responses on Ras/MAP kinase and/or phosphatidylinositol-3-OH kinase (PI3K) signaling (Zhang et al., 1998; Wong et al., 1999; Peron et al., 2001). Ras/MAP kinase signaling also induces expression of TGF-β1 which is further enhanced by TGF-β signaling (Yue et al., 2000; Owen & Ostrowski, 1990; Geiser et al., 1991; Van Obberghen-Schilling et al., 1988). This may explain the very often observed increase in expression of TGF-β1 by tumor cells. Cooperativity between Ras/MAP kinase signaling and TGF-β signaling in tumor cell differentiation and behavior (Lehmann et al., 2000; Oft et al., 1996) along with several additional mechanisms of crosstalk with Smad pathway suggest the important role of TGF-β signaling in cancer cells.

The activation of some signaling pathways, including epidermal growth factor (EGF) receptor activation (Afrakhte et al.,1998), interferon-γ signaling through STATs (Ulloa et al., 1999) and tumor necrosis factor-α induced activation of NF-κB (Bitzer et al., 2000) induces the expression of Smad7, which in turn inhibits TGF-β signaling through Smads. The activation of p38 MAP kinase and JNK can enhance Smad signaling through either Smad phosphorylation (Engel et al., 1999) or the phosphorylation of c-Jun and ATF-2 (Sano et al., 1999; Hanafusa et al., 1999; Hocevar et al., 1999), these transcription factors cooperate with Smad3 that results into functional crosstalk with Smad-mediated transcription at defined promoters.

2.8 TGF-β signaling in stem cells

Stem cells essentially represent primary cells of undifferentiated state with great potential to give rise to fully differentiated cell types having ability of self-renewal and expansion of the self population. Characteristics of stem cells share some common features with cancer cells, including ability of self renewal, expansion of the self population and the loss of contact inhibition. Moreover, cancer cells are presumed to be derived from cancer stem cells (Prindull et al., 2005). Thus, understanding stem cells may provide insight into cancer development and vice versa. Multiple signaling networks from early embryo formation to later life orchestrate the development and differentiation of ES cells and somatic stem cells into various functional lineages of neuronal, hematopoietic, mesenchymal and epithelial cells. Among these, signaling network activated by proteins of TGF-β family showed key roles in the self renewal and maintenance of stem cells as well as selection and the progression of differentiation along an individual lineage. Further, TGF-β family signaling maintains the differentiated phenotype of epithelial, mesenchymal and other cell types. Failure of TGF-β signaling is implicated in an undifferentiated phenotype and cancer development (Mishra et al., 2005).

Members of TGF-β family proteins work for both maintenance of undifferentiated cell and initiation of differentiation of cells. TGF-β family proteins, nodal and activin also have a role in human ES cell maintenance through Smads. TGF-β signaling in ES cells in *Xenopus* explants and dose dependent signaling of TGF -β family players mediate key decisions that specify germ layer differentiation. In mammals, this signaling pathway is presumably activated by nodal or related factors. Activin or TGF-β also induce mesoderm differentiation, whereas, BMP signals confer ectodermal and mesodermal differentiation of human ES cells. Conversely, in *Xenopus*, inhibition of activin or TGF-β as well as BMP signaling gives rise to neuroectoderm formation. Similarly, mouse ES cells in culture showed formation of neuroectoderm in the absence of these factors (Schuldiner et al., 2000). Thus, the presence or absence of TGF-β family signals is a determinant of both maintenance and initial specification of ES cells and of the primary cell fate decision in early embryogenesis that may give rise to multiple cell lineages and cell fates.

2.9 TGF-β signaling in haematopoietic and mesenchymal stem cells

TGF-β family proteins and their downstream signaling effectors, the Smads, have key roles in hematopoietic differentiation (Ruscetti et al., 2005; Scandura et al., 2004). In combination with cytokines, TGF-β, BMPs, promote hematopoietic specification, differentiation and proliferation of human ES cells (Park et al., 2004). Although, TGF-β showed negative regulation of hematopoietic progenitor and stem cells in vitro, impaired TGF-β signaling does not affect hematopoietic lineage selection in vivo (Larsson et al., 2005). TGF-β inhibits the proliferation of early multipotent hematopoietic stem cells but not that of later progenitors. The effects of TGF-β on more mature progenitor cells are complex and depend on the presence of other growth factors. TGF-β mediated signaling also has decisive effects on mesenchymal differentiation. It offers an ability to mesenchymal stem cells to differentiate into a variety of cell types comprising fibroblasts and highly specialized cell types such as skeletal muscle cells (myocytes), bone-matrix-depositing cells (osteoblasts), cartilage cells (chondrocytes) and fat cells (adipocytes). In a mouse model and cell culture systems autocrine and paracrine stimulation by TGF-β have shown its importance in the maintenance and expansion of the mesenchymal stem cell progenitor populations (Chen et al., 2004).

2.10 EMT during development

Embryonic development essentially required a sequence of well orchestrated differentiation events. While doing so, a sequential molecular interplay triggers the fate of cells either to differentiate along a specific lineages or to remain undifferentiated or partly differentiated during the formation of specific tissues, thus, allowing maintenance of stem cell and progenitor cell populations within a specified tissue (Deryck & Akhurst, 2007). Members of TGF-β family have versatile roles such as offering ability to self renewal, to endorse the embryonic and somatic stem cells with differentiation potential and selection of the lineage and progression along that lineage for differentiation. Understanding of their activities during developmental processes has provided insights into the ability of a cell to undergo differentiation (Deryck & Akhurst, 2007).

Process of EMT marks its presence very early during development. Formation of mesoderm during gastrulation may be considered as an earliest example of EMT in the embryo development. TGF-β family members nodal and activin act through Smads in concert with other signaling pathways (Gadue et al., 2006; Cordenonsi et al., 2007; Sirard et al., 1998) in the formation of the three primary germ layers from which the

embryo develops. Delamination of migratory neural crest cells from the neuroepithelial tube gives rise to mesenchymal and other cell types for the development of head structures and of many organs and tissues of the body (Wang et al., 2006). During cardiac development, several TGF-β family ligands particularly TGF-β2 and bone morphogenetic proteins (BMPs) as well as TGF-β family receptors through Smads induce EMT for the development of heart valves within the atrio-ventricular canal (Mercado-Pimentel et al., 2007a, 2007b; Desgrosellier et al., 2005). During craniofacial development, autocrine expression of TGF-β3 acts to transform midline epithelial stratum of the secondary palate to palatal mesenchyme, an essential process for palatal development in mice (Proetzel et al., 1995).

The inverse process of EMT known as mesenchymal-epithelial transition (MET) is implicated in generation of transitory mesenchymal cells into polarized epithelial cells after migration and homing at new sites of tissue formation. MET has been described in the context of embryonic development and in fibrotic disorders (Zeisberg et al., 2004, 2005; Kowanetz et al., 2004). Morphogenetic processes such as EMT or MET are governed by the functional interplay of many signaling pathways, typically initiated by secreted polypeptide factors such as members of bone morphogenetic protein (BMP) family, that regulate transcriptional and post-translational events for the generation of new cellular phenotypes during development and in fibrosis. BMP induces MET and thus antagonize the actions of TGF-β on fibrotic kidney epithelial cells (Zeisberg et al., 2005) as well as in mammary and lens epithelial cells in vitro (Kowanetz et al., 2004). The process of MET is an essential episode required in tissue formation during development.

2.11 EMT is important during embryogenesis

EMT is an integral process that marks developmental transitions like embryogenesis. This process is considered to be extremely critical for formation of the three germ layers, ectoderm, mesoderm and endoderm through gastrulation. During gastrulation movements of epithelial cell sheets leads to the formation of migratory mesenchyme that progresses along the primitive streak and populates new areas of the embryo that will develop into mesoderm and endoderm (Hay, 2005). Fibroblast growth factor (FGF) signaling through receptor tyrosine kinases (RTK) triggers migration of mesenchymal cells through the primitive streak and thus mesoderm formation (Ciruna et al., 2001). Apart from TGF-β signaling FGF-mediated signaling also recruits key regulatory

molecule Snail during gastrulation which directly represses expression of the E-cadherin, an integral component of adherens junctions in epithelial cells (Ciruna et al., 2001; Carver et al., 2001; Pera et al., 2003; Peinado et al., 2004). In addition to this, Wnt signaling through β-catenin along with its nuclear partner LEF1 is also attributed in the EMT process during gastrulation (Kemler et al., 2004; Mohamed et al., 2004; Sakai et al., 2005, 2006). Formation of other structures, such as the vertebrae, the cardiac valves, the craniofacial structures, the neural derivatives and the secondary palate as well as the disappearance of the male mullerian duct also take place through EMT.

Process of EMT continues through a later stages of embryogenesis, whereby epithelial cells from the neural crest, give rise to mesenchymal cells that migrate long distances to new tissue areas and differentiate into several other types of mesenchymal cells such as somites, bone and chondrocytes (Trainor et al., 2003; Tatjana et al., 2006). BMP a member of TGF-β super family has been implicated in neural crest EMT in dose -dependent manner along with a group of transcription factors, including paired-box, high-mobility group (HMG), winged-helix transcription factors and Snail (Meulemans et al., 2004). Interestingly, in vitro studies have shown that TGF-β signaling can also induce expression of Notch ligands, such as Jagged-1 that triggers Notch signaling to induce EMT and epithelial cell cycle arrest (Zavadil et al., 2004; Niimi et al., 2007). Overall, it provides a reflection on major developmental signaling pathways such as TGF-β, Wnt, RTK and Notch that enforce embryonic epithelial cells to convert into mesenchymal derivatives along with enhanced migratory and differentiation capacity critically required for embryogenesis (**Plate IV**).

2.12 TGF-β signaling in EMT during development

Members of the transforming growth factor-β superfamily have profound role in onset and manifestation of EMT during several inevitable events that execute development. Widespread studies in various models of EMT pointed out that TGF-β signaling plays prime role in EMT although, various EMT events may require different members to activate the signaling. During embryonic development in both *Xenopus* and zebrafish, induction of mesoderm mainly requires members of the Nodal subfamily of TGF-β (McDowell & Gurdon, 1999; Kimelman, 2006). Another member of Nodal family Vg1 is also known to play a critical role in mesoderm induction in *Xenopus* and chicken whereas, Gdf3 is involved in induction of mesoderm in mice since *Gdf3* null mutants

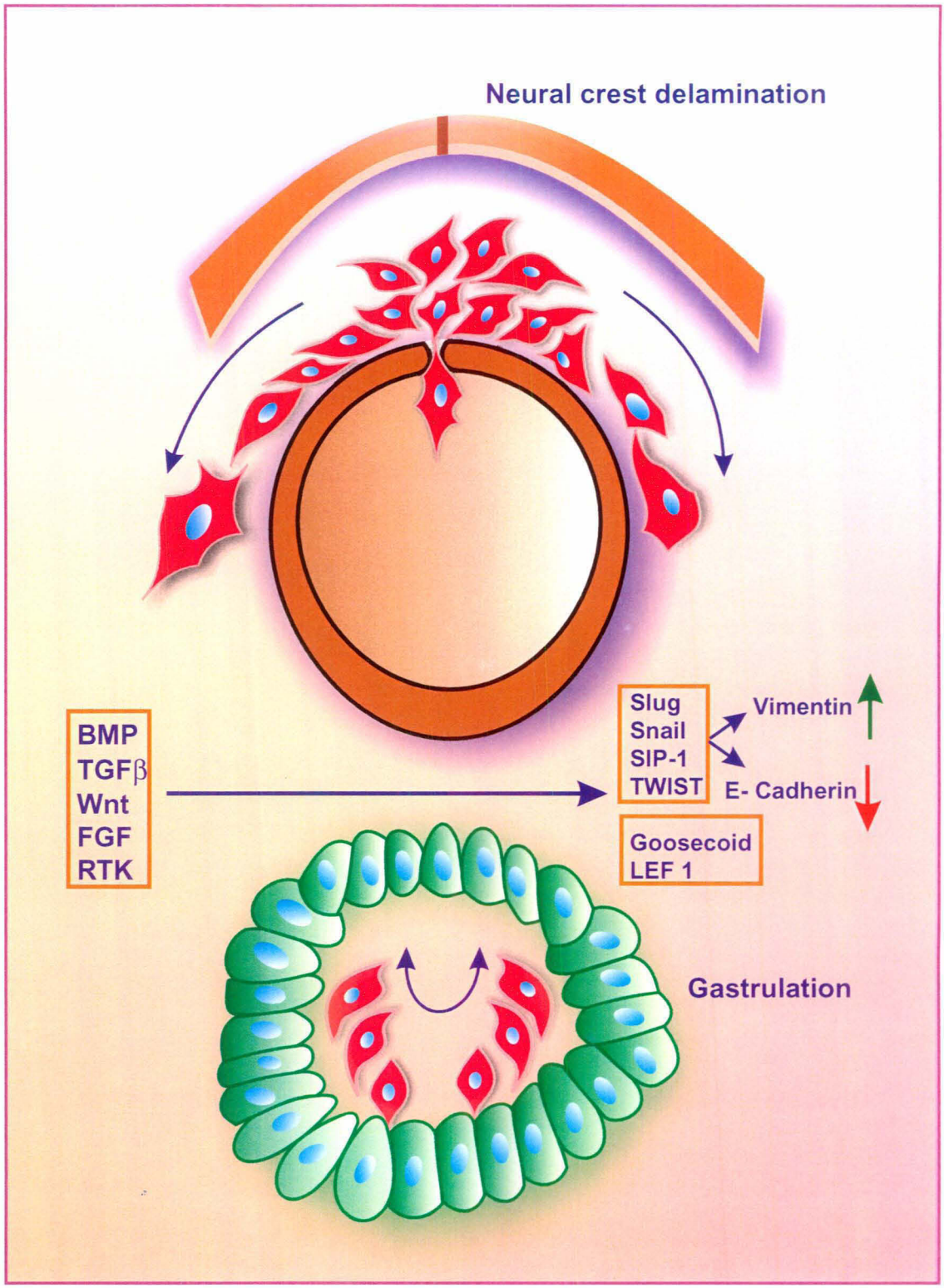


Plate IV: EMT during embryogenesis.

Members of TGF- β family and other cytokines mark their presence in early embryo development and recruit key regulatory molecules such as Snail, Slug, SIP-1, Twist and others. Downregulation of E-cadherin and upregulation of vimentin are another important events required to execute EMT. EMT is critically involved in delamination of nerual crest cells from neuroepithelial tube that give rise to mesenchymal cell and in formation of three germ layers during gastrulation.

displayed defective mesoderm formation (Chen et al., 2006). Activation of BMP signaling is required for neural crest formation. Differential activation of the BMP signal in *Xenopus* may require setting up a competency zone between neural plate and ectoderm from which the neural crest can be induced (Raible, 2006). Requirement of BMPs have also been seen in the induction and/or migration of neural crest cells in chickens and mice (Correia et al., 2007).

TGF-β signals also play critical roles in the activation of EMT during cardiac valve formation and secondary palate fusion. TGF-β2 seems to be essential for initiation of the EMT program and the separation of endothelial cells in chicken AV explants. On the contrary, TGF-β3 has been shown to be specifically required for the migration of the separated endothelial cells into ECM (Boyer et al., 1999). Involvement of TGF-β1, TGF -β2 and TGF-β3 is also seen in the EMT during cardiac morphogenesis and cleft palate phenotype in individual *TGF-β1* or *TGF-β2* and *TGF-β3* knockout mice models and heart explant culture (Mercado-Pimentel & Runyan, 2007; Ahmed et al., 2007). Comprehensive studies on EMT unanimously suggest the necessity of TGF-β signaling and crosstalk with other signaling pathways such as Wnt, Notch and receptor tyrosine kinase-mediated signaling to generate the specificities required for EMT in various morphogenetic steps. Future studies are needed to reveal how these complex networks interact to coordinate and specify individual steps of the EMT program (Yang & Weinberg, 2008).

2.13 Contribution of EMT in cancer progression and metastasis

In contexts with cancer progression, EMT has been referred to as oncogenic EMT whereby malignant cells loose epithelial characteristics and acquire migratory and highly matrix invasive cell phenotypes (**Plate V**) supported with self-sufficient autocrine loops of cytokine signaling and mechanisms to abolish apoptosis (Derynck et al., 2001; Gotzmann et al., 2004). Majority of human solid tumors are carcinomas of epithelial cell origin. Function of E-cadherin has been considered as a key gatekeeper of the epithelial framework, loss of E-cadherin has been associated with progression and poor prognosis in various human and mouse carcinomas (Berx et al., 2001; Vincent-Salomon & Thiery, 2003). Loss of E-cadherin expression at an early stage of tumor development exhibit permanent mesenchymal phenotype, acquired through irreversible EMT in several types of human carcinomas. Transcriptional regulation of EMT in the majority of human carcinoma cell populations causes the loss of E-cadherin. Several EMT-inducing

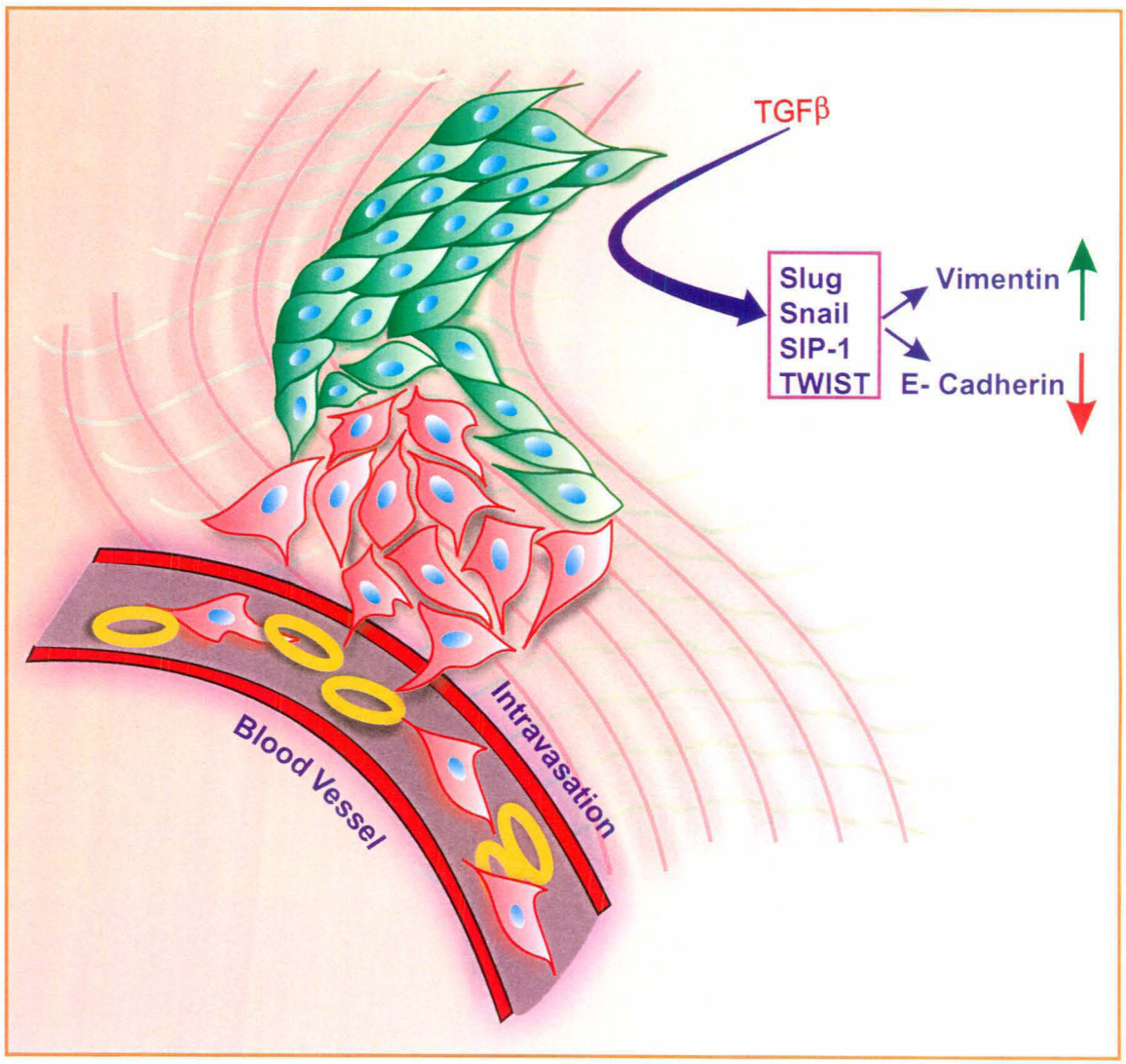


Plate V: EMT in progression of cancer.
Strikingly, TGF-b shows its presence in the progression of cancer. EMT program is generously used by cancerous cell. Molecules involved in developmental EMT similarly work for cancer progression. Downregulation of E-cadherin and existence of vimentin are the objectives executed by these molecules during EMT in cancer.

transcription factors including Snail, Slug, dEF1, SIP1, Twist1, FOXC2 and Goosecoid have been implicated in tumor invasion and metastasis of human carcinomas. Expression of Twist1 is necessary for mouse breast carcinoma cell line to metastasize from mammary gland to lung (Yang et al., 2004). Enhanced expression of FOXC2 or Goosecoid promotes dissemination of weakly metastatic human carcinoma cells (Hartwell et al., 2006; Mani et al., 2007). A recent study on a mouse model representing hepatocellular carcinoma progression and metastasis, have shown that TGF-β signaling is implicated in sequential manner to induce PDGF secretion and PDGF receptor activation, that cooperates with β-catenin signaling to give rise to population of carcinoma cells that seem to act as cancer stem cells (Fischer et al., 2007).

In vivo studies, blocking of the TGF-β receptors showed prevention of activation of EMT and metastatic and invasive properties of a variety of experimental cancers (Dumont & Arteaga, 2003; Ge et al., 2004; Subramanian et al., 2004; Yingling et al., 2004). Thus in oncogenic EMT role of TGF-β in cooperation with oncogenic Ras associated with cancer progression has been found to be important by a wide array of studies (Derynck et al., 2001; Dumont & Arteaga, 2003; Roberts & Wakefield, 2003; Gotzmann et al., 2004). In addition to ability to activate EMT, malignant cells acquire additional ability of intravasations and extravasations to continue metastatic growth. Metastatic potential of malignant cells is also aided by resistance to apoptosis, endothelial cell adhesivity and autonomous growth. Thus, manifestation of oncogenic EMT shows highly abnormal genetic and cellular signaling background distinct from nononcogenic EMT (Zavadil & Bottinger, 2005).

2.14 Contribution of EMT in fibrosis

The process of EMT also has been described in context with epithelial injury/stress in case of kidney, liver and lung. The molecular interplay involved in this situation is yet to be explored for various molecules already established in EMT. Evidence of nonmalignant EMT has been reported in genetically engineered mice (Iwano et al., 2002). Contribution of EMT may underlie in disintegration of mature epithelial structures and in giving rise to fibroblasts in chronic fibrotic disorders (Kalluri & Neilson, 2003). Importance of mesenchymal cells has been demonstrated in formation of the body plan and normal tissue remodeling during organogenesis. Excessive accumulation of aggressive fibroblasts in adult organs can have serious consequences for morbidity and mortality, as seen in idiopathic pulmonary fibrosis, renal tubulo-interstitial

nephritis, scleroderma and hepatic fibrosis. Significant contribution from mesenchymal cells arising through EMT has been attributed to many fibrotic conditions (Iwano et al., 2002). TGF-β have been involved in many of these manifestations such as idiopathic pulmonary fibrosis, fibrotic cardiac valve disease and in fibrotic liver, kidney and eye diseases (Kim et al., 2006; Willis et al., 2005; Paruchuri et al., 2006; Ju et al., 2006; Roberts et al., 2006).

In case of diseased human kidney, evidence for EMT has been supported with coexpression of epithelial and mesenchymal markers (Rastaldi et al., 2002; Vongwiwatana et al., 2005) whereas, in experimental models or in human samples of these diseases, evidence for EMT is rare (Oldfield et al., 2001; Rastaldi et al., 2002), although, in vitro studies have suggested the manifestation of EMT in nonmalignant epithelial cells of renal, pulmonary or hepatic origins in response to hypoxic (Manotham et al., 2004) or oxidative stress signals (Rhyu et al., 2005). Besides being important mediators of EMT during development and tumor progression, new role for TGF-β has been suggested in progression of chronic fibrotic disorders (Border & Noble, 1994; Derynck et al., 2001; Bottinger & Bitzer, 2002; Roberts & Wakefield, 2003).

2.15 Contribution of the tumor microenvironment in EMT and metastasis

Microenvironment of tumor surrounded by stromal cells such as cancer associated fibroblasts or myofibroblasts, immune cells and microvessels is also influenced by EMT. EMT has been suggested as a possible source of developmental origin of tumor associated fibroblasts. Human breast cancer cells that undergo EMT still retain cytokeratins as a characteristic of epithelial cells and function as direct 'feeders' of carcinoma cells (Petersen et al., 2003). The process of 'feeding' is associated with several chemokines, growth and angiogenic factors. TGF-β plays a prime role in triggering secretion of other cytokines by the fibroblasts (Micke et al., 2005). In addition to TGF-β, cancer associated fibroblasts also produce PDGF, basic FGF and connective tissue growth factor (CTGF). Such growth factors that signal via RTK account for mitogenic effects of TGF-β on fibroblasts or on neighboring cells. The stromal environment of the tumor also contains the myofibroblasts that are known for the expression of α-smooth muscle actin and the so called activated form of myoblasts have been proposed to provide migratory cues for metastatic carcinoma cells (De Wever et al., 2004). The invasive properties of the myofibroblasts have been characterized by the expression of N-cadherin. In the invasive front of squamous cell carcinomas, secretion of

TGF-β induces EMT in stromal myofibroblasts (Lewis et al., 2004). TGF-β signaling induces expression of HGF in the stromal myofibroblasts that further enhances proliferation and invasion of cancer cells. Further, knockout studies of the *TGFβRII* confirm this tumor model independently in fibroblast (Bhowmick et al., 2004). In addition to this, deletion studies on *TGFβRII* from the mammary gland fibroblasts have shown significant inhibition of normal mammary duct development with decrease in epithelial cells and increase in knockout fibroblasts cells (Cheng et al., 2005). A mixture of receptor knockout mammary fibroblasts with mammary carcinoma cells, when xenografted into mice, led to high tumor growth and invasion, which is correlated with over production of HGF, macrophage stimulating protein (MSP) and other mitogenic factors by the knockout fibroblasts. Overall, studies concentrating on cancer associated fibroblasts and their regulatory roles may hold a promise to design new anti-tumor therapeutics.

2.16 Contribution of TGF-β promoted angiogenesis in cancer progression

Tumor angiogenesis plays crucial role in tumor growth, invasion and finally metastasis as blood vessels deliver nutrients and oxygen to the tumor cells and allow intravasation of delocalized tumor cells which leads to landing at distant tissues and organs that result into invasion and metastasis. TGF-β induces expression of the angiogenesis-inducing vascular endothelial cell growth factor (VEGF) (Pertovaara et al., 1994). Capillary formation of endothelial cells cultured on a collagen matrix showed direct effects of TGF-β whereas, indirect stimulation of angiogenesis by TGF-β1 might occur through the potent chemoattractant activity of TGF-β for monocytes, which release angiogenic cytokines (Madri et al., 1988; Sunderkotter et al., 1991). Also, TGF-β induced expression of the metalloproteases MMP-2, MMP-9 and downregulation of the tissue inhibitor of metalloproteases (TIMP) in tumor and endothelial cells, provide a protease rich microenvironment conducive to the enhanced migratory and invasive properties of angiogenically active endothelial cells (Hagedorn et al., 2001). Thus, direct effects of TGF-β and indirect effects on the tumor microenvironment stimulate tumor angiogenesis. Many studies implicate the angiogenesis-inducing capabilities of tumor secreted TGF-β in accelerating tumor progression. In human breast tumors, high levels of TGF-β1 mRNA are associated with increased microvessel density and both parameters correlate with poor patient prognosis (de Jong et al., 1998). In other carcinomas, an association exists between high tumor burden and elevated circulating plasma levels of TGF-β1

along with enhanced tumor angiogenesis and poor patient prognosis (de Jong et al., 1998; Ito et al., 1995).

2.17 Contribution of TGF-β in EMT and metastasis

One aspect of tumor cell biology that is thought to contribute to metastasis is increased plasticity of cells that effectively undergo transient differentiation event called epithelial to mesenchymal transition that allows them to move out of the primary tumor into circulation (Thiery et al., 1999). TGF-β influences morphological changes that resemble EMT in normal and transformed mammary epithelial cells in culture (Oft et al., 1996; Miettinen et al., 1994; Hosobuchi & Stampfer, 1989). However, in mouse mammary tumors, cooperativity between the Ras and TGF-β signaling has been found to be frequently elevated during tumour progression (Oft et al., 2002; Janda et al., 2002). EMT from a squamous to fibroblastoid tumor is characterized by prominent changes in the expression of cell-adhesion molecules, such as downregulation of E-cadherin and a rearrangement and replacement of cytokeratin with vimentin as a cytoskeletal protein (Portella et al., 1998). TGF-β also induces further differentiation of fibroblasts into myofibroblasts along with expression of pro-metastatic factors, such as matrix metalloproteinases, interleukin-8, vascular endothelial growth factor and the chemokine receptor CXCR4. Reorganization of ECM facilitates the carcinoma cell towards enhanced migration, invasion and intravasation or extravasation within a circulatory system (Oft et al., 2002). Whereas, elevated expression of chemokine ligands and receptors, such as stem cell factor 1 (SCF-1) and CXCR4, may facilitate homing and survival of carcinoma cells at the metastatic site. EMT during spread of cancer in humans most often shows a transient form and not necessarily a complete or reversible form (Yang et al., 2004). Thus, EMT may provide an advantage to the metastatic cell, not only to migrate from the site of origin but also to establish at a favorable secondary site (**Plate V**).

In cancer, metastasis or migration and invasion of primary tumor cells to distant location in the body is a hallmark of mortality in cancer patient. TGF-β plays an important role in cancer progression (Akhurst, 2002, 2007). Studies on kidney transplant patients showed highly invasive skin tumors in response to treatment with immunosuppressive agents such as cyclosporine (Hojo et al., 1999). In vivo experiments in NUDE mice injected with MDA-MB-231 breast cancer cells that normally metastasize to bone, showed TGF-β as a major effecter of breast tumor metastasis. Blocking of TGF-

β signaling in MDA-MB-231 breast cancer cells by overexpressing a dominant negative *TGFβRII* mutant and injecting these cells in immunodeficient mice resulted in fewer tumors, with less osteoclast recruitment, less bone destruction at metastatic sites and prolonged host survival. Production of parathyroid hormone-releasing peptide (PTHrP) in a tumor, which is responsible for osteoclast recruitment and renal tubular resorption of calcium was found to be induced by TGF-β1. This study showed significant finding as women with PTHrP positive breast tumors are more likely to suffer metastatic disease than those with PTHrP negative tumors (Yin et al., 1999).

2.18 TGF-β mediated transcription regulation network in EMT

Regulation of E-cadherin is a central event of the EMT in the development and advancement of carcinoma. A wide array of transcription factors are involved in regulation of EMT. TGF-β is able to recruit key regulators in all types of EMT. Many of the transcriptional repressors are involved in repressor activities such as members of the zinc finger proteins of Snail family (Snail, Slug), two-handed zinc finger/homeodomain proteins (dEF1, SIP1), bHLH proteins (E12/E47, Twist) and high mobility group box-containing proteins-1 (LEF1) (Peinado et al., 2004, 2007). These repressors are known to recognize E-box DNA sequences located in upstream region of the E-cadherin gene along with the recruitment of transcriptional corepressors and histone deacetylases. TGF-β activates both Snail and Slug directly through Smad3; however, the pattern of activation is mutually exclusive and cell type dependent in multiple nonmalignant cell culture models of EMT (Zavadil et al., 2004). TGF-β mediated activation of Snail is also achieved via activation of the Erk and PI3K pathways (Peinado et al., 2003; Sato et al., 2003). Snail represses E-cadherin expression during mesoderm formation in early embryonic development (Carver et al., 2001) as well as in cultured cells (Batlle et al., 2000; Cano et al., 2000). Apart from TGF-β, other growth factors, such as FGF and HGF also induce the expression of Snail and Slug known to be responsible for repression of desmoplakin and desmoglein and eventually disassembly of desmosomes (Savagner et al., 1997; Grotegut et al., 2006).

Repression of E-cadherin in context with carcinogenesis related EMT is also executed by two zinc finger transcriptional repressors of the dEF family, dEF1 and Smad -interacting protein-1 (SIP1) (Comijn et al., 2001; Eger et al., 2005; Vandewalle et al., 2005). Like Snail and Slug, TGF-β activates SIP1 through Smad signaling in a cell type-dependent manner (Zavadil et al., 2004). SIP1 may associate with and regulate

transcriptional activity of Smad proteins (Postigo et al., 2003). Ectopic expression of SIP1 in MDCK cells eventually resulted into dissociation of adherens junctions and increased motility (Comijn et al., 2001). Similarly, dEF1 can directly interact with the E-cadherin promoter and its ectopic expression in mammary gland epithelial cells causes an invasive mesenchymal phenotype transition similar to that observed with ectopic expression of c-fos and oncogenic Ras (Eger et al., 2005). Recent study showed that dEF1 (ZEB1) promotes colorectal cancer cell metastasis and loss of cell polarity. dEF1 suppresses the expression of cell polarity factor Lgl2 which is critical for the epithelial phenotype and its loss might be involved in metastasis in colorectal and breast cancers (Spaderna et al., 2008).

Ectopic expression of Fos protein in inducible and sustained manner in mammary epithelial cells showed loss of cell polarity and increased invasiveness in collagen cultures (Reichmann et al., 1992). Sustained activation of Fos is required for maintenance of EMT depending on β-catenin/LEF1 signaling and autocrine production of TGF-β (Eger et al., 2000, 2004). On the contrary, short-term activation of Fos can result in reversible loss of cell polarity (Reichmann et al., 1992). Sustained EMT in human keratinocytes is also observed with rapid and transient induction of c-Fos in response to TGF-β stimulation (Zavadil et al., 2001). Thus, the functional role of c-Fos transcription factor appears to be associated in a context dependent manner in epithelial cells. Smad-mediated TGF-β signaling is also implicated in the expression of high mobility group factor, HMGA2, identified as a new regulator of EMT during embryogenesis (Thuault et al., 2006). The loss of HMGA2 prevents EMT in mammary epithelial cells whereas, its presence induces expression of the transcriptional regulators Snail, Slug and Twist and thus promotes EMT. A potential offered to a HMGA to induce key repressors through TGF-β suggests expression of these key repressors to be ensured by multiple pathways mediated through Smads. It further shows importance of the interplay mediated through these repressors during EMT.

2.19 Interplay of extra-cellular signaling in EMT

Molecular interplay involved in EMT comprises various extra-cellular signals, including components of the extra-cellular matrix (ECM), such as collagen and hyaluronic acid (Zoltan-Jones et al., 2003), as well as soluble growth factors, such as members of the TGF-β superfamily and fibroblast growth factor (FGF) families, epidermal growth factor (EGF) and SF/HGF. Further analysis of effect of cytokines on EMT suggests that it is

context-dependent e.g. scatter factor/hepatocyte growth factor (SF/HGF) induces EMT during somatogenesis, but inhibits EMT in other processes (Zavadil et al., 2005). Signaling through these ligands triggers the activation of downstream intracellular effector molecules, such as members of the small GTPase family- Ras, Rho, Rac and members of the Src tyrosine kinase family. The activation of signaling pathways results in the activation of transcriptional regulators such as snail and slug which enforce the transcriptional reprogramming needed for EMT. These effectors promote the disassembly of junctional complexes and the changes in cytoskeletal organization during EMT. Repression of the E-cadherin is considered as a central target of these transcriptional regulators. Downregulation of E-cadherin has several direct consequences that favor EMT. Limiting level of E-cadherin results in the loss of E-cadherin mediated intracellular junctional complexes. Whereas, loss of E-cadherin results in release of β-catenin from adherens junction. It is further exploited by Wnt signaling pathway and translocated into nucleus to cooperate transcriptional regulation through lymphoid enhancer-binding factor/T-cell factor-4 (LEF/TCF-4). In vitro studies show that several extra-cellular activators are involved in the regulation of EMT. Extensive crosstalk exists between the signaling pathways that activate and repress EMT with many common endpoints, including downregulation of E-cadherin and expression of vimentin.

2.20 TGF-β associated context dependent network and cross-signaling in EMT

Recent insights into the signaling networks that regulate EMT during embryological processes and progression of carcinoma show synergy with TGF-β signaling. Functional coopratívity of several pathways with TGF-β signaling contributes to a fundamental process called EMT. TGF-β marks its presence in the very beginning of the interplay and gradually recruits key molecular players specialized in reprogramming and remodeling the cellular architecture, inherently attributed for the development. While doing so, TGF-β extends its functionality in terms of establishing a contextual cooperation with several other molecular players working for other signaling pathways and specialized in specific functions, collectively required to achieve the same developmental objective. Cancer cells conveniently exploit the molecular interplay supposed to work exclusively for development (**Plate VI**).

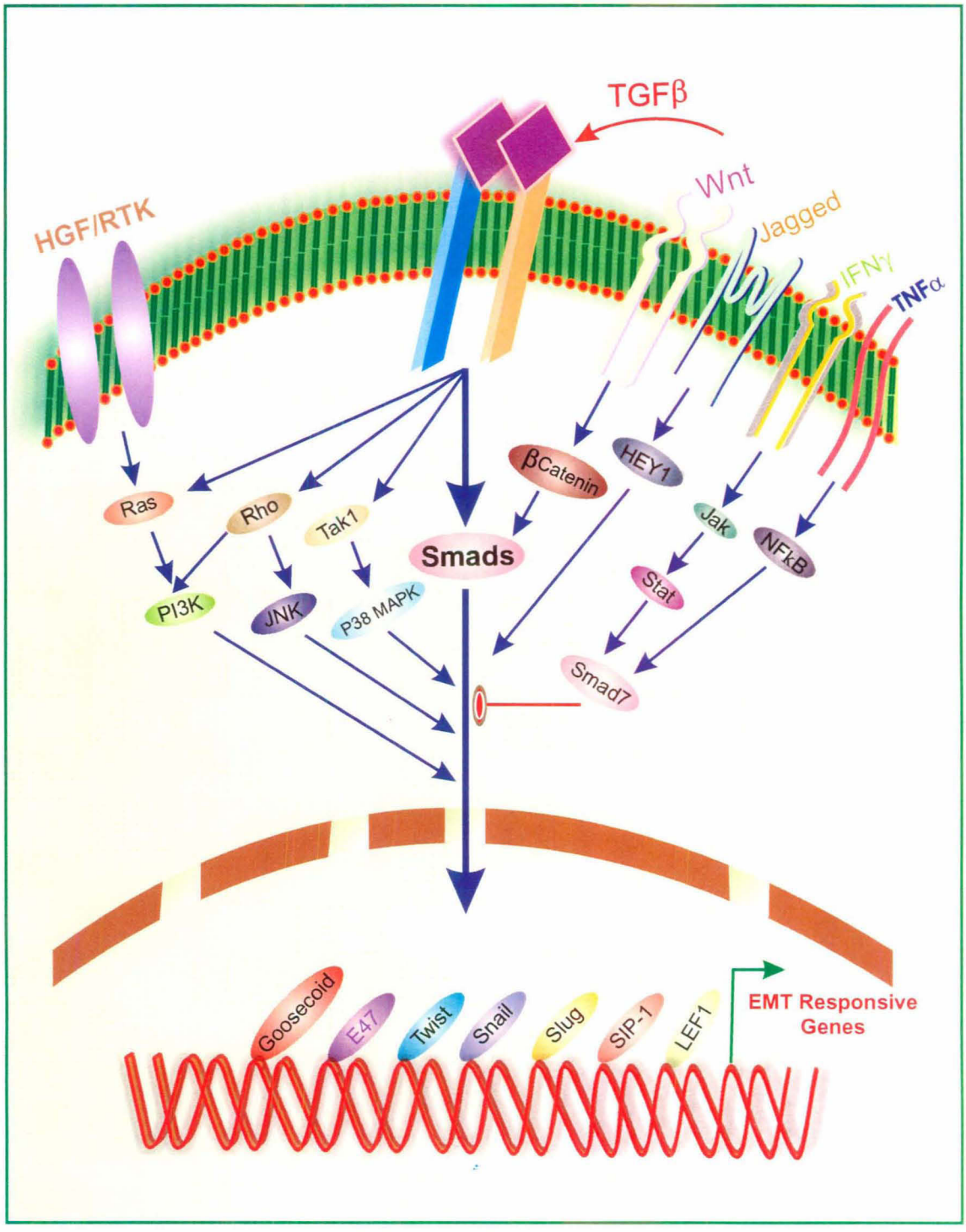


Plate VI: TGF-β associated cross signaling network in EMT.
Several signaling pathways are known to be involved in EMT during development and cancer progression. TGF-β extends its functional network during EMT. Smads-mediated signaling converge with other signaling pathways to induce EMT responsive genes.

2.20.1 Ras/Erk MAPK and p38 MAP kinase

Various studies reveal complex functional relationship between Ras and TGF-β in EMT signaling (Oft et al., 2002; Gotzmann et al., 2004). Cooperative signaling between TGF-β and Ras/Raf/Mek/MAPK is required for maintenance of complete EMT in various epithelial cell types. In mammary epithelial cells, autocrine production of TGF-β and its signaling through TGFβRI promotes EMT through activation of mutant Ha-Ras. Activated Raf can also induce secretion of TGF-β leading to autocrine TGF-β stimulation and maintenance of irreversible invasive phenotypes in vitro (Lehmann et al., 2000) whereas, hyperactive Raf/MAPK activity is required for metastatic features of EMT in vivo (Oft et al., 1998; Janda et al., 2002).

Several evidences strongly suggest for crosstalk and cooperation between TGF-β and mitogen-activated protein kinase (MAPK) Erk. Erk (p44MAPK) is rapidly activated by TGF-β in the context with growth arrest (Hartsough & Mulder, 1995). Erk function is required for disassembly of adherens junctions as well as cell motility in response to TGF-β-induced EMT program. A transcriptome screen of TGF-β-induced EMT in HaCaT keratinocytes with or without inhibitor of Mek/Erk MAPK identified nearly 80 EMT related targets of Erk MAPK with defined roles in cell–matrix interactions, cell motility and endocytosis, suggesting that Erk controls cell motility and disruption of adherens junctions (Zavadil et al., 2001). TGF-β stimulates Erk activity in culture models of EMT (Ellenrieder et al., 2001; Zavadil et al., 2001; Xie et al., 2004). Components of MAPK signaling including Ras, Mek1/2, Erk1/2 and activator of Erk1 (p42MAPK) (Xie et al., 2004) are transcriptionally induced by TGF-β in mammary gland epithelial cells undergoing EMT (Xie et al., 2003).

The p38 MAPK is involved in TGF-β induced EMT in mammary gland epithelium and colon cancer. Activation of MKK3/6-p38MAPK-ATF2 pathway in cultured mouse mammary epithelial cells (NMuMG) required TGFβRI and TGFβRII receptor kinase activities (Bakin et al., 2002) as well as integrin-mediated cell adhesion (Bhowmick et al., 2001b). The p38 is also required for TGF-β induced EMT and apoptosis (Yu et al., 2002). Bates and Mercurio, (2003) also showed requirement of p38 for synergic activation of MAPK by TNF-α and TGF-β signaling to promote a rapid morphological conversion of a colonic carcinoma epithelium to a mesenchymal phenotype.

2.20.2 Jagged/Notch

Several reports suggest functional interactions between TGF-β/Smad and Notch signaling in various tissues, either by hierarchical activation of one pathway by the other or by coordinate regulation of common target genes. Functional integration of TGF-β/Smad and Jagged1/Notch is involved in skin cancer progression, as both TGF-β and Notch may exert overlapping oncogenic activities characterized by EMT (Cui et al., 1996; Weijzen et al., 2002). TGF-β induced EMT in keratinocytes showed activation of Notch pathway downstream of TGF-β with early upregulation of Notch ligand Jagged1 and Notch target genes *HES1* and *TLE3* as well as basic-helix–loop–helix (bHLH) in a panel of epithelial cells from mammary gland, kidney tubules and epidermis. On the contrary, TGF-β-induced EMT is prevented by silencing of *HEY1* or *Jagged1* and by chemical inactivation of Notch. Overall, these findings suggest functional integration of TGF-β/Smad and Jagged1/Notch signaling in EMT (Zavadil et al., 2001, 2004).

2.20.3 Wnt/GSK3/ β -catenin

β-catenin represents an essential component of adherens junctions. It associates with E-cadherin and mediates its contact with actin cytoskeleton. It also acts as a transducer and transcriptional coactivator of Wnt signaling. Transcriptional activity of β-catenin was found to be concomitantly associated with loss of E-cadherin and activation of autocrine TGF-β in maintenance of the mesenchymal phenotype (Eger et al., 2004). Glycerol synthase kinase (GSK3) represents an essential component of the inhibitory complex that targets β-catenin for degradation in the absence of Wnt signaling. It also shows its involvement in EMT by inhibiting the activity of zinc-finger transcription factor Snail, a key repressor of E-cadherin expression. Overall, GSK3 shows its role in maintenance of the integrity of adherens junctions and of epithelial phenotypes (Zhou et al., 2004; Bachelder et al., 2005).

2.20.4 PI3K/ NF-kB

TGF-β activates phosphatidylinositol-3-OH kinase (PI3K) in a RhoA-dependent manner during EMT in mammary epithelial cells. PI3K-Akt signaling is required for deregulation of ZO-1 and disassembly of cell-cell junctions and thus migration of breast cancer cells (Bakin et al., 2000). In a model of multistep carcinogenesis, TGF-β induces EMT in cooperation with active oncogenic Ras. On the contrary, PI3K protects cells from TGF-β activated apoptosis (Janda et al., 2002). Also, PI3K is not required for

c-Raf1 activated and Ras-induced EMT (Lan et al., 2004) thus, suggesting its limited role as an effector of TGF-β induced EMT. Cooperation of TGF-β, Ras and NF-kB is critically involved in epithelial plasticity induced by EMT. NF-kB shows key modulation of TGF-β-induced EMT in Ras overexpressing mammary epithelial cells (Huber et al., 2004). In these cells inhibition of NF-kB shows prevention of EMT. Ectopic activation of NF-kB induces mesenchymal phenotype independently of TGF-β whereas, inhibition of NF-kB in mesenchymal cells shows restored phenotype of the epithelial cells.

2.21 Interdisciplinary approach to insight into EMT

EMT is a dynamic process executed by many overlapping regulatory pathways that influence various intra- and intercellular events, an interdisciplinary approach may provide insight into this complex regulation. Recently, biophysical approaches together with high-throughput screening have shed light on the EMT related modulation of junctional complex integrity. Signaling pathways that control EMT essentially converge on to the regulation of E-cadherin, the prototypic epithelial adhesion molecule in adherens junctions.

In a recent high-throughput screening of protein–protein interactions, TGFβRI interacting proteins that regulate the assembly of tight junctions in the process of EMT have revealed occludin, a structural component of tight junctions found to be interacting with TGFβRI (Barrios-Rodiles et al., 2005; Ozdamar et al., 2005). Mutation in the binding site showed that the TGFβRI–occludin interaction is critical for TGF-β mediated tight junction dissolution during EMT. Furthermore, PAK1 (Yang et al., 2005) was also found to physically associate with TGFβRI, although, the significance of this interaction is yet to be assessed. PAR6, which functions as a scaffold for the assembly of polarity regulating proteins such as Rho, aPKC and PAR3 was also found to interact with TGFβRI and thus, orchestrates the assembly of tight junctions (Ozdamar et al., 2005). PAR6 is also a component of TGFβRI–occludin complex and is phosphorylated by TGFβRII. It is also involved in regulation of local RhoA degradation along with SMURF1, an E3 ubiquitin ligase and found to be necessary for tight-junction dissolution (Ozdamar et al., 2005; Wang et al., 2003). Ser mutation at TGFβRII mediated phosphorylation site on *PAR6* cause abrogation of tight-junction dissolution suggesting PAR6 phosphorylation in response to TGF-β to be one of the important events regulated by TGF-β during EMT.

2.22 Future perspectives

The prime objective of EMT during advanced tumor progression and metastasis seems to be productive expansion of tumor cells through invasiveness and intravasation to the neighboring vasculature (Christiansen et al., 2006). Recent advances in imaging technology and availability of transgenic mouse models would promise the possibilities to study transitory mesenchymal cells in vivo in tumor-related EMT derived from carcinomas (Wang et al., 2005).

High-throughput studies (Barrios-Rodiles et al., 2005; Ozdamar et al., 2005), have raised the hope to unravel the multiple facets of EMT by identifying the potential candidate genes involved in this interplay. In addition, the extensive screening of RNA interference (RNAi) libraries in the fruit fly and in zebra fish would provide insight into interaction networks of signaling pathways that regulate EMT. In vitro studies due to their simplified environment would allow the relatively easy identification of pathways involved in the EMT. However, in contexts with EMT in vitro studies are limited, because very few immortalized epithelial cell lines show ability to undergo EMT. Therefore, validations of in vitro observations need to be tested in an experimental animal model or a better in vitro model. EMT does not show confinement to the time and space therefore the kinetics of EMT varies considerably and spans over hours to a week. Differential gene expression profile during EMT is likely to mask information of expression of some of the key molecules that are commonly involved in other cellular programs. Better in vitro models can exclusively attribute the molecules to induce EMT without sharing other cellular processes. The use of reporter genes under the control of EMT regulated promoters may serve the purpose to know the critical steps in the execution of the EMT program.

In cancer patient, blocking of TGF-β signaling to inhibit EMT may provide two positive effects such as a restriction on metastatic spread and an enhanced uptake of administered drugs delivered to the tumour cells simultaneously (Salnikov et al., 2005). Intra-tumoral drug delivery against TGF-β signaling may also provide inhibition of the vascular system, particularly restricting the cellular differentiation of tumour vessel walls along with their association with pericytes and smooth muscle cells (Kano et al., 2007). Recently, nanoparticle encapsulated cancer drugs along with administration of TGF-β inhibitors have shown markedly enhanced uptake and efficacy in mouse tumour allograft model.

The process of EMT involves or withstands functioning of various signaling factors which have been explored in greater depth and unanimously suggested the necessity of TGF-β signaling and crosstalk with other signaling pathways such as Wnt, Notch and receptor tyrosine kinase mediated signaling to generate the specificities required for EMT in various morphogenetic steps. Future studies are needed to reveal how these complex networks interact to coordinate and specify individual steps of the EMT program (Yang & Weinberg, 2008). Further, a future challenge would be to understand the multiple signaling pathways that establish EMT in order to design the regime of drugs that affect TGF-β, Notch, Wnt and RTK signaling in anticancer therapy. Understanding of EMT would promise new drugs against cancer cell invasiveness and metastasis, as well as against tissue fibrosis.

Materials & Methods

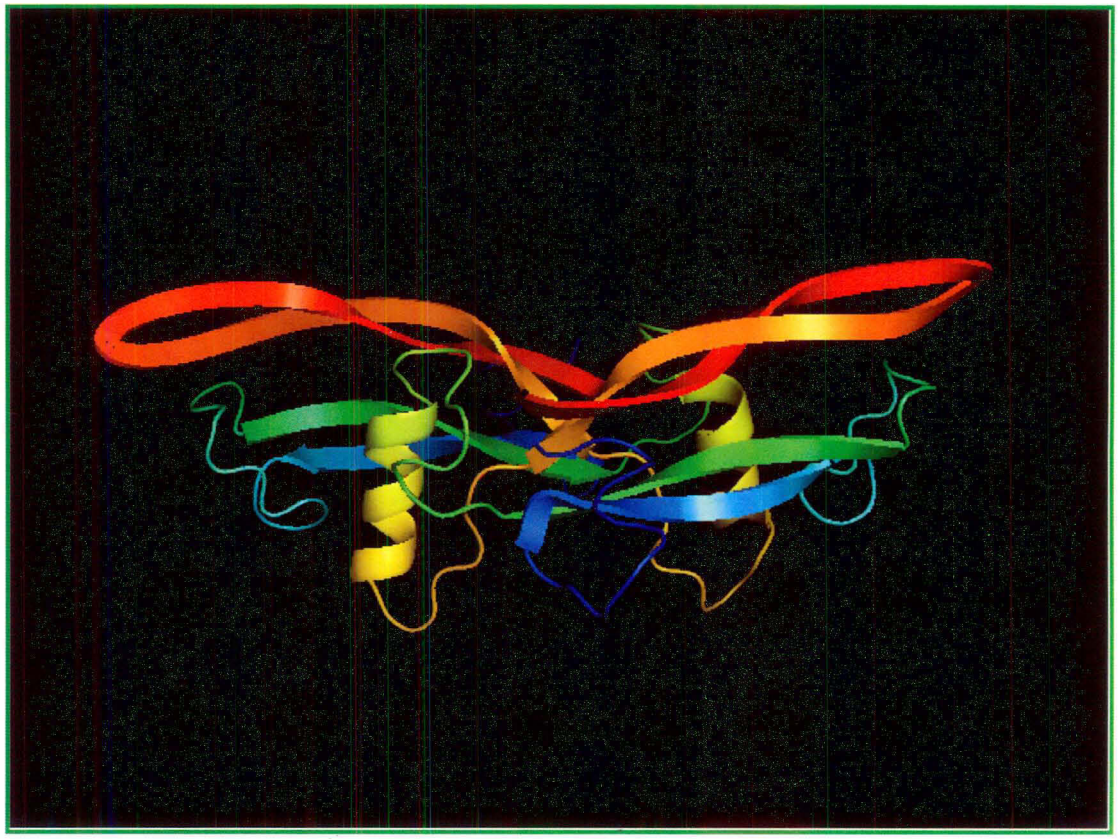


Plate VII: 3-Dimentional ribbon structure of TGF-β1 dimer protein.
The molecule has been used in present study on EMT in various experiments. The picture has been adopted from studies on TGF-β1 solution structure from Hinck et al., 1996.

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals/Consumables

Analytical and guaranteed reagent grades of the following chemicals were obtained from the mentioned sources for the preparations of various compositions of reagents and buffers.

Acrylamide (Sigma)

Acetic acid glacial (Merck-GR, Qualigens-AR), concentration 99.7%. Stored at room temperature.

Acetone (Qualigens)

Agarose (Pronadisa, Promega)

Ammonium persulphate (Sigma, Qualigens)

Antibodies: Mouse Anti-vimentin (MAB1633), Mouse Antikeratin-18 (MAB1600), Chemicon International, 100µl each, Goat antimouse IgG HRP conjugated (Genei, Lot-61035), stored at -20°C. Goat antimouse IgG FITC conjugated (Genei, Lot-31115), 1ml each, stored at 4°C.

bis-Acrylamide (Sigma)

β-Mercaptoethanol (Sigma, M Merck-GR), stored at 4°C.

Boric acid (Qualigens)

Bromophenol blue (Sigma)

Calcium chloride dihydrate (Sigma)

Cell lines: MCF-7, MDA-MB-231, NCCS, Pune.

Chloroform (Qualigens, AR grade from E.Merck, Germany, Cat. No. 2445)

DAPI (Sigma) stored at -20°C.

Diethyl pyrocarbonate (DEPC) (Sigma, D-5758), stored at 4°C.

DNA molecular weight markers: 100 bp ladder (NEB), stored at -20°C.

Dulbecco's Modified Eagle Medium (DMEM) (Sigma, D-5523), stored at 4°C.

Dimethyl sulphoxide (DMSO) (Sigma, D-2650), stored at room temperature.

Ethanol (Merck-GR 1.00983.0511, Bengal Chemicals and Pharmaceuticals, Ltd)

Ethylene diaminetetra acetate (EDTA) (Qualigens)

FCS (Gibco-BRL), stored at -20°C.

Formaldehyde solution (37%) (Qualigens-ExcelaR), stored at room temperature. Formamide (Sigma, F-7508, Merck 1.12027.0100), deionised by DOWEX MR-3 mixed bed resin (Sigma, ml-005), filtered through Whatman No.1 and stored at -20°C.

Geimsa Stain (Qualigens stains)

Glycerol (Sigma, G-5150, Qualigens AR)

Haematoxylin (Sigma)

Hydrogen Peroxide (Merck, 17544), 30% purified solution. Made fresh dilutions in H_2O and discarded after use.

Iodoacetamide (Amersham-GE Healthcare), 250mg of iodoacetamide was dissolved in freshly prepared equilibration buffer.

Liquid Nitrogen: Central instruments facility (CIF), School of Life Sciences, JNU, New Delhi.

Manganese chloride (Qualigens-AR)

Methanol (Qualigens-ExcelaR)

Membranes: Millipore GS WP-02400- Pore size 0.22 micron filters for sterilization (Millipore Corporation, USA). Stored in a clean dry place at room temperature. Trans blot transfer medium (BIO-RAD, Cat.162-0115). Pure Nitrocellulose Membrane 0.45 μm. Stored in a clean dry place.

Nucleotides (Labeled): [γ^{32}P] ATP: BRIT, LCP 101, 1 mili Curie, 5000/3000 Ci/mmole, 10 mCi/ml. Stored at -20°C protected.

Penicillin G: sodium salt of benzylpenicillin (Sigma, P-3032), added to a final concentration of 100 U/ml.

Phenol (Ranbaxy-AR): Double-distilled, added hydroxyquinoline to a final concentration of 1%, equilibrated with 0.5M Tris.Cl (pH 8.0) and stored in 200 ml aliquots at -20°C. Working solution was stored at 4°C.

Protein Ladder: Page ruler Protein ladder (Fermentas, SM0661). Prestained Protein ladder (Fermentas, SM0671) 250μl each. Stored at -20°C.

Reverse Transcription Premix kit (Maxime RT Pre Mix Kit, 25082) stored at -20°C.

Silver Nitrate (Qualigens-ExcelaR)

Sodium Carbonate anhydrous salt (Qualigens AR)

Sodium Chloride (Qualigens-AR)

Sodium hydrogen carbonate (E.Merck, India, Cat.17520), stored in a cool, dark place.

Sodium thiosulphate (Qualigens-SQ)

Streptomycin (Sigma, S-9137), added to the final concentration of 100 mg/ml.

Taq DNA polymerase recombinant (Gibco-BRL), 5U/μl, stored at –20°C. 10X buffer: 200mM Tris.Cl (pH 8.4), 500mM KCl. Stored at -20°C.

T4 Polynucleotide kinase Recombinant (NEB, Lot 88), 500 Units.

TEMED: N, N, N', N', Tetra methyl ethylene diamine (Sigma, T-7024), stored at 4°C.

TGF-β1 (Human) recombinant protein (R&D System, 240B), 1μg/ml, stored at -20°C.

Triton X-100 (Sigma, T-8787)

Trypan Blue Stain (Sigma, T-6146), made in 1X PBS at a final concentration of 0.4%, stored at room temperature.

Tween-20 (Promega)

Water: Milli Q Water (Millipore deionizer) or quartz double-distilled, autoclaved H_2O.

Whatman Paper: Whatman 3 MM Paper (3030917), stored in a clean dry place.

Xylene cyanol (Sigma)

Zinc Sulphate ($ZnSO_4$) (Qualigens-AR)

3.1.2 Probes used for EMSA

Double stranded 29mer oligonucleotide probes were taken from selected region of the natural promoters of TGF-β1, Snail and E-Cadherin gene. E-cadherin probe has been taken from literature as a reference probe (Battle et al., 2000). Normal sequence probe and mutant sequence probe was designed and used in EMSA study.

	Name	Sequence	Resource
1	TGF-β1 Probe Sequence	5'CCCTTCCATCCCT**CAGGTG**TCC TGT TGCC 3' 3'GGGAAGGTAGGGA**GTCCAC**AG GACAACGG 5'	Microsynth, Switzerland
2	Snail Probe Sequence	5'GCAGCCGGCG**CACCTG**CTCGG GGAGTGGC 3' 3'CGTCGGCCGC**GTGGAC**GAGCC CCTCACCG 5'	Microsynth, Switzerland
3	E-Cadherin Probe sequence	5'GGCTGAGGGTT**CACCTG**CCGG CCACAGCC 3' 3'CCGACTCCCAA**GTGGAC**GGCC GGTGTCGG 5'	Microsynth, Switzerland

4	Normal Sequence (4-Times repeat)	5'**CACCTGATCACCTGAT CACC TGATCACCTG** 3' 3'**GTGGACTAGTGGACTAGTGG ACTAGTGGAC** 5'	Microsynth, Switzerland
5	Mutant Sequence (4-Times repeat)	5'**AACCTAATAACCTAATAACCT AATAACCTA** 3' 3'**TTGGATTATTGGATTATTGGA TTATTGGAT** 5'	Microsynth, Switzerland

3.1.3 Primers used for RT-PCR

The 20mer oligonucleotide primers used for RT-PCR were taken from CDS sequences of E-cadherin, TGF-β1and Cytokeratin 18 genes.

	Name	Sequence	Resource
1	E-Cadherin	Left- 5' TGCCCAGAAAATGAAAAAGG 3' Right- 5'GTGTATGTGGCAATGCGTTC 3'	Microsynth, Switzerland
2	TGF-β1	Left- 5'GGGACTATCCACCTGCAAGA 3' Right- 5'CCTCCTTGGCGTAGTAGTCG 3'	Microsynth, Switzerland
3	Cytokeratin18	Left- 5'CACAGTCTGCTGAGGTTGGA 3' Right- 5'GAGCTGCTCCATCTGTAGGG 3'	Microsynth, Switzerland

3.1.4 Reagents/Buffer recipes

Following reagents and buffers were prepared and used for various experiments in present study.

Acrylamide 30%

29g Acrylamide (Sigma, A-9909) + 1g N, N-methelyene-bis-acrylamide (Sigma, M-7256) dissolved in 60 ml H_2O, warmed on the magnetic stirrer to dissolve and final volume made up to 100 ml; acrylamide solution was stirred for an hour, filtered through Whatman 1MM filter paper and

	stored at 4°C in dark brown bottles.
Agarose gel	Type V High melt (Sigma, A-3768) or Type VII Low melt (Sigma, A-40180) or Agarose, LMP (Promega, V283A). Typically 0.8-2.5% agarose gels in 1X TAE were used with a final concentration of 0.5 μg/ml Ethidium bromide.
APS 10%	0.10g Ammonium per sulphate (Sigma, A-9164) was dissolved in 1ml H_2O to get a 10% solution. Prepared fresh just before use.
Benzamidine 250 mg/ml	Hydrochloride: Hydrate (Sigma, B-6506) Peptidase inhibitor. Prepared in sterile distilled H_2O and stored at -20°C. Used at a final concentration of 0.5 mg/ml.
Binding Buffer (10X) (EMSA)	20mM HEPES (pH 7.6),150mM KCl, 3mM $MgCl_2$, 0.2mM $ZnSO_4$, 0.3mg BSA, 10% glycerol, 1μg Salmon Sperm DNA added freshly before use.
Bradford's Reagent	Coomassie Brilliant Blue G-250 (Merck-GR, Ger.) 10mg dissolved in 5 ml Ethanol + 10 ml conc. H_3PO_4, made a volume to 100 ml with distilled H_2O.
BSA 10mg/ml	Dissolved 100 mg BSA (Fraction V, Sigma, A9647) in H_2O and stored as 10 ml aliquots at -20°C.
Carrier DNA	Salmon sperm DNA, sodium salt (Sigma, D-1626) 10mg/ml in H_2O, added NaCl to a final concentration of 0.1M, sonicated and ethanol precipitated. Dissolved the sheared DNA in H_2O to a final concentration of 10mg/ml. The aliquots were stored at -20 °C.
Coomassie Brilliant Blue R-250 dye	Dissolved 0.25gm Coomassie Brilliant Blue-R250 (SRL) in MeOH:H_2O:AcOH::45:45:10. Filtered

	through Whatman No.1 filter to remove any particulate matter.
DAB System	3-3'-Diaminobenzidinetetrahydrochloride (Genei, 51125) was used as a substrate to develop a colored precipitate. Stored at 4°C.Working concentration: DAB-6mg, Dilution Buffer-10ml, hydrogen peroxide-6µl.
DAPI solution	1mg of DAPI was dissolved in 1ml of PBS and aliquots were wrapped in aluminum foil and stored at -20°C.
DNA loading dye (6X) (EMSA)	25 mg Xylene cyanol, 25mg bromophenol blue, 7ml H_2O, 3ml sterile glycerol. Mixed well and stored at 4°C in 1 ml aliquots.
DTT 1M	D-L-Dithiothreitol (Sigma, D-9779) dissolved in deionised H_2O and kept frozen as aliquots (100 µl) at -20 °C.
EDTA 0.5M (pH 8.0)	Added 93.05g of di-sodium ethylenediaminetetra-acetate.$2H_2O$ (Qualigens-ExcelaR, Sigma, E-5134) to distilled H_2O and adjusted pH to 8.0 with 5M NaOH, the final volume made up to 500ml with distilled H_2O, autoclaved and stored at room temperature.
EGTA 0.5M (pH 7.0)	Ethylene glycol-bis-[B-amino ethyl ether]-N, N, N', N', tetraacetic acid (Sigma, E-3889). Added 1.9092 g EGTA to 35 ml H_2O and adjusted the pH to 7.0 by adding 10M NaOH dropwise while stirring. Made up the volume to 50 ml with H_2O, sterilised by autoclaving and stored at room temperature.

EMSA Buffer (5X)	Dissolved 151.425g Trizma (Sigma, T-1503) and 750g Glycine (Merck-GR) in 4.5 litre H_2O. Added 100ml 0.5M EDTA (pH 8.0) and made up the volume to 5 litre. Checked the pH -should be 8.5. Sterilized by autoclaving. Stored at room temperature.
Equilibration Buffer (2-D Electrophoresis)	6M Urea, 75mM Tris.Cl (pH8.8), 30% v/v Glycerol, 2% w/v SDS, 0.002% Bromophenol blue were added and dissolved in 100ml H_2O. DTT (100mg) and Iodoacetamide (250 mg) were added freshly and separately before use.
Ethidium bromide 10mg/ml	Dissolved 100mg ethidium bromide (Sigma, E-8751) in 10ml sterile H_2O, stored as 1ml aliquots at 4°C in dark coloured Eppendorf tubes. Working concentration: 0.5 μg/ml.
Fixation solution	7.5% v/v Acetic acid, 10% v/v Methanol. For 100ml solution, 7.5ml of Acetic acid, 10ml of Methanol was added and volume was made with H_2O. Prepared freshly before use.
Formaldehyde Stock Solution (2%)	2g formaldehyde was added to 100ml 1X PBS solution and heated at 70°C in a fume hood until the formaldehyde dissolved in solution. Allowed to cool at room temperature and pH adjusted to 7.4 using 0.1M NaOH/0.1M HCl. The solution was filtered and stored at 4°C.
HEPES 1M	(Sigma, H-1016), dissolved 23.83g HEPES in 75 ml H_2O and adjusted the pH to 7.9 with NaOH, made up the volume to 100 ml and stored at room temperature.

HEPES Buffer (2X)	Dissolved 1.19g HEPES, 1.64 g NaCl in 80 ml H_2O, added 1 ml 100X Phosphate Buffer and adjusted the pH to 7.05 with 1N NaOH. Made the volume to 100 ml and rechecked the pH. Sterile filtered and made aliquots of 5 ml. Stored at room temperature.
KCl 1M	Potassium chloride (Qualigens-AR). Dissolved 7.45 g KCl in 100 ml H_2O and autoclaved. Stored at room temperature.
Loading Buffer (6X) for DNA	0.25% Bromophenol blue (Sigma, B-7021), 0.25% Xylene cyanol FF (Sigma, X-4126) in 30% glycerol, stored at 4°C as 1 ml aliquots.
Lysis Buffer (whole cell lysate)	1X PBS with 1% Triton X-100. Just prior to use added the protease inhibitors: 1mM DTT, 0.5mM PMSF, 1 µg/ml Leupeptin, 1 µg/ml Aprotinin, 0.5 mg/ml Benzamidine, 1 mM Sodium ortho-vanadate.
Lysis Buffer (EMSA)	10mM HEPES (pH 7.9), 10mM KCl, 0.1mM EDTA (pH 8.0), 0.1mM EGTA (pH 7.0). Stored at –20°C. Protease inhibitors added just prior to use to a final concentration of 1mM DTT, 1mM PMSF, 2 µg/ml aprotinin, 2 µg/ml leupeptin, 0.5 mg/ml benzamidine.
$MgCl_2$ 1M	Magnesium chloride, hexahydrate (Qualigens-ExcelaR). Dissolved 20.33g of $MgCl_2.6H_2O$ in 80 ml of H_2O and the volume adjusted to 100 ml. Sterilised by autoclaving.
$MgSO_4$ 1M	Dissolved 24.65g of $MgSO_4.7H_2O$ (Qualigens-ExcelaR) in 100 ml H_2O and sterile filtered through 0.22 µM filter and stored at 4°C.
Normal saline	0.9% NaCl solution in sterile H_2O.

NP-40 10%	(Sigma, I-3021. IGEPAL CA-630), Nonionic detergent (Octylphenoxy) polyethoxyethanol or Nonidet P-40. Dissolved 1 ml 100% Nonidet P-40 in 9 ml H_2O and mixed gently by inverting the tube. Stored at –20°C.
Nuclear Extraction Buffer (EMSA)	20mM HEPES (pH 7.9), 400mM NaCl, 1mM EDTA (pH 8.0), 1mM EGTA (pH 7.0). Stored at –20°C. Protease inhibitors added just prior to use to a final concentration of 1mM DTT, 0.5 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 0.5 μg/ml benzamadine.
Polyacrylamide gel electrophoresis (PAGE)	Stacking gel: 5% Polyacrylamide in Tris.Cl (pH 6.8). Resolving gel: 8 to 10% Polyacrylamide Tris.Cl (pH 8.8).
PBS (10 X)	1.3M NaCl, 20 mM KCl, 78 mM $Na_2HPO_4.2H_2O$, 14 mM KH_2PO_4, autoclaved aliquots were stored at 4°C.
PBST	1X PBS (pH 7.6) containing 0.1% Tween-20.
Phosphate Buffer (100X)	Added 2.67g $Na_2HPO_4.2H_2O$ to sterile H_2O and made up the volume to 100 ml. The final 100X solution is 150 mM. Sterile filtered and stored at room temperature.
PMSF 100 mM	Phenylmethylsulphonyl fluoride (Sigma, P-7626). Dissolved in isopropanol to get the desired concentration. Stored at -20°C.
PNK Buffer (1X)	(NEB, Cat.M02015), 70mM Tris.Cl, 10mM $MgCl_2$ 5mM DTT (pH7.6) stored at -20°C.
Rehydration Buffer (2-D Electrophoresis)	7M Urea, 2M Thiourea, 4%CHAPS, 2% IPG Buffer, 40mM DTT (added freshly before use). Aliquots were made and kept in -80°C.

Running Buffer 5X (EMSA)	54g Tris base, 27.5g Boric acid dissolved in1L H_2O to make 5X buffer. Autoclaved and stored at room temperature.
Sample Buffer (2X) (SDS-PAGE)	100 mM Tris.Cl pH 6.8, 4% (w/v) SDS, 0.2% (w/v) bromophenol blue, 20% glycerol, 200 mM DTT in H_2O. DTT is added just prior to use. Stored at room temperature.
SDS 10%	Lauryl sulfate-sodium salt (Sigma, L-4390) 10% (w/v) SDS dissolved in 100ml distilled H_2O. Stored at room temperature.
Sephadex G-50	Sephadex G-50 (Sigma, G-50-80), 10g soaked and washed several times in excess H_2O to remove all traces of dextran, re-suspended in 100 ml 1X TNE, autoclaved and stored at 4°C.
Sodium acetate 3M	Dissolved 24.6g sodium acetate.$3H_2O$ (Qualigens-ExcelaR) in 80 ml H_2O and the pH adjusted to 5.2 with glacial acetic acid, volume made up to 100 ml. Aliquots were autoclaved and stored at room temperature. For use with RNA, the reagent was prepared in DEPC treated water.
Sodium chloride 5M	Dissolved 29.2g Sodium chloride (Qualigens-ExcelaR) in 100ml H_2O, autoclaved, stored at room temperature.
TAE Buffer (50X)	242g Tris base (Qualigens-ExcelaR), mixed with 57.1 ml glacial acetic acid, 100 ml 0.5M EDTA (pH 8.0) and the volume made up to 1L with H_2O, autoclaved and stored at room temperature.

TBE Buffer (5X)	54g Tris base, 27.5g boric acid (Qualigens-Excela R) and 20 ml 0.5M EDTA (pH 8.0), added and dissolved in 1L H_2O to make 5X buffer. Autoclaved and stored at room temperature.
TE buffer	10mM Tris.Cl pH 7.5 or 8.0 and 1mM EDTA dissolved in sterile distilled H_2O, stored at room temperature.
TNE (10X)	0.5M Tris.Cl (pH 7.5), 1M NaCl and 0.05M EDTA in H_20, pH was 7.4 -7.5 in a 1/10 dilution, autoclaved and stored at room temperature.
Transfer Buffer (Western)	For every 1L of the buffer, 200 ml 5X Tris-glycine Buffer (Western) and 200 ml methanol were adjusted to 1L with sterile distilled H_2O. Final concentration: 25 mM Tris base, 0.2 M glycine, 20% Methanol (pH 8.5).
Tris.Cl 1M pH 7.5	(Qualigens-ExcelaR, Sigma, T-1503). Tris or Trizma base 121.2g dissolved in H_2O, pH adjusted to 7.5 or 8.0 with concentrated HCl, autoclaved and stored at room temperature.
Tris-Glycine (5X) (Western)	15.1g Tris base, 75.07g glycine was dissolved in sterile distilled H_2O and adusted to the 1L of the final volume.
Tris-glycine SDS Buffer (5X) (SDS-PAGE)	15.1g. Tris base (Qualigens-ExcelaR), 94g Glycine (Merck-GR), 50ml 10% SDS (Qualigens-ExcelaR), dissolved in sterile distilled H_2O and the final volume was made up to 1L.

3.2 Methods

3.2.1 Cell culture and maintenance of the cells

Human breast carcinoma cell lines MCF-7 and MDA-MB-231 were obtained from NCCS, Pune, India. The cells were cultured in DMEM (Gibco-BRL) supplemented with 10% Fetal Calf Serum (Gibco-BRL), 100U/ml penicillin and 100µg/ml streptomycin (Sigma) at 37°C under 5% CO_2. When grown up to 80% of confluency in T_{25} flasks, sub culture was made in 1:3 ratio. Saline was used to wash the cells twice before trypsinization with gentle rinsing. To this washed flasks, 100µl of trypsin-EDTA solution (Stock 100X Gibco-BRL) was added along with 0.9ml of saline and kept for 30 seconds. The trypsin was removed and flasks were kept in hood till the appearance of rounded form and dislodging of the cells from culture flask was observed under microscope. The flask was flushed with medium and the cell suspension was transferred to fresh falsks and volume was made up with complete medium. The flasks were labeled with date and the name of cell line and kept in CO_2 incubator programmed to maintain 5% CO_2. For cell counting purpose, homogeneous suspension of cells were made and appropriate volume of cell suspension along with trypan blue was taken on to haemocytometer and cell count was made under microscope.

3.2.1.1 Cryopreservation of cells

The flasks with 70-90% of confluency of cells with healthy morphology were considered for preservation. Cells were trypsinized and homogeneous cell disaggregation was made with suspension in 2ml complete medium. The cell suspension was centrifuged at 1000 rpm under aseptic conditions for 5 minutes. The cells were harvested and transferred to the sterilized cryo-vials along with 90% FCS and 10% DMSO as freezing medium. The vials with 1ml aliquot were wrapped in cotton and kept at -20°C for an hour, then kept at -80°C overnight and transferred to liquid nitrogen container for long term storage.

3.2.1.2 Revival of the cells

The stored cryo-vials were taken from liquid nitrogen container and immediately kept in water bath preset at 37°C. The thawed cell suspension were transferred to new sterile centrifuge tube and centrifuged to remove the medium and washed twice with complete medium to remove the residual DMSO, gently resuspended in small amount of complete medium with 10-20% FCS and seeded in to fresh culture flasks and kept in CO_2 incubator. After 24 hours, the medium was changed and antibiotics were added in order to avoid contamination. The normal growth of the cells was observed under microscope

through subsequent days. Flasks with normal growth with appropriate confluency were continued for subculture.

3.2.2 TGF-β1 treatment

Cells under culture were treated with TGF-β1 with the optimized concentration of 5ng/ml for immunofluorescence, western blot, flowcytometry, EMSA, 2D electrophoresis and 3ng/ml for time kinetics studies in RT-PCR.

3.2.3 Total RNA isolation

The trypsinized cells were washed twice with cold PBS and 2ml of Trizol (for 5-10 $x10^6$cells) (Gibco/Invitrogen) was added into the T_{25} flask. Cells were disrupted by continuous pipetting and kept on ice for 5 minutes. Chloroform (200µl) was added and vortexed or shaken vigorously for 15-20 seconds and kept on ice for 15 minutes. The samples were centrifuged at 12000 rpm for 15 minutes at 4°C. The aqueous phase was collected into fresh tubes. Isopropanol (0.5 to 1.0 ml) was added and mixed gently and incubated at -20°C for 1hr and then centrifuged at higher speed (12000 rpm) for 15 minutes at 4°C. The supernatant was discarded and the pellet was washed with 70% ethanol and dried at room temperature. The pellet was reconstituted with DEPC treated water and kept at 4°C for 15 minutes and taken for qualitative and quantitative analysis.

3.2.4 Reverse transcription -PCR

Total RNA was isolated from MCF-7cells using Trizol (Gibco/Invitrogen) and quantitated spectrophotometrically. Synthesis of cDNA was carried out with 0.2-1.0 µg of total RNA with random primers. The RT-PCR was performed with cDNA (2µl) and target specific sense and antisense primers. The amplification conditions used for RT-PCR were, initial denaturation at 94°C for 5minutes followed by 40 cycles of denaturation at 94°C for 1minute, annealing for various primers (Cytokeratin 18, Ecadherin and TGF-β1) at 50°C, 47°C and 57°C respectively for 1 minute, extension at 72°C for 1 minute and final extension at 72°C for 5 minutes. The PCR product was electrophoresed in 2.5% agarose gel along with 100bp DNA ladder.

3.2.5 Immunocytochemistry for adherent cells

Confluent MCF-7 cells were trypsinized. Approximately 200 µl of cell suspension was plated on a coverslip put into six-well plates. Cells were grown to approximately 80%

confluency. Culture medium was carefully aspirated and cells were rinsed with PBS. Methanol was used as a fixative and added for 1minute at room temperature. Immediately, cells were washed twice with PBS for 5minutes. Cells were blocked with 5%BSA in PBS and incubated for 1hr at room temperature in a humidity chamber. This was followed by wash with PBS for 5 minutes and incubation with primary antibody reconstituted with 1%BSA in PBS (1:500 dilution) for 2 hrs at room temperature. Washing was performed after incubation three times with PBS for 5 minutes. Cells were incubated with HRP-conjugated anti-mouse IgG with 1%BSA in PBS (1:2000 dilution) for 1hr at room temperature. Washing was performed three times for 5 minute duration each. Haematoxylin was used for nuclear staining. Coverslips were mounted on slides and examined for staining under inverted light microscope.

3.2.6 Immunofluorescence

Six-well plate of cells was taken out at room temperature, culture medium was drained off and cells were washed thrice with PBS for 5 minutes each. Chilled methanol was used to fix the cells and kept at -20°C for 10 minutes. Methanol was removed and PBS was added and kept at room temp for 5minutes. PBS was replaced by 5% BSA and the plate kept in humidity chamber for 2hrs. Blocking solution was discarded and cells were incubated with primary antibody (1:500 dilution) with 5% BSA in humidity chamber overnight at 4°C. Next day primary antibody solution was discarded and cells were washed with PBS three times for 5 minutes each. Next, secondary antibody (1:1000dilution) with 5% BSA, labeled with FITC was added along with DAPI solution and kept in humidity chamber at room temperature for 1 hr. After incubation with secondary antibody the solution was discarded and cells were washed thrice with PBS for 5 minutes each. Coverslips were mounted on glass slides and examined under fluorescence microscope.

3.2.7 Intracellular antigen staining for flowcytometry (Indirect staining)

Cells were trypsinized and disaggregated into single cell suspension by repeated pipetting and gentle vortexing. Cells were washed with PBS and pelleted. The pellet was resuspended in a 0.875ml of cold PBS and mixed gently. Then, 0.125ml of 2% formaldehyde solution was added and vortexed briefly. The suspension was incubated for 30 minutes at 4°C and centrifuged at 250g. The supernatant was removed and cells gently resuspended in 1ml of Tween-20 solution (0.2% in PBS) and incubated at 37°C in

water bath for 15 minutes. Incubation buffer (1ml) (PBS + 2% FCS + 0.1% sodium azide) was further added into tubes after incubation and spun for 20 minutes at 250g. Supernatent was removed, pellet resuspended in 50µl of HAB (Human AB serum, heat inactivated) for 1minute and 50 µl of incubation buffer and primary antibody added. Tubes were vortexed briefly and kept for incubation for 30minutes. Cells were washed twice with 1ml of 0.2% Tween-20 solution and cells centrifuged at 250g for 5 minutes. Cell pellet was resuspended initially in 50µl of HAB for 1 minute, FITC-conjugated secondary antibody added and briefly vortexed. This was incubated for 30 minutes in the dark at 4°C. After incubation, cells were washed twice and centrifuged at 250g for 5 minutes. The sample was resuspended in 1ml buffer for flowcytometric analysis.

3.2.7.1 Acquisition and analysis of samples by flowcytometry

The detection of intracellular staining was performed by flowcytometry (FACS Calibur, BD Biosciences). The samples were acquired and analyzed by using Cellquestpro software. Templates of acquisition dot plot (FScVs SSc), quadrant dot plot (FL-1-H Vs FL-2-H) and histogram plot (Count Vs FL-1-H) were made for acquisition. The samples were run at slow flow rate mode as a single cell to generate enough signals when exposed to laser beam. Initially the control samples were run to acquire homogeneous cell population in acquisition dot plot by means of adjusting the voltage parameter for FSc and SSc. Similarly, the voltage was adjusted for FL-1-H and FL-2-H to localize the unstained population at lower left region of quadrant plot. The same settings were used for query samples. Acquisition of control samples (autofluorescence as well as non specific staining) and of query samples was performed and during analysis, acquisition templates were changed into analysis template using gate R1 in analysis of dot plot FSc Vs SSc, quadrant plot and histogram plot as G1=R1.

3.2.8 Cytosolic and nuclear protein extraction

Cells were collected after trypsinization and centrifuged at 1000 rpm for 5 minutes. Lysis buffer (100-200µl) along with protease inhibitors was added and kept on ice for 10 minutes. After lysis buffer treatment, cells were centrifuged at 12000 rpm for 10 minutes at 4°C. The supernatant of cytosolic protein was collected in fresh tube. The pellet of nuclei was further dissolved with 50-100µl ice cold nuclear extraction buffer along with protease inhibitors and incubated for 30 minutes at 4°C. After incubation the lysate was centrifuged at 12000 rpm for 10 minutes at 4°C. The supernatant was transferred in fresh tube and both cytosolic and nuclear protein estimation was done by Bradford method.

3.2.9 SDS-PAGE

SDS-PAGE gels were prepared as per Laemli's method. 30% stock solution of degassed acrylamide was mixed with 1.5 M Tris.Cl pH 8.8, water and 10% SDS. The solution was swirled gently to avoid the formation of bubbles and freshly prepared 10% APS and TEMED were added. All ingredients were added as per the requirements of a 10% acrylamide gel. The solution was poured into sealed glass plates. A layer of isopropanol was poured on the resolving gel and the gel allowed to polymerize at room temperature for one hour. The isopropanol layer was removed completely and the stacking gel which should be at least 1 cm below the bottom of the comb was poured. The stacking gel was allowed to polymerize for an hour. The wells were washed carefully after removing the comb to remove any traces of unpolymerized acrylamide. The gel was placed in the vertical gel apparatus, ensuring that there were no air-bubbles trapped between the agarose and the buffer at the bottom of the gel. The protein sample (25 µl) was loaded into separate wells and electrophoresed in 1X Tris-glycine SDS buffer at 80Volts for 2 hrs.

3.2.9.1 Fixing and staining of SDS-PAGE gel using Coomassie Brilliant Blue R-250

Polypeptides resolved in SDS-PAGE gel was fixed with 50% (v/v) methanol, 10% (v/v) acetic acid and stained with Coomassie Brilliant Blue R-250. The gel was immersed overnight in 5 times its vol. of Coomassie Brilliant Blue R-250 with slow agitation. Background of the gel was destained by soaking and shaking it in 50% (v/v) methanol, 10% (v/v) acetic acid, changing the solution till the background became clear. The next day the gel was stored in 20% glycerol.

3.2.10 Western Blotting

3.2.10.1 Preparation of SDS-PAGE stacking and resolving gel

Typically 8-10% polyacrylamide was used as resolving gel with stacking gel of 4% polyacrylamide was made. Electrophoresis was performed using Tris-glycine buffer.

3.2.10.2 Loading of sample and electrophoresis

The protein sample was mixed with equal volume of 2X sample buffer, denatured in boiling water bath for 10 minutes, cooled at room temperature and loaded in the gel along with prestained marker in a separate well. Electrophoresis was carried out at 80 Volts till the dye front reached the end of stacking gel and the further electrophoresis was carried out at 120 Volts at room temperature.

3.2.10.3 Transfer of protein from gel to membrane

After electrophoresis the gel was washed with distilled water and then immersed in transfer buffer for 30 minutes. Similarly, the blotting tissue pad and PVDF/Nitrocellulose membrane was soaked in transfer buffer for 30minutes.The membrane was placed on gel in between two tissue pads and kept in Bio-Rad semidry transfer unit and the transfer was made at 15 Volts for 1hr.

3.2.10.4 Immunoblotting

The PVDF/Nitrocellulose membrane was immersed in blocking solution PBST+3%BSA for 2-3 hours at room temperature with constant shaking. The membrane was washed three times with PBST for 5-10 minutes and incubated with primary antibody (1:1000 dilution) for 1hr on a rocker at room temperature. The membrane was washed three times with PBST for 5-10 minutes each and immediately incubated with horseradish peroxidase coupled secondary antibody at optimized dilution (1:3000) for 1hr at room temperature. The membrane was washed four times, 5-10 minutes each with PBST and developed with DAB as a substrate for a colored precipitate.

3.2.11 Electrophoretic mobility shift assay (EMSA)

3.2.11.1 Labeling of probes at 5'-end

The double-stranded oligonucleotides used as probes in EMSA experiments.

Labeling of the probes at 5'-end was made with following reaction:

Chemicals	**Volume (µl)**	**Final concentration**
Probe (1 pmole/ µl)	3.0	3 pmoles
Buffer (10X)	2.0	1X
T4 polynucleotide Kinase (NEB)	1.0	10U
γ^{32}P-ATP (BRIT) 10 µCi/µl, 5000Ci/mmole	2.0	2 mCi/ml
Sterile Distilled H_20	12.0	-
Total	**20.0µl**	

The reaction was incubated at 37°C for 30 minutes in a water bath. The reaction was stopped by adding 2µl of 0.5M EDTA.

3.2.11.2 Sephadex-G_{50} column purification of the labeled oligo

Sterile syringe of 1ml was used to prepare a column, the nozzle of syringe was plugged with sterile glass wool and packed with Sephadex-G_{50}. The column was washed 5 times with 200µl of double distilled sterile water by centrifuging in a clinical centrifuge at 2000 rpm for 2 minutes and the volume of eluate was adjusted to 200 µl. To the column the 100 µl annealing reaction was added and the eluate (~100 µl) was collected in an eppendorf tube. The eluate (1µl) was used to take Cerenkov counts to estimate the labeling efficiency. The specific activity of the oligo was typically $1X10^6$ cpm/pmole.

3.2.11.3 Annealing of the complimentary strand

Complimentary strand (100 pmole/µl) was added to the labeled probe. The mixture was brief spun and heated at 85°C in water bath for 10 minutes and kept for cooling overnight at room temperature.

3.2.11.4 Casting the acrylamide gel

A 7.0 to 7.5% polyacrylamide gel was cast prior to setting up the binding reaction. The gel was prepared by mixing the following solutions:

Stock solutions	**Volume (ml)**
H_2O	27.06
5X EMSA Buffer	10.0
30% acrylamide solution	12.5
10% APS	0.40
TEMED	0.04
Total volume	**50.0ml**

The gel was allowed to polymerize for 45 minutes at room temperature. After polymerization, slot former was removed, wells were thoroughly rinsed with buffer and the gel was fixed with the buffer tank. EMSA buffer was added and the wells were flushed clean with the buffer using a 5 ml hypodermic needle and syringe. A pre-run was carried out at 120V at room temperature for at least 30 minutes immediately after starting the binding reaction.

3.2.11.5 Binding reaction

Following components were mixed on ice in a microfuge tube for the binding reaction:

Stock solution	**Volume**
^{32}P oligonucleotides [2ng]	-
10X binding buffer	2 µl
Salmon sperm DNA (1 µg/µl)	2 µl
10% NP-40	2 µl
H_20	To make up the volume to 20µl
Nuclear Extract (0.5-1.0 µg/µl)	-
Total	**20 µl**

The reaction mix was given a pulse spin at 4°C and incubated at 4°C for 30 minutes on ice. After incubation the DNA loading dye was added to each sample. Pre-run was stopped and the samples were loaded. The gel was electrophoresed at 120 Volts till the bromophenol blue migrates 1-2 cm from the bottom of the gel. The glass plates were separated by applying a twisting pressure with a thin wedged shaped article between them. The orientation of the gel was marked and the gel transferred to a piece of 3MM filter paper. The gel was fixed with 1% acetic acid, covered with saran wrap and kept on the gel dryer for drying at 80°C for 1 hour under vaccum suction. The dry gel was exposed on a Fujifilm Phosphorimager screen and scanned by using the program 'Image reader' in a Fujifilm, FLA-5000 Phosphorimager.

3.2.12 Two dimensional gel electrophoresis (2DE)

3.2.12.1 Protein extraction and rehydration

For 2DE, protein extraction was done in lysis buffer containing low salt concentration (10mM KCl). Protein concentration with extract was estimated and required amount (45-60µg for 7cm IEF strip and 200µg for 18cm IEF strip) was mixed with absolute acetone and kept at -20°C for 1hr. The mixture was centrifuged at 12000rpm at 4°C for 10minutes. The pellet obtained was further washed thrice with 80% acetone and centrifuged at 12000rpm at 4°C for 10minutes after every washing. The pellet was air dried and dissolved in rehydration buffer and kept at room temperature for complete dissolution.

3.2.12.2 Isoelectrofocussing (IEF)

The rehydrated protein sample was loaded on IEF strips and kept 12-22 hrs at room temperature. The swollen or rehydrated strips were transferred to IEF apparatus in manifold with the orientation of + end of strip towards anode of the apparatus. The IEF was performed at 50 Ampere per strip at 20°C using following programs.

For 7cm linear strip (3-10)

Steps	Volt	Time(h:min)
S1-Step1	300V	0.30hr
S2-Gradient	1000V	0.30hr
S3-Gradient	5000V	1.20hr
Step4	5000V	0.30hr

For 18cm linear strip (3-10)

Steps	Volt	Time(h:min)
1-Step&hold	500V	1.00hr
2-Gradient	1000V	3.00hr
3a-Gradient	8000V	3.00hr
4a-Step&hold	8000V	3.00hr
3b-Gradient	10000V	3.00hr
4b-Step&hold	10000V	1.00hr

After the completion of IEF, strips were taken from the apparatus and stored at -80°C. Next day the strips were taken from -80°C and kept at room temperature. A freshly prepared equilibration buffer was used to equilibrate the strips.

3.2.12.3 SDS-PAGE electrophoresis

Before equilibrating the IEF-strips, 12% SDS-Polyacrylamide gel was cast and allowed to polymerize for 2hrs. The IEF-strips were moistened with running buffer and inserted into the plates horizontally residing onto a gel. Protein marker was loaded on whatman strip and inserted separately into the plate beside the IEF-strip. The air gaps between gel and strips were removed and the strips were sealed with 0.2% agarose containing bromophenol blue dye. For 7cm IEF-strip the gel was run at 15mA per gel for 30minutes and 30mA per gel for rest of the run. For 18 cm IEF-strip the gel was run at 5mA per gel

for 30 minutes and 15mA per gel for rest of the run. After completion of the run gels were taken out and washed with Mili-Q water for 5 minutes. The gels were further kept in fixative overnight at room temperature.

3.2.12.4 Silver staining

Next day before proceeding for silver staining gels were washed thrice with Mili-Q water for 15 minutes each. The silver staining was performed through following three steps.

Step1- Sensitization:

Sodium thio sulphate solution (0.02%) was used to treat the gels for one minute. The gels were washed thrice with Mili-Q water for 30 seconds each.

Step2-Staining:

Gels were treated with solution (40mg/100ml) of silver nitrate for 30 minutes with continuous shaking on rocker. The gels were washed thrice with Mili-Q water for one minute each.

Step3-Developing:

Color development of the gel proteins was achieved by developing solution containing 6g/100ml of anhydrous sodium carbonate, 2ml of 0.02% sodium thio sulphate and 40µl /100ml of formaldehyde solution. The gels were kept on gentle shaking for approximately 10 minutes till the spots were appeared prominently without background staining. The developing reaction was stopped by adding 6% acetic acid in Mili-Q water.

3.2.13 In-gel digestion and peptide extraction

In-gel digestion of proteins was carried out using different concentrations of trypsin. Spots in the gel were washed in 100µl destaining solution containing 1:1 100mM NH_4HCO_3 (ammonium bicarbonate) (Sigma chemicals) and 100% ACN (Acetonitrile) till the spots appear colorless. Finally gels were dehydrated in 100% ACN. Reduction of proteins was carried out in100µl of 10mM DTT in 50mM NH_4HCO_3 for 45 minutes at 56°C. Alkylation of proteins was done with 100µl of 55mM Iodoacetamide (IAA) prepared in 50mM NH_4HCO_3 for 30 minutes in dark. The gels were washed in 100µl of destaining solution containing 1:1 ratio of 50mM NH_4HCO_3 and 100% ACN. Final dehydration of gels was done in 100% ACN for 15 minutes. The gels were centrifuged and supernatants were discarded and gels were completely dried in speed Vac (Thermo Savant, USA) for 20 minutes. Five different concentrations of trypsin (Sigma) viz. 2.5 ng/µl, 5ng/µl, 10ng/µl, 20ng/µl and 30ng/µl were chosen. Trypsin was prepared in 25 mM NH_4HCO_3. Trypsin solution (10µl) was added and kept at 40°C for 30 minutes for

absorption. This was followed by further addition of 25 mM NH_4HCO_3 solution (20 µl). The samples were kept at 37°C for 12-16 hrs for digestion. After digestion, samples were centrifuged and supernatants containing peptides were collected and transferred to fresh microfuge tube rinsed with 100% ACN. Further, 0.1% Triflouroacetic acid (10 µl) and 100% ACN (10 µl) were added to the samples and sonicated for 15 minutes at room temperature. The samples were centrifuged and the supernatants were collected and mixed with the previous tubes. These supernatants containing peptides were dried completely in speed vac and stored at -80 °C until given for MS analysis. Before MS analysis peptides were resuspended in 0.1% TFA (10µl) in 30% ACN.

3.2.14 MALDI-TOF analysis of peptides and database search

Matrix was prepared by dissolving alpha cyano-hydroxy cinnamic acid (Bruker Daltonics, Bremaen, Germany) in 1:2 ACN and 0.1%TFA to make a 1mg/ml solution. It was sonicated for 15 minutes and the solution was centrifuged at 10,000 rpm for 5 minutes. The mixture of peptides (2µl) was mixed with equal volume of matrix clear supernatant and 0.5 µl of this was spotted on Maldi ground steel target plate (Bruker Daltonics, Germany). Samples and calibration standards with the same matrix composition were spotted adjacent to each other on the target plate for optimal calibration and enhance mass accuracy. Spectra were externally calibrated with the calibration standard of following peptide masses:

Angiotensin_II_ [M+H] +/- mono = 1046.54180

Angiotensin_I_ [M+H] +/- mono = 1296.68480

Substance_P_ [M+H] +/- mono =1347.73450

Bombesin [M+H] +/- mono =1619.82230

ACTH_Clip_ (1-17) [M+H] +/- mono = 2093.08620

Somatostatin (28) [M+H] +/- mono = 3147.47100

Mass spectra were obtained on a Bruker autoflexII MALDI-TOF mass spectrometer equipped with a pulsed N2 laser (337nm). Operating conditions were as follows: ion source1 = 19.00KV, ion source 2 = 16.50KV, Reflector voltage = 20.00KV, optimized pulsed ion extraction time = 120 ns, matrix suppression = 400Da and positive reflectron mode. Around 500 laser shots were collected from one spot from five different positions with 100 shots per position. Peptides mass fingerprinting (PMF) spectra were searched online against NCBInr and MSDB using the Mascot search engine (Matrix Sciences, London) with 100ppm mass tolerance and 0-2 missed cleavage.

Results

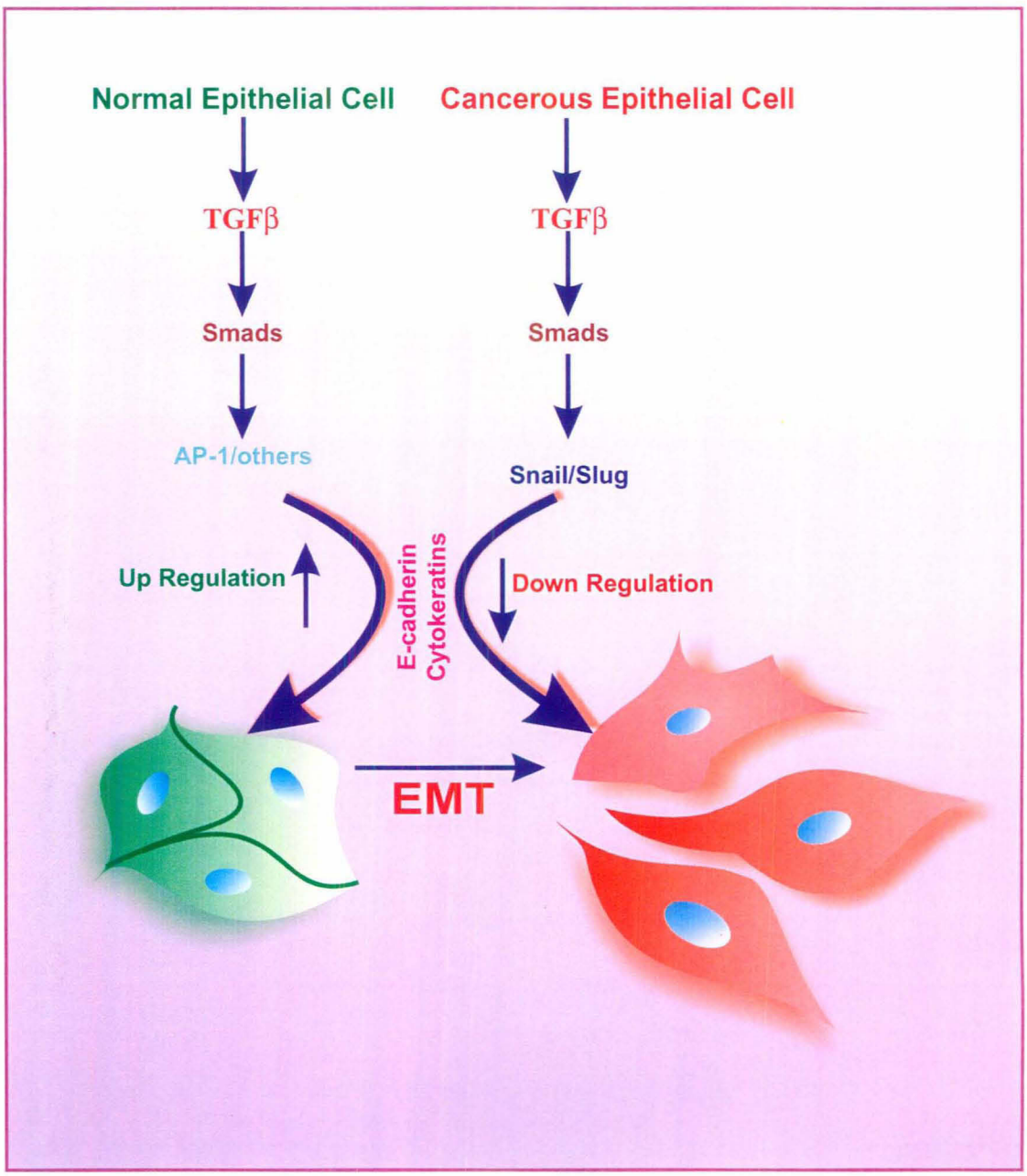


Plate VIII: Context dependent dual nature of TGF-β action.

TGF-β is a potent inhibitor of normal epithelial cell proliferation, while reverse happens in context with cancerous cell. Normal cells do respond to inhibitory action of TGF-β through Smads/AP-1 mediated signaling whereas, cancerous cell exploits proliferatory action of TGF-β through Smads/Snail/Slug mediated signaling.

4 RESULTS

4.1 Phase contrast images of MCF-7 and MDA-MB-231 cells show uniform pattern of morphology

MCF-7 cells and MDA-MB-231 cells (**Fig 1**) were grew as adherent cells in culture with typical morphological pattern with active cell division without any sign of refractile or granular structure. MCF-7 cells showed epitheloid morphology with healthy growth (**Fig 1-A, B**) and this cell line was used throughout the present study on EMT. MDA-MB-231cells showed spindle shape morphology with healthy growth (**Fig 1-C, D**) and these cells were used in EMSA study against TGF-β1 probe.

4.2 Morphological variant in normal culture condition may result from low frequency EMT like phenomenon

In normal culture conditions (**Fig 2**), proliferative MCF-7 cells that represent adenocarcinoma of mammary gland, showed striking morphological changes that represent loss of cell-cell junctions and loss of typical epithelial pattern over certain passages. This primary observation initiated with a small number of cells which increased over consecutive passages. This observation indicates that endogenous expression of various molecules involved in molecular interplay followed by EMT are sufficient to induce morphological changes like EMT in MCF-7 cells.

4.3 Expression of vimentin characterizes the cells forming a migratory front

Consistent with primary observation of striking EMT like changes under normal culture condition over certain passages (**Fig 3**), cells were probed for mesenchymal specific marker protein called vimentin employing monoclonal antibody against vimentin. HRP-conjugated secondary antibody was used against primary antibody. Haematoxylin was used for nuclear staining (**Fig 3-A, B**) and DAB mediated color precipitation was done to visualize cytosolic localization of vimentin. Dispersed cells from main population appeared as migratory front and showed vimentin expression prominently, suggestive of EMT.

4.4 Observations of CACCTG motif in promoter regions of TGF-β1 and Snail gene

The bioinformatics exercise was done with the sequence of natural promoter region of TGF-β1gene and Snail gene to locate occurrence of E-box containing CACCTG motif

which corresponds to binding site for Snail/Slug proteins. The highlighted sequence (**Fig 4, Fig 5**) containing CACCTG/CAGGTG motif was selected and used as a TGF-β1 probe and Snail probe respectively in EMSA-experiments.

4.5 Epithelial to mesenchymal transition in MCF-7 cells in response to TGF-β1 treatment

TGF-β induces epithelial to mesenchymal transition in breast cancer cells (**Fig 8**). Loss of cell-cell junctions e.g. adherens junctions, desmosomal junctions are striking features of EMT and were observed in TGF-β1 treated cells. TGF-β1 (5ng/ml concentration) was used to induce EMT in MCF-7 cells. Increase in dissociation of cell-cell connections were observed at various time points. Untreated MCF-7 cells (**Fig 8-A, B**) showed morphology characteristic of epithelial cells. TGF-β1 treated cells after 24 hrs showed loss of various junctions and significant dissociation of cells (**Fig 8-C, D**). Further increase in dissociation was observed with 36 hrs exposure (**Fig 8-E, F**). Photomicrography of untreated cells were done at 20X objective to show intact cellular junctions with epithelial morphology whereas, TGF-β1 treated cells at various time points were photographed at half the magnification covering maximum cells undergoing EMT.

4.6 Nuclear localization of vimentin in MCF-7 cells

Cytoskeletal rearrangement is an essential event in the manifestation of EMT. Mesenchymal specific intermediate filament vimentin shows marked expression in cells undergoing EMT (**Fig 13**). Immunofluorescence studies in MCF-7 cells without treatment showed cytosolic localization (**Fig 13-D, E**) consistent with our observation of EMT in normal culture without treatment (**Fig 2**) that was confirmed with probe against vimentin employing specific antibody (**Fig 3**). After treatment with TGF-β1 (5ng/ml) with the exposure of 48 hrs, cells prominently showed nuclear localization in addition to cytosolic localization (**Fig 13-F**). FITC-labeled secondary antibody was used against vimentin specific monoclonal antibody. DAPI was used as a nuclear counter stain (**Fig 13-A**). Primary antibody negative cells were observed in bright field (**Fig 13-B**) and same area was observed for nonspecific FITC signal (**Fig 13-C**) as an internal control.

4.7 TGF-β1 induces cytosolic expression of vimentin

Immunofluorescence study of vimentin expression in both cytosol and nucleus was further examined by western blotting (**Fig 14**). Total cytosolic protein was extracted from untreated cells, whereas total cytosolic and nuclear protein were extracted from TGF-β1 treated cells (5ng/ml, 48 hrs) and 10 μg estimated protein of each were loaded per well. HRP-conjugated secondary antibody was used against vimentin antibody and DAB as a substrate for colored precipitation. Total cytosolic fraction from untreated and treated cells showed expected band of 52Kd of vimentin (**Fig 14-lanes 2, 3**), whereas nuclear fraction of treated cells prominently showed expression of vimentin suggesting shuttling or trafficking of vimentin in nucleus (**Fig 14-lane 4**).

Further investigation of the finding of nuclear localization of vimentin was done in subsequent western blot (**Fig 15-A**) that clearly showed increased cytosolic expression of vimentin in response to TGF-β1 and nuclear expression of vimentin independent of TGF-β1 treatment. Total cytosolic and nuclear proteins were extracted from untreated and treated (5ng/ml, 72 hrs) cells. Protein from treated and untreated cells (12μg estimated protein of each) was loaded separately per well for SDS-PAGE. HRP-conjugated secondary antibody was used against vimentin antibody and DAB as a substrate for colored precipitation. Cytosolic fraction from treated cells showed increased expression of vimentin compared to cytosolic fraction from untreated cells (**Fig 15-A-lanes 2, 4**), whereas nuclear fraction from untreated cells showed presence of vimentin irrespective of TGF-β1 treatment (**Fig 15-A-lane 3**) similar to nuclear fraction from treated cells (**Fig 15-A-lane 5**). Profiling of total cytosolic and nuclear proteins from TGF-β1 treated and untreated cells was done (**Fig 15-B**) to demonstrate equal loading in support of findings obtained in western blotting (**Fig14, 15**).

4.8 DNA-protein interactions support probable interplay of TGF-β1 and TGF-β1 induced transcription repressors

4.8.1 TGF-β1 DNA undergoes electrophoretic mobility shift with nuclear extract of MCF-7 cells

Gel shift assay was performed for TGF-β1 probe in untreated MCF-7 cells undergoing EMT and in MDA-MB-231 cells showing no signs of EMT (**Fig 6**) with the presumption of expression of proposed transcription repressors and their probable interaction with TGF-β1 promoter having E-box core consensus. We did obtain electrophoretic mobility

shift. Nuclear extracts from MDA-MB-231 and MCF-7 cells (5μg) were used. Various concentrations of KCl (150mM and 300mM) were kept to examine maximum binding of regulatory factors. Band shift was observed at 300mM concentration of KCl (**Fig 6-Lane1**), whereas, no shift was observed at 150mM concentration of KCl (**Fig 6-Lane2**) in MDA-MB-231 cells. Mobility shift was observed at both 300 mM and 150 mM concentration of KCl in MCF-7 cells (**Fig 6-Lanes3, 4** respectively). Prominent shift in MCF-7 cells for TGF-β1 may suggest the induction and presence of regulatory factors (Snail, Slug, SIP1 and Twist) and their probable interaction with TGF-β1 promoter in context with EMT as they are well known for their interactions with E-box in context with EMT and consequently metastasis. Probe sequence containing CAGGTG motif present in TGF-β1 promoter region was used for assay.

4.8.2 Snail probe shows electrophoretic mobility shift with nuclear extract from MCF-7 cells

Gel shift assay was performed for Snail probe in MCF-7 cells undergoing EMT (**Fig7**). Binding reaction was carried with nuclear extract from MCF-7 cells (5μg) and Snail probe (2ng). Probe sequence containing CACCTG motif in Snail promoter region was used for assay. Prominent shift was observed with Snail probe (**Fig7-Lane1**). The observed shift may suggest binding of regulatory factors viz. Snail, Slug, SIP1 and Twist. It has documented that Snail has stronger binding affinity towards E-box containing CACCTG motif.

Binding of these regulatory factors to TGF-β1 probe and Snail probe may indicate that MCF-7 cells, a representative form of aggressive carcinoma, express EMT inducing growth factors and transcription repressors essential for EMT over certain passages.

4.8.3 MCF-7 cells with intact junctions show low or no binding with TGF-β1 or Snail probe

In another EMSA experiment (**Fig12**), two sets of MCF-7 cells were used. One set was kept untreated ensuring intact cell-cell junctions and typical epitheloid morphology while another one was treated with TGF-β1 (5ng/ml) for 48 hrs. During this period of exposure to TGF-β1, cells consistently showed certain changes. These cells showed marked dissociation after 24 hrs while significantly increased after 48 hrs with the manifestation of EMT. Significantly, fewer changes were observed in untreated cells with no visible

alteration in cellular morphology and a much smaller number of cells undergoing dissociation. Nuclear extract from both sets of cells were prepared and incubated (5μg protein) with TGF-β1 probe and Snail probe respectively. TGF-β1 probe with nuclear extract from untreated cells showed no prominent shift (**Fig12-Lane2**). Prominent shift was observed for TGF-β1 probe with nuclear extract from treated cells (**Fig12-Lane3**). Similarly, Snail probe with nuclear extract from untreated cells showed no sign of shift (**Fig12-Lane5**). More than one shift was observed for Snail probe with treated nuclear extract. Prominent shift (upper one) followed by less prominent shift (lower one) are suggestive of more than one transcription factor/repressor interaction with Snail probe (**Fig 12-Lane 6**).

4.8.4 E-cadherin probe also shows electrophoretic mobility shift with nuclear extract from TGF-β1 treated and untreated MCF-7 cells

Snail is a well known repressor of E-cadherin. Probe sequence containing CACCTG motif in E-cadherin promoter region taken from literature as a reference probe was used for assay (**Fig 9**). Two sets of MCF-7 cells were used for assay, one set of untreated cells undergoing EMT was as such used for assay and another one was treated with TGF-β1 (5ng/ml) ensuring maximum EMT with the exposure to TGF-β1 for 72 hrs. Nuclear extract from both sets of cells were prepared and incubated (11μg) separately with E-cadherin probe in binding reaction. E-cadherin probe showed mobility shift with nuclear extract from both untreated as well as treated cells (**Fig 9-Lane2, 3**). Regulation of E-cadherin by Snail is a well studied and established fact in context with EMT.

4.8.5 A mutant sequence probe seems to undergo shifts more prominently than the wild type sequence probe

Stringency of binding to a consensus motif was examined with a probe containing four times CACCTG repeat (**Fig 10**). Two sets of MCF-7 cells were used for assay, one set of untreated cells undergoing EMT was as such used for assay and another one was treated with TGF-β1 (5ng/ml) ensuring maximum EMT with the exposure of 72 hrs. Nuclear extract from both sets of cells were prepared and incubated (11μg protein) separately with sequence repeat probe in binding reaction. Shift was observed for both treated and untreated nuclear extract (**Fig 10-Lanes2, 3**) respectively.

Another EMSA experiment was performed with sequence mutant probe containing four times AACCTA repeat (**Fig 11**) under similar conditions as for sequence

repeat probe. Shift was observed for both treated and untreated nuclear extract (**Fig 11-Lanes2, 3**) respectively. Mutant sequence probe seems to undergo more prominent shift compared to normal sequence probe. The binding stringency and affinity towards probe might involve a space between two motifs and orientation of sequence with core binding nucleotides.

4.9 Flowcytometric analysis of vimentin and cytokeratin expression

Studies on expression of EMT markers was performed along a time course and in situ localization visualized by flowcytometry for vimentin and cytokeratin18. MCF-7 cells were treated with TGF-β1 (5ng/ml) for 72 hrs. The control cells and treated cells were further probed for expression of markers of transition by employing primary antibody against vimentin and cytokeratin18. The staining was achieved by using FITC-labeled secondary antibody. The autofluorescence and non specific FITC fluorescence was examined for MCF-7 cells (**Fig 16**). Cell populations were selected from gate R1 for dot plot analysis (**Fig 16-A, B**). In quadrant dot plot, 0.66% of selected unstained cell population represents autofluorescence (**Fig 16-C**). Non specific FITC staining without primary antibody showed 2.93% signal (**Fig 16-D**). Histogram plot of autoflourescence and FITC staining (**Fig 17**) showed marker M1 that represents selected unstained population, whereas M2 showed cells among population with autofluorescence (**Fig 17-A**) and non specific FITC staining with slight increase in fluorescence (M2) in comparison to autoflourescence (**Fig 17-B**). Overlay plot (**Fig 17-C**) represented overall picture of expression indicate nonsignificant signal of both autoflourescence and nonspecific FITC staining.

4.9.1 Expression analysis of vimentin

Cell population (**Fig 18**) from gate R1 was selected for dot plot analysis from untreated and treated cells respectively (**Fig 18-A, B**). Quadrant plot showed expression of vimentin in terms of fluorescence intensity in untreated cells (2.31%) and treated cells (3.05%) (**Fig 18-C, D**). Histogram plot corresponding to vimentin expression (**Fig 19**) showed no significant shift of vimentin stained cells in M2 region (2.31%) from unstained M1 region (97.64%) of control cells (**Fig 19-A**). Similarly, insignificant shift of vimentin stained cells were observed in M2 region (3.05%) of treated cells (**Fig 19-B**). Overall, overlapping plot showed absence of any considerable change in vimentin expression in treated as compared to control cells (**Fig 19-C**).

4.9.2 Expression analysis of cytokeratin 18

Cell population (**Fig 20**) selected from gate R1 for dot plot analysis from control (**Fig 20-A**) and treated cells (**Fig 20-B**) showed marginally higher expression of CK18 in terms of fluorescence intensity of 36.15% in TGF-β1 treated cells (**Fig 20-D**) in comparison to untreated cells with intensity of 33.26% (**Fig 20-C**). Histogram plot corresponds to cytokeratin18 expression (**Fig 21**) showed considerable shift of CK18 stained cells in M2 region (33.26%) from unstained M1 region in control cells (**Fig 21-A**). TGF-β1 treated cells showed marginal shift of CK18 stained cells in M2 region (36.15%) in comparison to CK18 staining in control cells (**Fig 21-B**). Overall, overlapping plot showed marginal change in CK18 expression in both treated and control cells (**Fig 21-C**).

4.10 Proteomic study of markers of EMT and differentially expressed proteins

Epithelial to mesenchymal transition involves versatile molecular interplay that essentially begins with downregulation of cell adhesion molecules such as E-cadherin, remodeling of cytoskeletal structure and rearrangement of extra-cellular matrix. Many of the epithelial specific structural proteins such as intermediate filaments e.g. various cytokeratins, desmoplakins and structural stability associated proteins such as α and β actins are influenced by transcriptional reprogramming. TGF-β recruits key regulatory factors that enforce structural alterations essential for EMT. To understand this interplay of various molecules at the level of proteomics, 2D gel electrophoresis was performed and the differentially expressed proteins were given for MALDI-TOF analysis.

4.10.1 MALDI-TOF analysis indicates signs of TGF-β1 mediated EMT in MCF-7 cells

MCF-7 cells represent aggressive form of breast carcinoma and respond very well to TGF-β1-mediated EMT accompanied with differential expression of Heat shock protein-27. In an experimental query (**Fig 22, 23, 24**), MCF-7 cells were treated with TGF-β1 (5ng/ml) up to 72 hrs ensuring maximum dissociation of the cells from cell-cell junctions. Whereas untreated cells showing normal epithelial pattern without much loss of cell-cell contacts were taken for this study. Total cytosolic protein was isolated from untreated and TGF-β1 treated cells. 60μg of estimated protein of each sample was loaded on 7 cm, pH 3-10 linear IEF-strip separately for electrofocussing. SDS-PAGE (12%) was run for second dimension along with marker proteins.

4.10.2 PMF spectra and MS/MS spectra corresponds to expressed proteins

In 2D gels spots showing differential expression (highlighted with arrow head) and probable markers of EMT under study (highlighted with circles) were screened and given for MALD-TOF analysis. On the basis of molecular weight and PMF spectra, analysis was performed. PMF spectrum of the spots **C1** and **T1** were identified as enolase from untreated and TGF-β1 treated cells respectively. PMF spectrum of the spot **T2** represent heat shock protein-27 (HSP-27) with differential expression in treated cells. HSP-27 PMF spectrum (**Fig 25**) along with sequence coverage map showed intensity coverage-53.5%, sequence coverage- 46.3%, pI-6.0 and kDa-22.8. MS / MS spectrum further confirmed the matched sequences: LFDQAFGLPR of m/z1163.681 corresponds to HSP-27 (**Fig 26**). PMF spectrum of the spots named **C4, T4** and **T3** in figure-24 belongs to various cytokeratins. Most of the proteins under study fall within 4.5 to 6.0 pH range and need to be separated further by using 4-7 pH gradient IEF-strip. In this profiling PMF spectrum of the spots **C1** and **T1** (**Fig 27, 28**) mentioning enolase were taken as a reference to ensure the accuracy of the detection made by MALDI-TOF and MS/MS analysis.

With the feedback of cytoskleletal rearrangement in MCF-7 cells undergoing EMT in response to TGF-β1, another experiment of 2D electrophoresis was performed to explore markers of EMT and differentially expressed proteins in connection with EMT (**Fig 29, 30, 31**). Similar conditions of treatment described as in previous set of 2D experiment were used. MCF-7 cells were treated with TGF-β1 (5ng/ml) for 72hrs. Total cytosolic protein was isolated from untreated and TGF-β1 treated cells. Estimated protein (200μg) of each sample was loaded on 18 cm, pH 3-10 linear IEF-strip separately for electrofocussing. SDS-PAGE (12%) was run for second dimension along with marker proteins.

4.10.3 Mass analyses of proteins indicate special roles for cytoskeletal rearrangement and structural stability associated proteins

Commonly expressed proteins in 2D gel (**Fig 29, 30, 31**) were highlighted by encircling, while differentially expressed proteins were highlighted by arrow heads and given for MALD-TOF analysis. An analysis was performed on the basis of molecular weight and PMF spectra. Consistent with previous 2D profile, PMF spectrum of the spots **C1** and **T1** correspond to enolase from untreated and TGF-β1 treated cells respectively. PMF spectrum of the spots **C2** and **C5** correspond to α and β tubulin respectively. PMF

spectrum of the spots **C3** and **T3** suggest calreticulin precursor protein. PMF spectrum of the spots **C4** and **T4** suggest β actin protein. PMF spectrum of the spots **C7** and **T7** suggest TUBB protein. PMF spectrum of the spots **D1, D2, D3, D4, D5, D6** and **D7** represents differentially expressed proteins. PMF spectrum of the spots **D1** and **D2** correspond to triose phosphate isomerase 1 isoform and **D3** to heat shock protein-27. PMF spectrum of the spot **D4** belongs to tyrosine3/tryptophan5-monooxygenase activation protein, ζ polypeptide. PMF spectrum of the spot **D5** suggest cytokeratin16. PMF spectrum of the spot **D6** suggest transcription factor elongin A2. PMF spectrum of the spot **D7** suggest ubiquitin protein.

Consistent with 2D profile shown and described in figure-24, differential expression of HSP-27 in cells undergoing EMT in response to TGF-β1, marked significant role in offering structural stability by maintaining unaltered conformation of actin in cells undergoing rearrangement of cytoskeletal proteins. Expression of cytokeratin16 along with β actin, α and β tubulin collectively signifies their importance in structural stability and loss of the same in context with EMT. Overexpression of HSP-27, isoforms of triose phosphate isomerase 1 and ubiquitin prominently indicates aggressive form of carcinoma that MCF-7 cells undergo after TGF-β1 treatment.

4.10.4 PMF spectra of respective spots corresponds to cytoskeletal rearrangement and structural stability associated proteins with sequence coverage map

MALDI-TOF analyses of screened spots with their PMF spectra (**Fig 32-47**) are compiled in **Table 1**. PMF spectrum corresponding to ubiquitin was further confirmed with matched sequence IQDKEGIPPDQQR of m/z1523.73 corresponding to ubiquitin by MS/MS analysis (**Fig 48**).

Table 1: PMF Spectra of screened spots given for MALDI-TOF analysis with intensity and sequence coverage (See Figures 29-31 for 2D gels and Figures 32-47 for mass analysis)

Fig No.	Protein suggested by PMF spectra	Intensity Coverage %	Sequence Coverage %	pI pH	MW kD
Fig. 32	Enolase (C1)	77.1	62.2	7.7	47.4
Fig. 33	α Enolase (T1)	79.0	43.0	7.7	47.4
Fig. 34	α Tubulin (C2)	21.5	32.5	4.8	50.5
Fig. 35	β Tubulin (C5)	76.0	43.6	4.6	50.3
Fig. 36	Calreticulin precursor protein (C3)	35.3	26.6	4.1	47.1
Fig. 37	β Actin (C4)	27.1	24.7	5.5	40.5
Fig. 38	β Actin (T4)	54.3	30.0	5.5	40.5
Fig. 39	TUBB protein (C7)	68.6	38.5	4.6	38.7
Fig. 40	TUBB protein (T7)	68.6	38.5	4.6	38.7
Fig. 41	Triose phosphate isomerase1 isoform (D1)	7.7	38.1	5.6	31.1
Fig. 42	Triose phosphate isomerase1isoform (D2)	3.8	37.4	5.6	31.1
Fig. 43	Heat shock protein-27 (D3)	15.9	29.3	6.0	22.8
Fig. 44	14-3-3ζ protein (D4)	13.8	33.6	4.6	30.1
Fig. 45	Type 1 Keratin 16 (D5)	22.5	30.2	4.8	51.5
Fig. 46	Transcription factor Elongin A2 (D6)	26.0	14.5	10.4	84.5
Fig. 47	Ubiquitin (D7)	43.8	50.0	7.6	8.5

pI- Isoelectric point

kD- Molecular weight in kilo Dalton

C- Untreated T- Treated D-Differential expression

4.11 Time course of expression of EMT markers

Expression profile of cytokeratin 18, E-cadherin and TGF-β1 was viewed at different time points (24 hrs, 48 hrs, 72 hrs) following TGF-β1 treatment (3ng/ml). Total RNA was isolated from TGF-β1 treated cells (3ng/ml) and untreated cells. RT-PCR was performed for negative control, untreated sample and samples corresponding to each time point. DNA ladder of 100bp was used as a marker.

4.11.1 Cytokeratin 18 shows consistent expression at different time points

RT-PCR analysis of cytokeratin 18 expression (**Fig 49**) was examined at different time points. In comparison to expression in untreated cells (**Fig 49-Lane3**) expression at 24 hrs (**Fig 49-Lane4**), 48hrs (**Fig 49-Lane5**) and at 72 hrs (**Fig 49-Lane6**) showed no noticeable changes in cytokeratin 18 expression.

4.11.2 Expression of E-cadherin shows gradual decrease with time

Downmodulation of E-cadherin is a hallmark of onset and progression of EMT. TGF-β modulates E-cadherin expression in context with EMT. Consistent with established behavior of E-cadherin in EMT, MCF-7 cells showed downregulation of E-cadherin with time, post TGF-β1 treatment.

Expression of E-cadherin transcript was examined by RT-PCR at different time points after TGF-β1 treatment (**Fig 50**). Signal intensity for 200bp amplicon decreased gradually at 24hrs (**Fig 50-Lane4**), 48hrs (**Fig 50-Lane5**) and at 72hrs (**Fig 50-Lane6**), in comparison to expression in untreated cells (**Fig 50-Lane3**). The observed downfall in E-cadherin expression with increase in time in response to TGF-β1 induced EMT correlates with the pattern observed for Snail in EMSA studies.

4.11.3 Expression profile of TGF-β1 shows correlation with the presumed interplay with Snail in auto-regulation

TGF-β1 has an auto-regulatory mechanism through transcription factor AP-1. Another mechanism in TGF-β1 regulation is suggested to be added through the interaction of Snail with E-box motif in TGF-β1 promoter on the basis of observations in EMSA studies. Consistent with EMSA studies, TGF-β1 showed gradual decrease and regain of expression in MCF-7 cells with time points, post TGF-β1 treatment (**Fig 51**).

Expression of TGF-β1 was examined by RT-PCR at different time points after TGF-β1 treatment. Visibly significant intensity and size of the band corresponding to

234bp of amplicon was observed in untreated sample (**Fig 51-Lane3**), whereas, the intensity was drastically decreased at 24 hrs (**Fig 51-Lane4**); moreover, further gradual decrease was observed at 48 hrs (**Fig 51-Lane5**). At 72 hrs, regain of expression in terms of increased intensity of the band was observed (**Fig 51-Lane6**). Observations in TGF-β1 expression with time points strikingly match with presumed regulatory interplay of TGF-β1 and Snail in feedback manner in context with EMT.

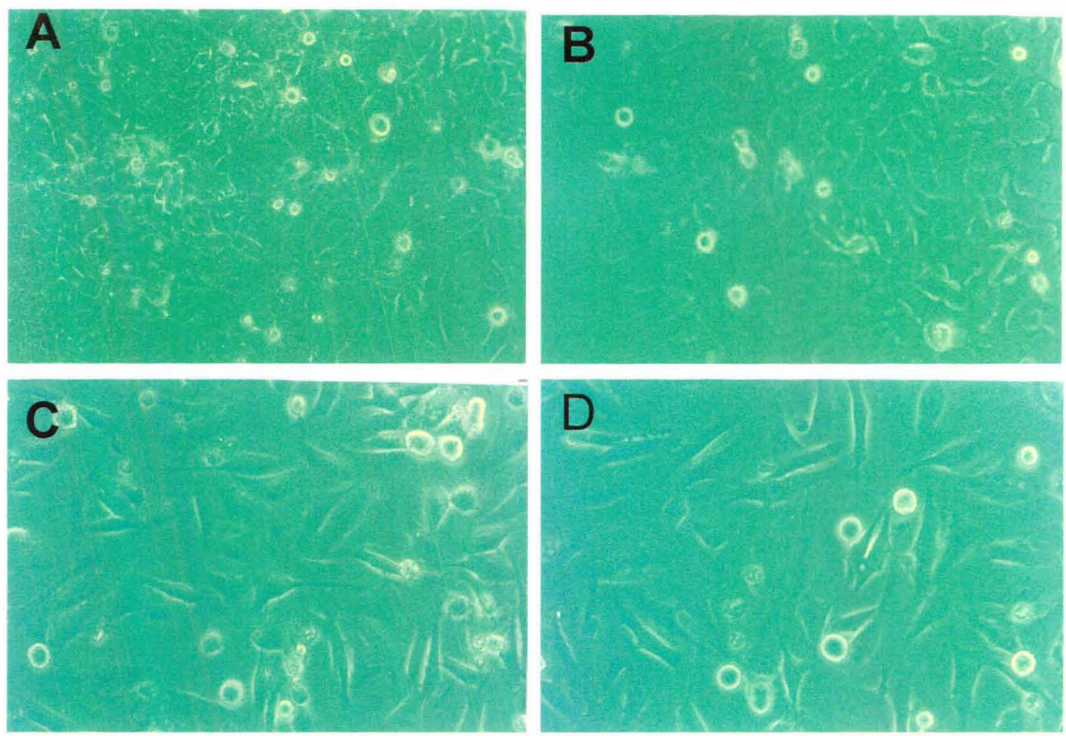


Fig.1: Phase contrast images of MCF-7 and MDA-MB-231 cells. A, B, MCF-7 cells, showing compact epitheloid morphology under normal proliferative conditions. **C, D,** MDA-MB-231cells, showing spindle shape morphology under similar conditions.

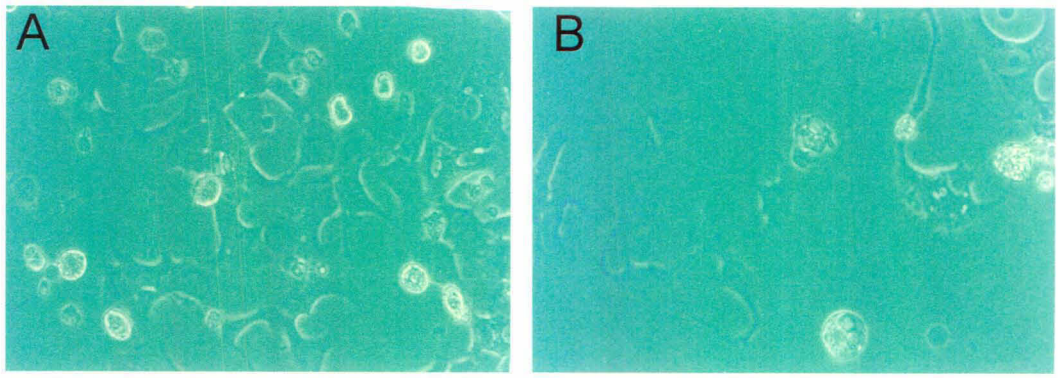


Fig.2: Normal culture of MCF-7 cells showing morphological features suggestive of partial epithelial to mesenchymal transition (EMT) by showing loss of cell-cell junctions. A, B, MCF-7 cells observed under 32x magnification.

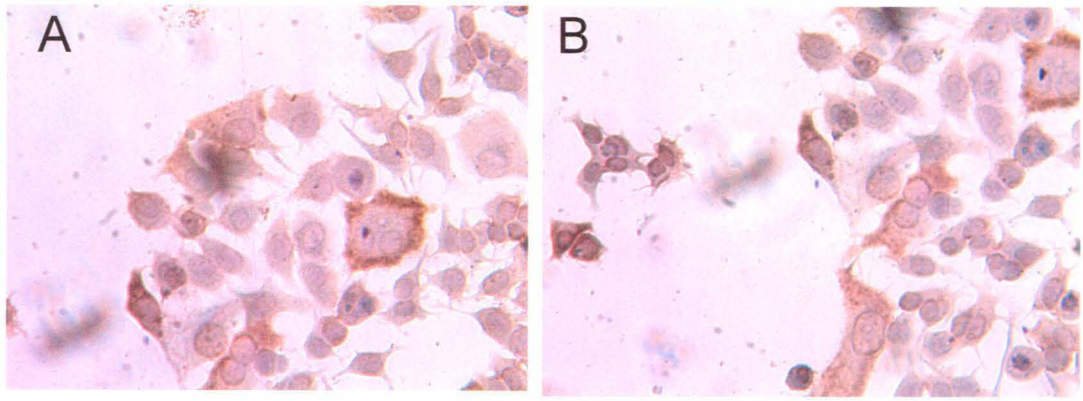


Fig. 3: Localization of vimentin in MCF-7 cells.
HRP-conjugated secondary antibody was used against vimentin antibody. Haematoxylin was used for nuclear staining. **A, B,** DAB-mediated color precipitation shows expression of vimentin among MCF-7 cells.

TGF-β1 Promoter

GGATCCTTAGAGGGGAGTAACATGGATTTGGAAAGATCACTTTGGCTGCTGTGTGGGGATAGA
TAAGACGGTGGGAGCCTAGAAAGGAGGCTGGGTTGGAAACTCTGGGACAGAAACCCAGAGAGG
AAAAGACTGGGCCTGGGGTCTCCAGTGAGTATCAGGGAGTGGGGAATCAGCAGGAGTCTGGTC
CCCACCCATCCCTCCTTTCCCCTCTCTCTCCTTTCCTGCAGGCTGGCCCCGGCTCCATTTCCA
GGTGTGGTCCCAGGACAGCTTTGGCCGCTGCCAGCTTGCAGGCTATGGATTTTGCCATGTGCC
CAGTAGCCCGGGCACCCACCAGCTGGCCTGCCCCACGTGGCGGCCCCTGGGCAGTTGGCGAGA
ACAGTTGGCACGGGCTTTCGTGGGTGGTGGGCCGCAGCTGCTGCATGGGGACACCATCTACAG
TGGGGCCGACCGCTATCGCCTGCACACAGCTGCTGGTGGCACCGTGCACCTGGAGATCGGCCT
GCTGCTCCGCAACTTCGACCGCTACGGCGTGGAGTGCTGAGGGACTCTGCCTCCAACGTCACC
ACCATCCACACCCCGGACACCCAGTGATGGGGGAGGATGGCACAGTGGTCAAGAGCACAGACT
CTAGAGACTGTCAGAGCTGACCCCAGCTAAGGCATGGCACCGCTTCTGTCCTTTCTAGGACCT
CGGGGTCCCTCTGGGCCCAGTTTCCCTATCTGTAAATTGGGGACAGTAAATGTATGGGGTCGC
AGGGTGTTGAGTGACAGGAGGCTGCTTAGCCACATGGGAGGTGCTCAGTAAAGGAGAGCAATT
CTTACAGGTGTCTGCCTCCTGA**CCCTTCCATCCCTCAGGTGTCCTGTTGCC**CCCTCCTCCCAC
TGACACCCTCCGGAGGCCCCCATGTTGACAGACCCTCCTTCTCCTACCTTGTTTCCCAGCCTG
ACTCTCCTTCCGTTCTGGGTCCCCCTCCTCTGGTCGGCTCCCCTGTGTCTCATCCCCCGGATT
AAGCCTTCTCCGCCTGGTCCTCTTTCTCTGGTGACCCACACCGCCCGCAAAGCCACAGCGCAT
CTGGATCACCCGCTTTGGTGGCGCTTGGCCGCCAGGAGGCAGCACCCTGTTTGCGGGGCGGAG
CCGGGGAGCCCGCCCCCTTTCCCCCAGGGCTGAAGGGACCCCCCTCGGAGCCCGCCCACGCGA
GATGAGGACGGTGGCCCAGCCCCCCCATGCCCTCCCCCTGGGGGCCGCCCCCGCTCCCGCCCC
GTGCGCTTCCTGGGTGGGGCCGGGGGCGGCTTCAAAACCCCCTGCCGACCCAGCCGGTCCCCG
CCGCCGCCGCCCTTCGCGCCCTGGGCCATCTCCCTCCCACCTCCCTCCGCGGAGCAGCCAGAC
AGCGAGGGCCCCGGCCGGGGGCAGGGGGGACGCCCCGTCCGGGGCACCCCCCCGGCTCTGAGC
CGCCCGCGGGGCCGGCCTCGGCCCGGAGCGGAGGAAGGAGTCGCCGAGGAGCAGCCTGAGGCC
CCAGAGTCTGAGACGAGCCGCCGCCGCCCCCGCCACTGCGGGGAGGAGGGGGAGGAGGAGCGG
GAGGAGGGACGAGCTGGTCGGGAGAAGAGGAAAAAAACTTTTGAGACTTTTCCGTTGCCGCTG
GGAGCCGGAGGCGCGGGGACCTCTTGGCGCGACGCTGCCCCGCGAGGAGGCAGGACTTGGGGA
CCCCAGACCGCCTCCCTTTGCCGCCGGGGACGCTTGCTCCCTCCCTGCCCCCTACACGGCGTC
CCTCAGGCGCCCCCATTCCGGACCAGCCCTCGGGAGTCGCCGACCCGGCCTCCCGCAAAGACT
TTTCCCCAGACCTCGGGCGCACCCCCTGCACGCCGCCTTCATCCCCGGCCTGTCTCCTGAGCC
CCCGCGCATCCTAGACCCTTTCTCCTCCAGGAGACGGATCTCTCTCCGACCTGCCACAGATCC
CCTATTCAAGACCACCCACCTTCTGGTACCAGATCGCGCCCATCTAGGTTATTTCCGTGGGAT
ACTGAGACACCCCCGGTCCAAGCCTCCCCTCCACCACTGCGCCCTTCTCCCTGAGGAGCCTCA
GCTTTCCCTCGAGGCCCTCCTACCTTTTGCCGGGAGACCCCCAGCCCCTGCAGGGGCGGGGCC
TCCCCACCACACCAGCCCTGTTCGCGCTCTCGGCAGTGCCGGGGGGCGCCGCCTCCCCCATG

Fig.4: Natural promoter nucleotide sequence of TGF-β1 gene.
The highlighted sequence was used as a probe in EMSA-experiments. The promoter was examined for CAGGTG motif corresponds to the binding site of Snail/Slug proteins with bioinformatics exercise before probe designing.

SNAIL promoter

GGATCCTGATTGGAGCTAAATTGACACGGGACGGGGAGTATTCCGCTTAATGACTGCTTACTG
CCTCTGTCCCCCACCCTTACTGCCCGCTCTCCAGCTAGAACCAGGGGAGGACGATTTTGTTCA
CGGCTGTCACACCCGGCACCAAGTGACTAAACAGACAGTAGTTCTGCCCTTCAGGTTGGTGGT
GGTGGTGGTGTGGGGTGCTTATAGGTTTTTATAGTCTTCCTTCACAGGTGGAAACAAGAACGG
TGAGACCTGTGAGGTTTATTTCAGAGCCACCCCCGGGCCTGGTACGCCTCCCAGGGAAGCTGG
GGTGGGGGTGCAGCTGCCCCTCACTTGGGCCCACCCCACATGCATTCAGTCAGGGCCCCTATG
GAGCCGTGTTACAGCCTTTAGACACAGGATCGAGGATCTTCACGGGGTGAGGAGACACGCCCG
GCCCATGCCTTCTTTCTCCCACCAGCCTACAGGGATCTTTTTCAACGAAACTCTAACCAGGTC
CCTCCTCAGCTGAAAATCCTTCGGTGGCTCCCCAGTGCCCTTGAGGCAAAGTCCAAACTCCTA
CGAGGCCCTGGGGCCCAGCACATCTGACCCCTCCGGGCATGCCCATCCCACCCCATCCCTGGA
AGCTGCTCTCTAGGAGTTACTCTGAAGCAGTTGCCACTTCTTCCCTCGGGCCTTTTCCCTTGA
TAATTCTTCACTTCCTCTGGGAAGTCACCCCGACCCCCTGTCAGGTGACCCGCCTCTTAACGG
TCGCCGCGTCCCGTCTCTCCCCCACCAAAGCACACTTCCCTTTGCATTGTAATTATCTGTTTA
CTTCGTCTGTCTCCCTCACTGGACCAGAAGCTACCCTTCGGGAGAGGCTCTGAGTGTTCTGTC
CGGGGCTGTGCCCTGGCCCCAGGTACAGTGCCCCACACGTGCTGGGCGCTCCGTAAACACTGG
ATAAGGGAAGGAACGGGTGCTCTTGGCTAGCTGGGCCAGGCTGCTTTGCAAAAAGGCCGTGGC
ATTTCAAGCCGCCGAGAGCCACGTGCGGTGTCCCTTTCCTCGCTTCCTCCCCAGTGATGTGCG
TTTCCCTCGTCAATGCCACGCTCTCCAGGCGCCAGCCGGGCGGAGGAAATTTCCGCCCCCTCC
CAAGCCCGAGGCGGGGGCGGGCGTCGGAAGGTCAGGTGTCCCGGCCGGCGCGCAGCGCCAGGG
GGCGTCAGAAGCGCTCAGACCACCGGGCGCTGAGCCGGTGGGCGCGCGGCGTCCTGCCGGGGT
CCCACCTCGCAGAGGCCTCGCTTCGCTCGACGTCCCGCCCCGGACAGCCCCAGCACCGGGGAC
GACCCGCGCTGCGCCAGCGAACCCCGCCTCGGAGGAGTCCCCGCCCGGGCTCTCACCGCCACG
CGGCGCGAGCCCGGCCA**GCAGCCGGCGCACCTGCTCGGGGAGTGGC**CTTCGGCGGAGACGAGC
CTCCGATTGGCGCGGAGGTGACAAAGGGGCGTGGCAGATAAGGCNCCGGCCCCTCCCACCCCC
CACCACCCCCCGGAGTACTTAAGGGAGTTGGCGGCGCTGCTGCATTCATTGCGCCGCGGCACG
GCCTAGCGAGTGGTTCTTCTGCGCTACTGCTGCGCGAATCGGCGACCCCAGTGCCTCGACCAC
TATGCCGCGCTCTTTCCTCGTCAGGAAGCCCTCCGACCCCAATCGGAAGCCTAACTACAGCGA
GCTGCAGGACTCTAATCCAGGTGCGTTGGAGGGGTTCTGGGCTCCAGGAGGTTTGGGGGAGAC
AGGCGAAGGCTGCGTGGGGGGCACCTGAGGGAGGCG

Fig.5: Natural promoter nucleotide sequence of SNAIL gene.
The highlighted sequence was used as a probe in EMSA-experiments. The promoter was examined for CACCTG motif corresponds to the binding site of Snail/Slug proteins with bioinformatics exercise before probe designing.

Probe Sequence: 5' c c c t t c c a t c c c **t c a g g t** g t c c t g t t g c c 3'
3' g g g a a g g t a g g g **a g t c c a** c a g g a c a a c g g 5'

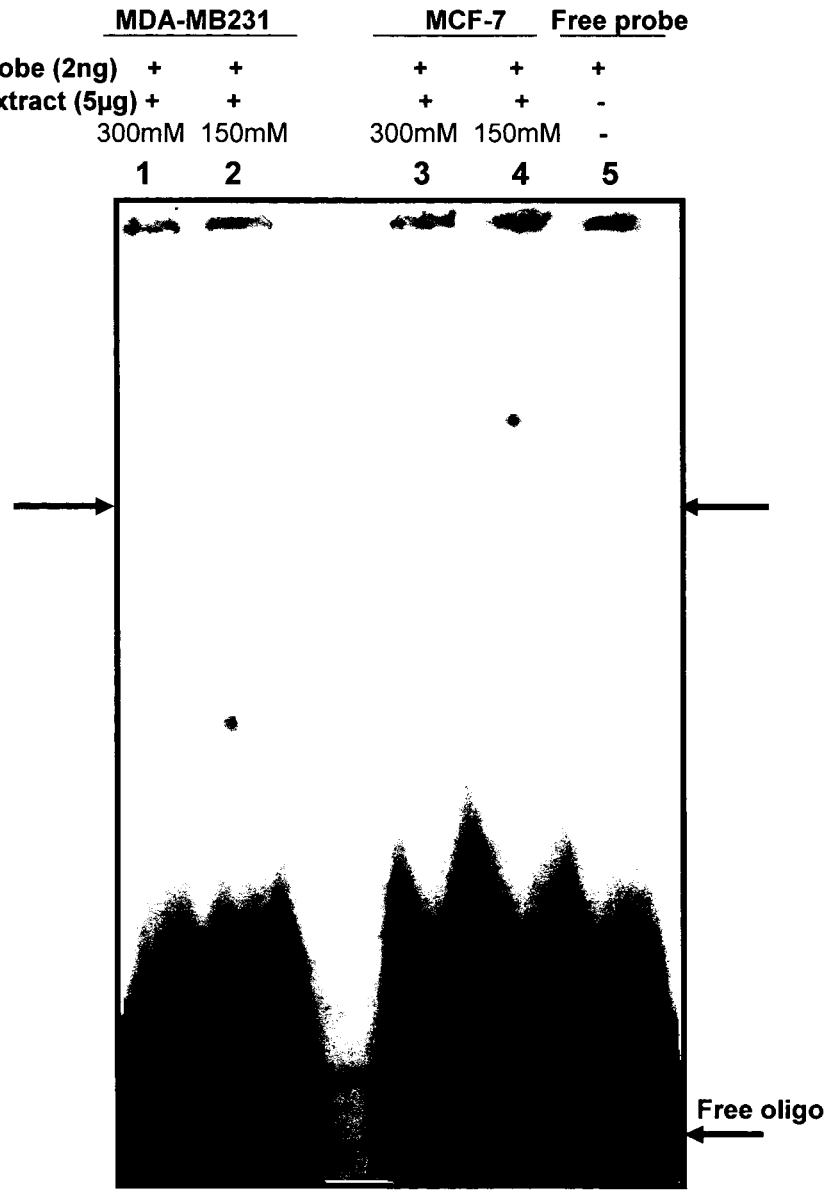


Fig.6: EMSA profile of TGF-β1 with various concentration of KCl. Band shift was observed at various concentration of KCl **Lanes1, 2,** Nuclear extract from MDA-MB-231 cells (5μg) was used, band shift was observed at 300mM concentration of KCl **Lane 3, 4,** Nuclear extract from MCF-7 cells (5μg protein) was used, band shift was observed at both 300 mM and 150 mM concentration of KCl respectively **Lane 5,** Free TGF-β1 probe (2ng). The results suggest presence of TGF-β1 regulatory factors in both cell lines.
Probe sequence containing CAGGTG motif present in TGF-β1 promoter region was used for assay.

Probe Sequence: 5' g c a g c c g g c g c a c c t g c t c g g g g a g t g g c 3'
3' c g t c g g c c g c g t g g a c g a g c c c c t c a c c g 5'

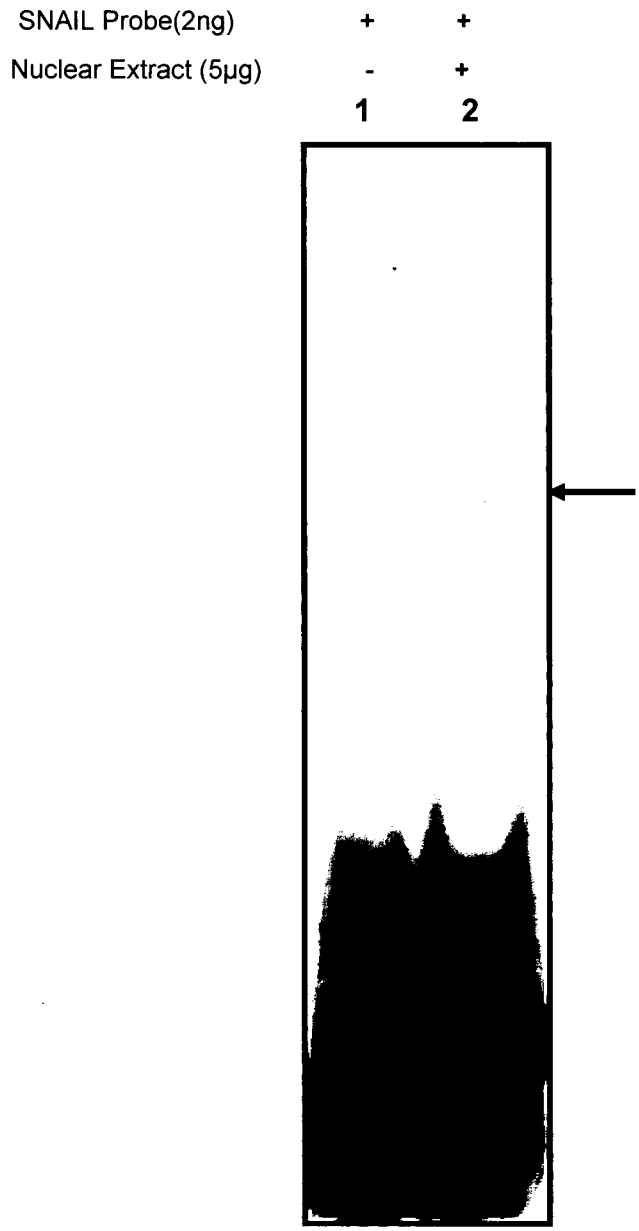


Fig.7: EMSA for SNAIL probe in MCF-7 Cells. Lane1, Free SNAIL probe. **Lane2,** Nuclear extract from MCF-7 cells (5µg) and SNAIL probe (2ng) were used to obtain band shift. Probe sequence containing CACCTG motif in SNAIL promoter region was used for assay.

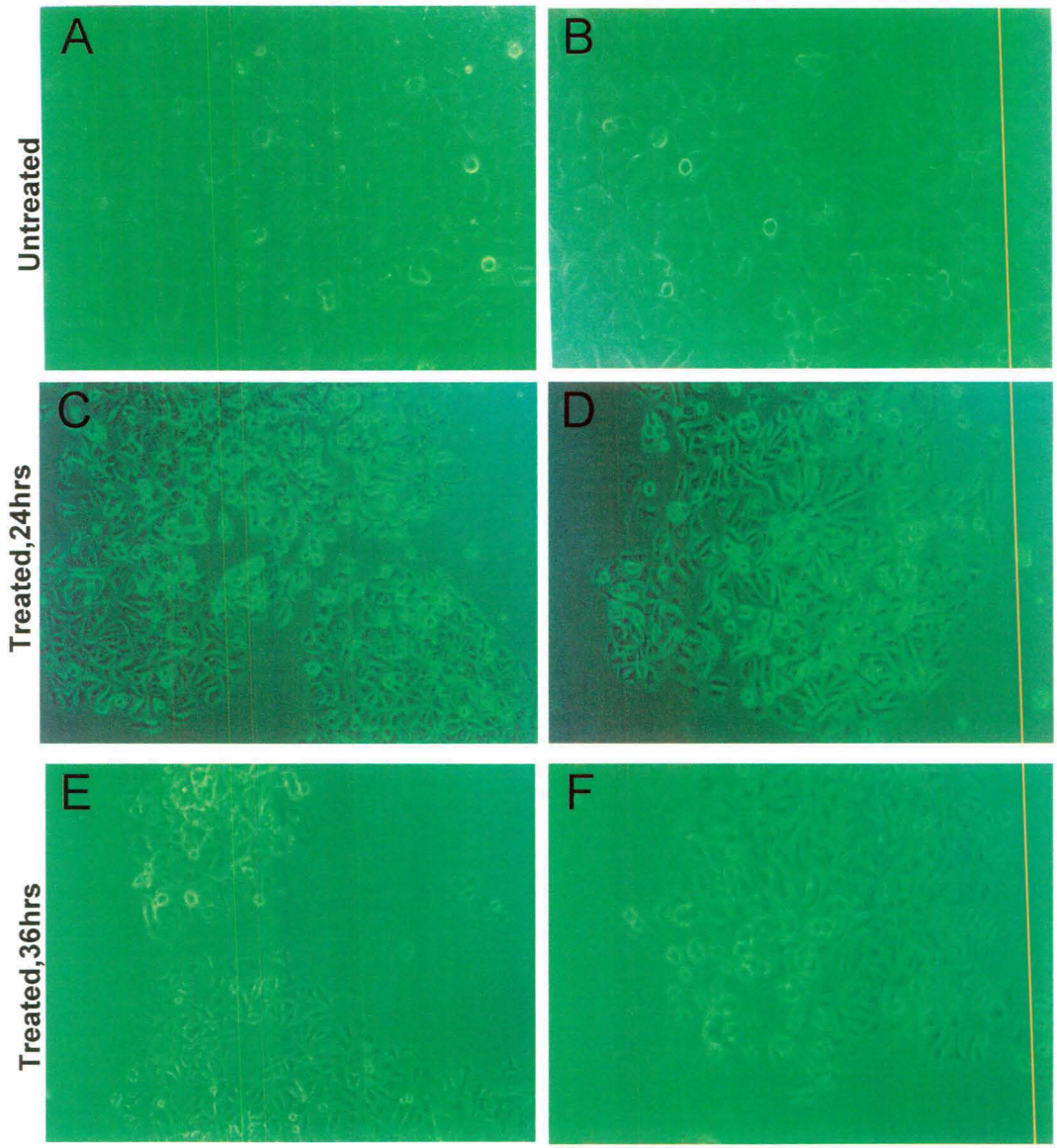


Fig.8: Epithelial to mesenchymal transition in MCF-7 cells in response to TGF-β1 treatment. A, B, Untreated MCF-7 cells in culture show intact cell-cell junctions (20x magnification). **C,D,E,F,** Cells undergoing EMT are seemingly loosing their cell-cell junctions after 24hrs and 36hrs of TGF-β1 treatment (5ng/ml) respectively (10x magnification).

Probe Sequence: 5' g g c t g a g g g t t **c a c c t g** c c g g c c a c a g c c 3'
3' c c g a c t c c c a a **g t g g a c** g g c c g g t g t c g g 5'

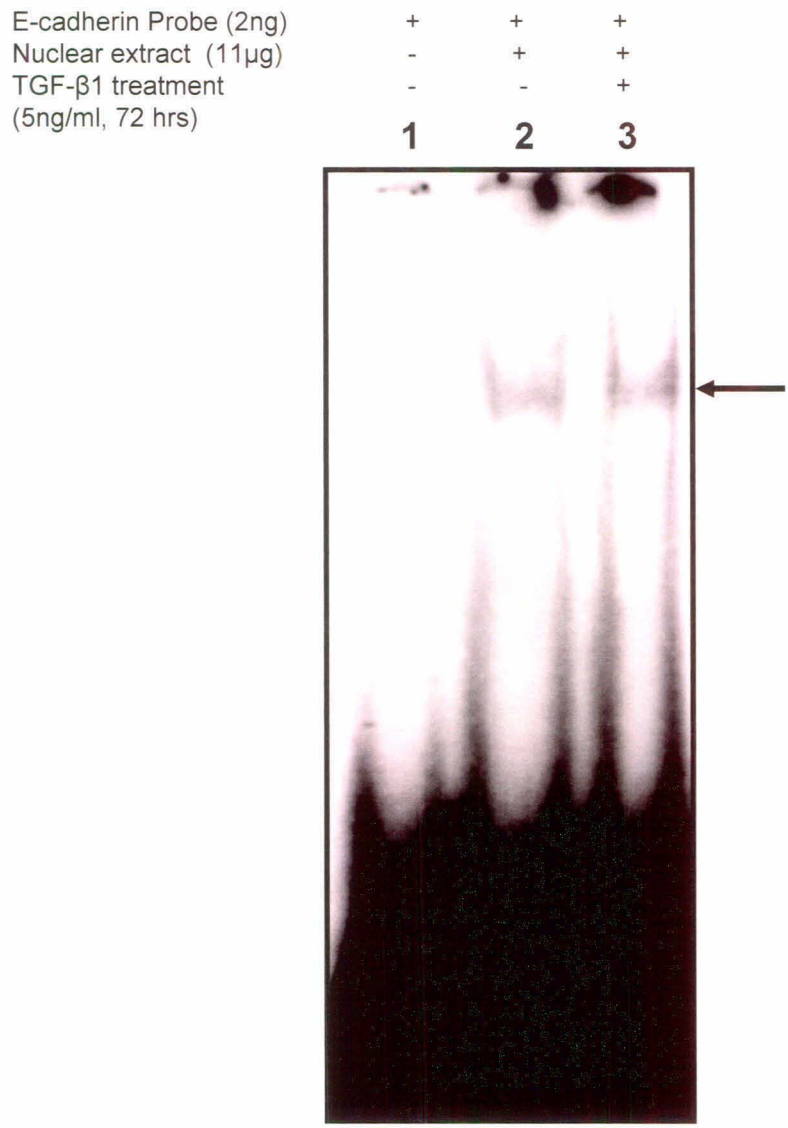


Fig.9: EMSA for E-cadherin probe with nuclear extracts of TGF-β1 treated and untreated MCF-7 cells. Two sets of MCF-7 cells were used for assay, one was kept untreated and other one was treated with TGF-β1(5ng/ml) for 72hrs. Nuclear extract (11µg protein) from both cells were incubated separately with E-cadherin probe for 30 minutes on ice. **Lane1,** Free E-cadherin probe. **Lane2,** Nuclear extract from untreated cells with E-cadherin probe showed prominent binding. **Lane 3,** Treated nuclear fraction showed binding with E-cadherin probe.
Probe sequence containing CACCTG motif in E-cadherin promoter region was used for assay and was taken as such from literature as a reference probe.

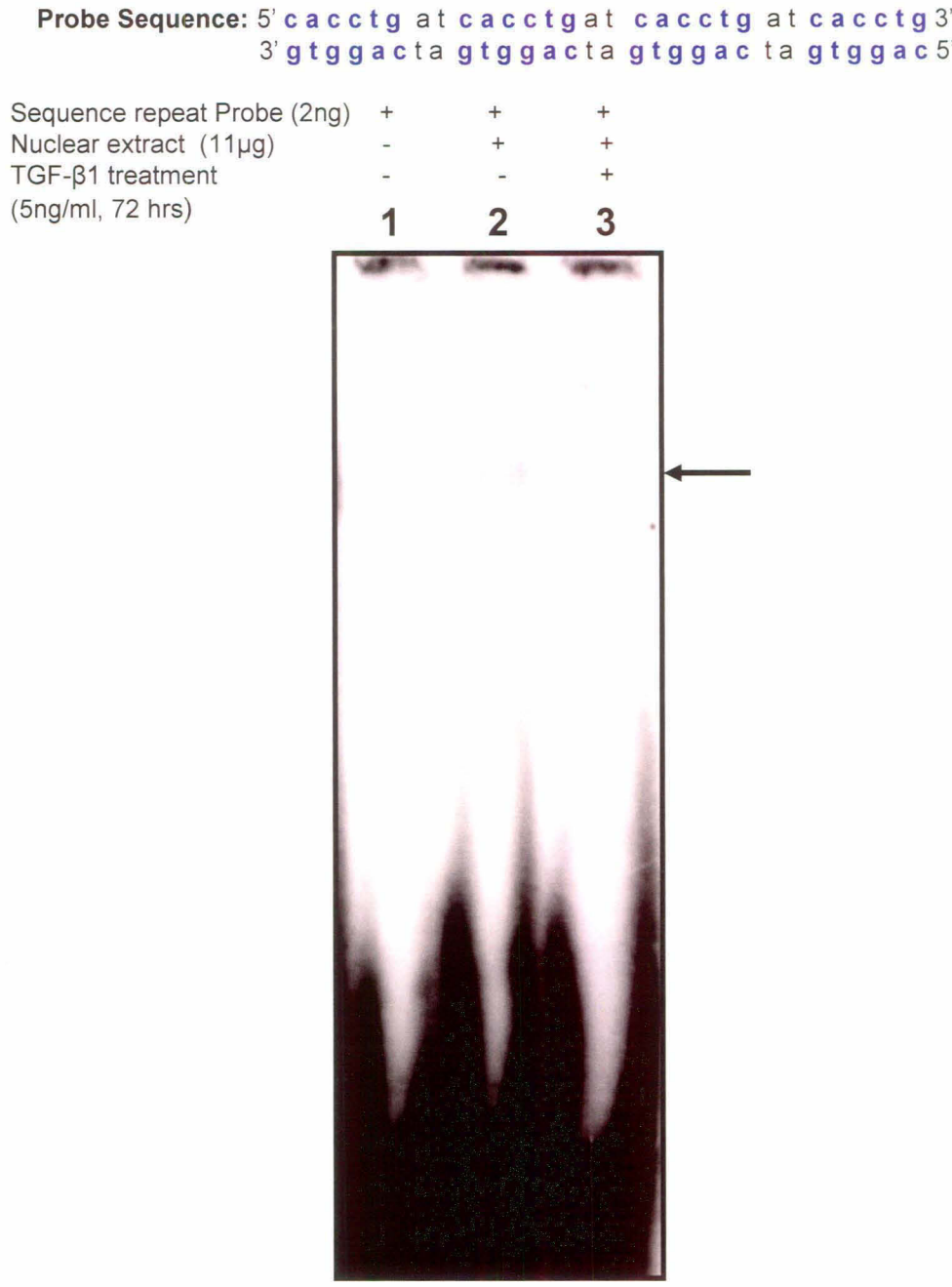


Fig.10: EMSA for CACCTG Sequence repeat (4 times) probe in both TGF-β1 treated and untreated nuclear fraction of MCF-7 cells.

Two sets of MCF-7 cells were used for assay, one was kept untreated and other one was treated with TGF-β1(5ng/ml) for 72hrs. Nuclear extract (11µg protein) from both cells were incubated separately with sequence repeat probe for 30 minutes on ice. **Lane1,** Free probe. **Lane 2,** Shift was observed for nuclear extract from untreated cells. **Lane3,** Nuclear extract from treated cells showed binding with probe.
Probe sequence containing CACCTG repeat (4 times) was used for assay.

Probe Sequence: 5' **a a c c t a** a t **a a c c t a** a t **a a c c t a** a t **a a c c t a** 3'
3' **t t g g a t** t a **t t g g a t** t a **t t g g a t** t a **t t g g a t** 5'

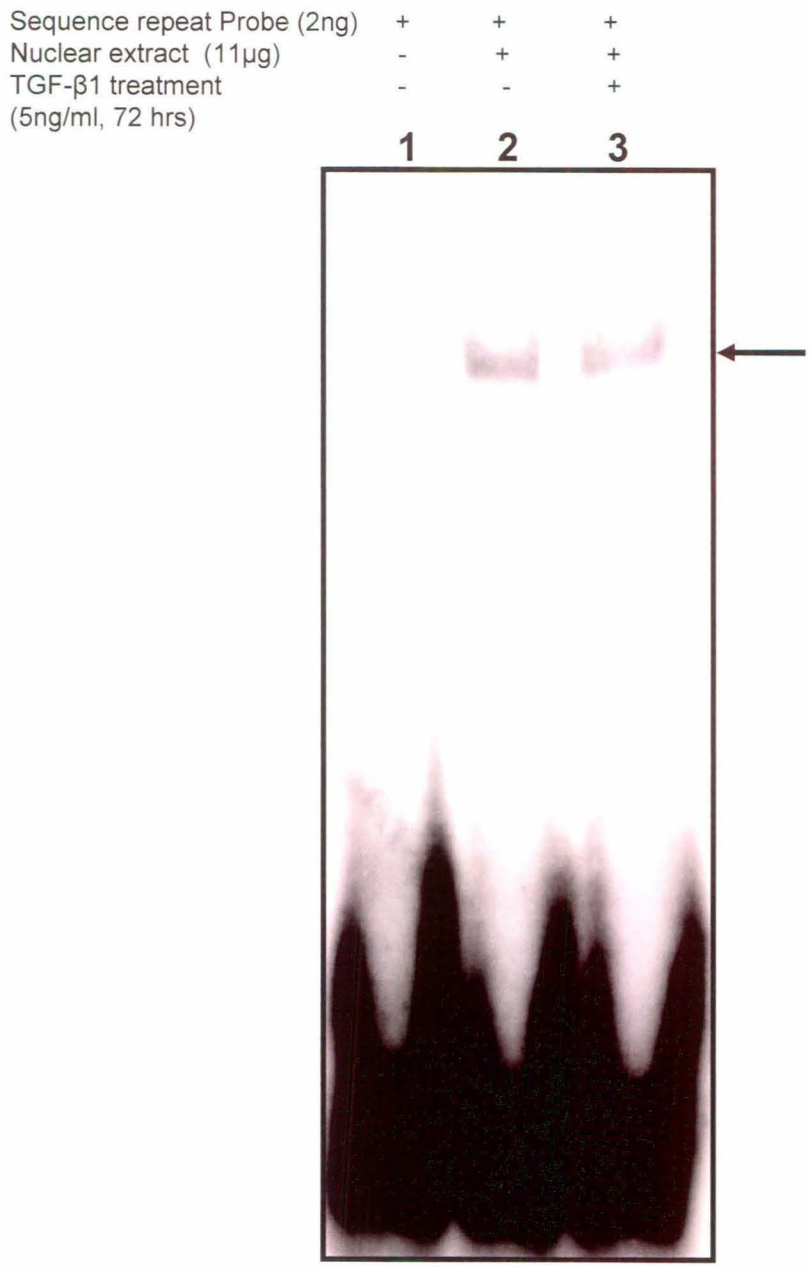


Fig.11: EMSA for AACCTA Sequence repeat (4 times) probe in both TGF-β1 treated and untreated nuclear fraction of MCF-7 cells.

Two sets of MCF-7 cells were used for assay, one was kept untreated and the other one was treated with TGF-β1 (5ng/ml) for 72hrs. Nuclear extract (11µg protein) from both cells were incubated separately with sequence repeat probe for 30 minutes on ice. **Lane1,** Free probe. **Lane 2,** Shift was observed for nuclear extract from untreated cells. **Lane3,** Nuclear extract from treated cells showed binding with probe.

Probe sequence containing AACCTA repeat (4 times) was used for assay.

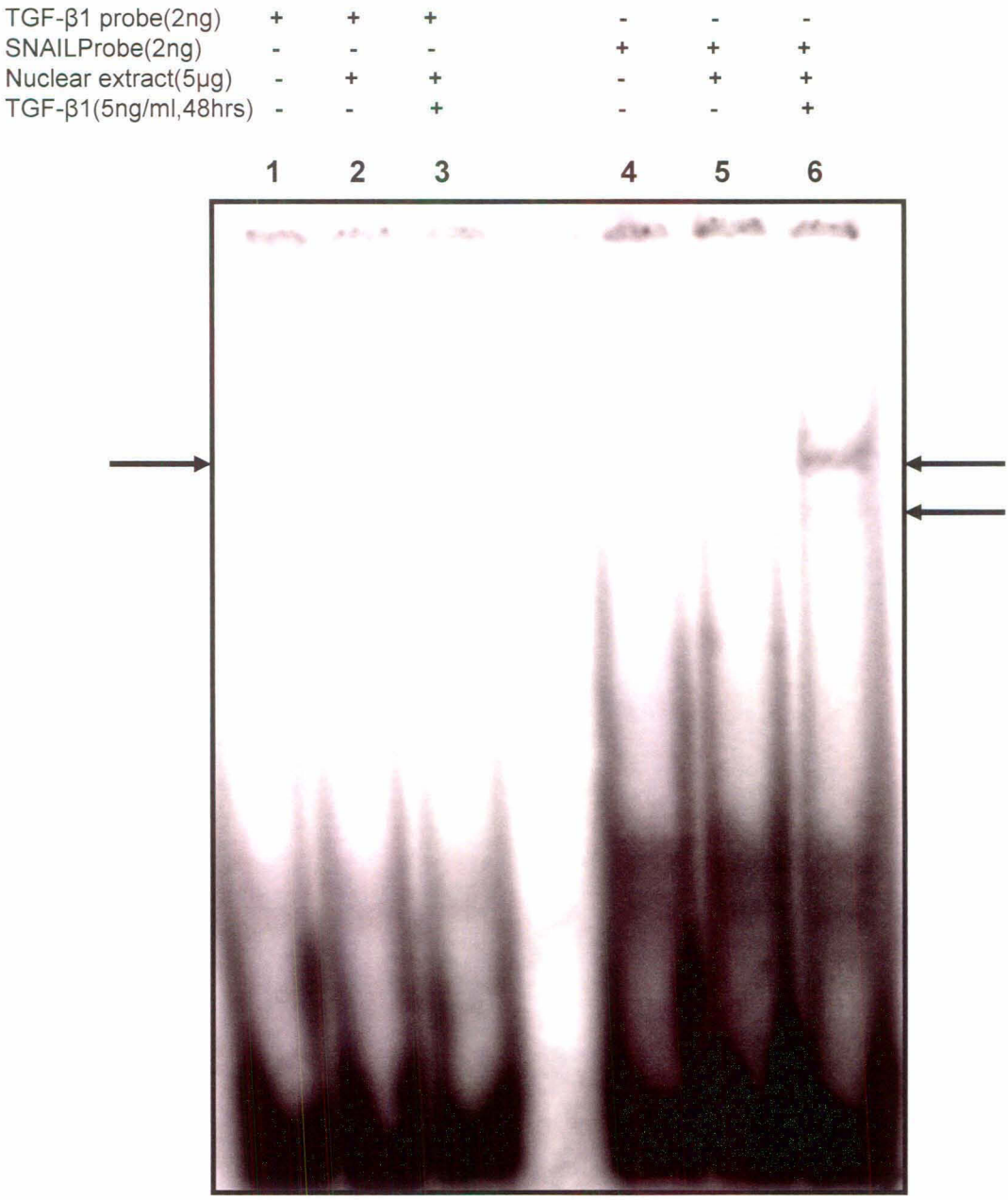


Fig.12: EMSA profile of TGF-β1 and SNAIL probes with and without treatment of TGF-β1. Two sets of MCF-7 cells were used for assay, one was kept untreated and the other one was treated with TGF-β1(5ng/ml) for 48hrs. Nuclear extract (5μg protein) from both cells were incubated with TGF-β1 probe and SNAIL probe respectively for 30 minutes on ice. **Lane1,** Free TGF-β1 probe. **Lane2,** TGF-β1 probe with nuclear extract from untreated cells showed no prominent shift. **Lane3,** Prominent shift was observed for TGF-β1 probe with nuclear extract from treated cells. **Lane4,** Free SNAIL probe. **Lane5,** SNAIL probe with nuclear extract from untreated cells showed no prominently visible shift. **Lane6,** More than one shift were observed for SNAIL probe with nuclear extract from treated cells. Prominent shift (upper one) followed by less prominent shift (lower one) are suggestive of more than one transcription factor/repressor interaction with SNAIL probe.
Shift of both probes may depend upon TGF-β1 inducible factors.

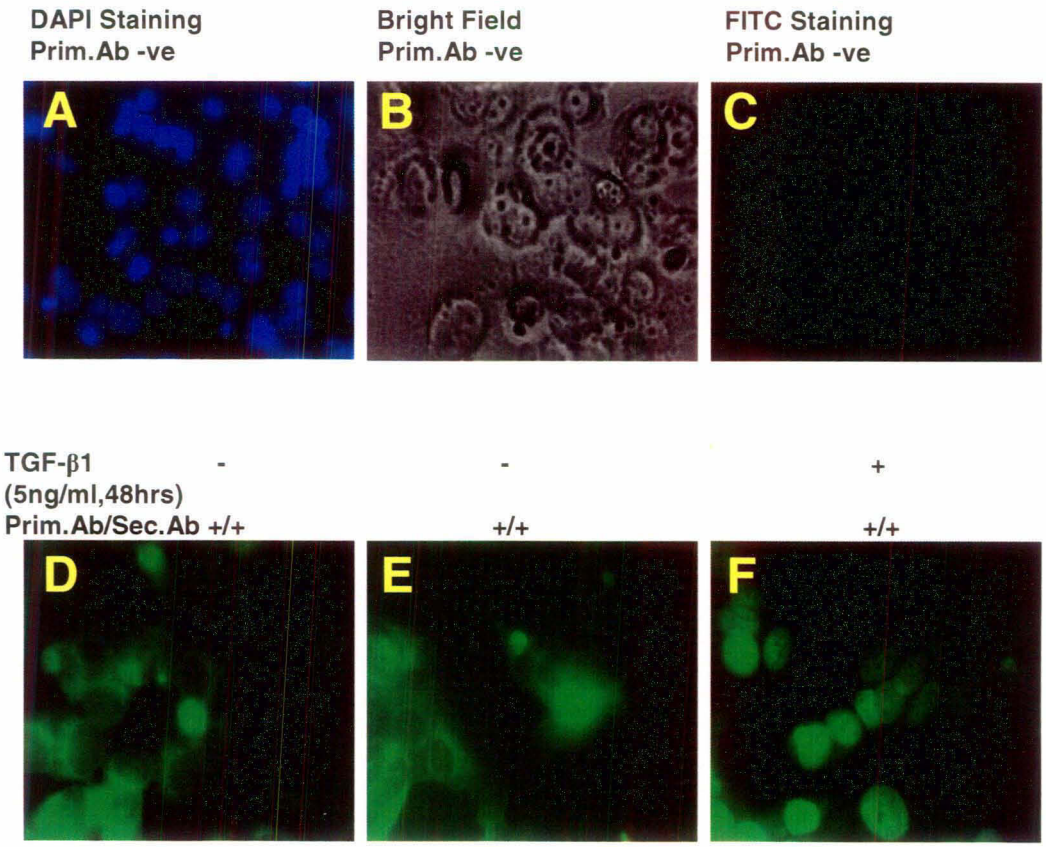


Fig.13: MCF-7 cells show nuclear localization of vimentin following TGF-β1 treatment. Cells were probed with primary monoclonal anti-vimentin and FITC-labeled secondary antibody. **A,** DAPI staining. **B,** Bright field image. **C,** ICC control, primary Ab negative cells observed for nonspecific FITC signal. **D, E,** cytosolic localization of vimentin in TGF-β1 untreated cells. **F,** nuclear localization was observed in TGF-β1 treated cells.

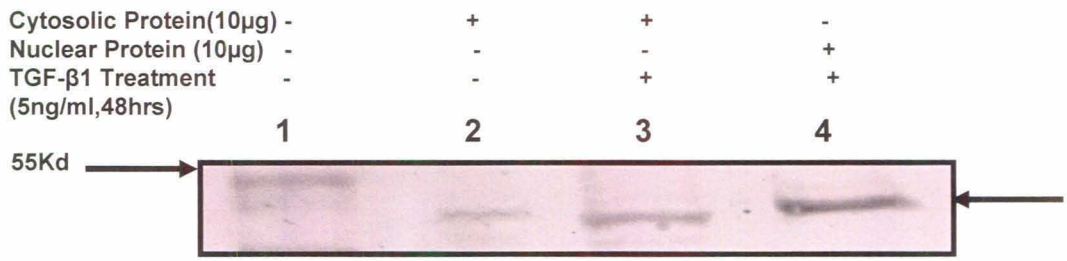


Fig.14: Western blot shows expression of vimentin in nuclear fraction of MCF-7 cells after treatment with TGF-β1. Total cytosolic proteins were extracted from untreated cells, whereas total cytosolic and nuclear proteins were extracted from TGF-β1 treated cells (5ng/ml, 48hrs) and 10µg estimated protein of each were loaded per well. HRP-conjugated secondary antibody was used against vimentin antibody and DAB as a substrate for colored precipitation. **Lane-1,** Pre-stained protein marker. **Lane-2,** Total cytosolic fraction from untreated cells shows vimentin expression. **Lane-3,** Total cytosolic fraction from treated cells shows vimentin expression. **Lane-4 ,** Shows expected band of 52Kd vimentin protein in nuclear fraction.

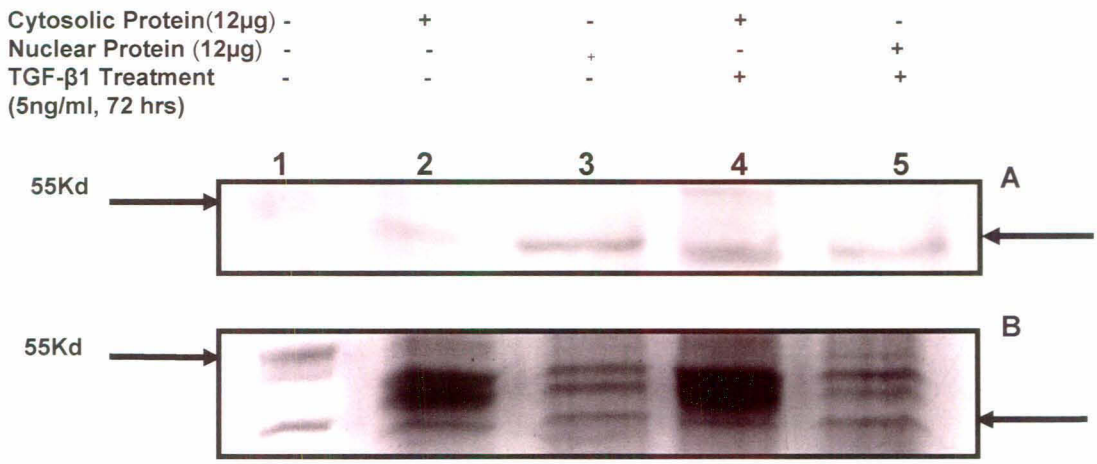


Fig.15: Western blot shows increased cytosolic expression of vimentin in TGF-β1 treated cells whereas nuclear fraction shows TGF-β1 independent expression of vimentin in MCF-7 cells. A, Total cytosolic and nuclear proteins were extracted from untreated and treated (5ng/ml,72hrs) cells. Estimated protein (12µg) of each were loaded separately per well. HRP-conjugated secondary antibody was used against vimentin antibody and DAB as a substrate for colored precipitation. **Lane-1,** Pre-stained protein marker. **Lane-2,** Untreated cytosolic fraction shows expected band of 52Kd of vimentin. **Lane-3,** Untreated nuclear fraction shows presence of vimentin expression irrespective of TGF-β1 treatment. **Lane-4,** Total cytosolic fraction from treated cells shows increased expression of vimentin compared to cytosolic expression from untreated cells. **Lane-5,** Total nuclear fraction from treated cells shows expression of vimentin. **B,** SDS-PAGE(10%) profile of nuclear and cytosolic proteins in TGF-β1 treated and untreated MCF-7 cells. Marker protein of 55Kd was taken as a reference for the expected band of 52 Kd vimentin protein in western blotting.

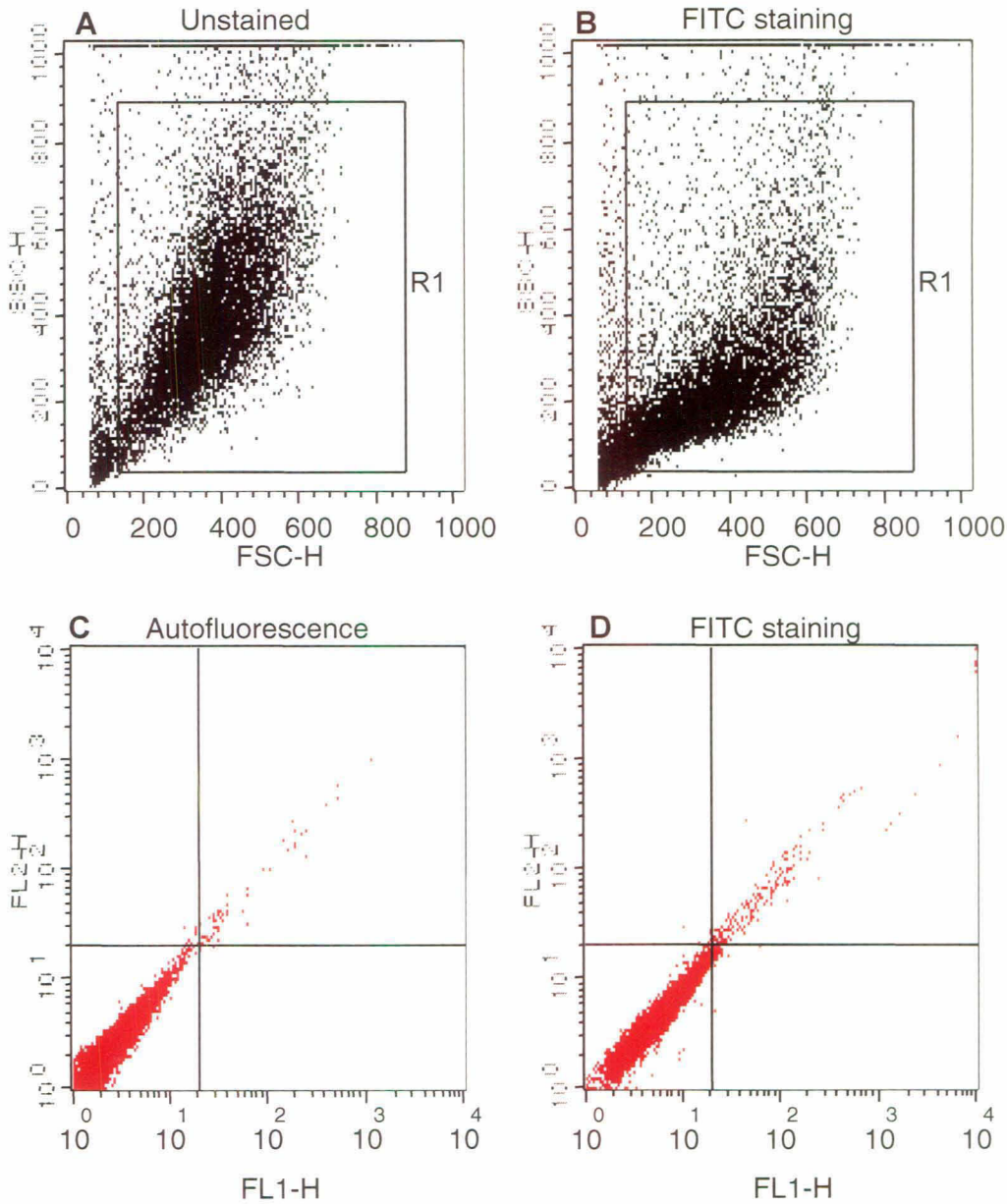


Fig.16: Flowcytometric analysis of MCF-7 cells for autofluorescence and non specific FITC fluorescence.

A, **B**, Cell populations were selected from gate R1 for dot plot analysis. **C**, Quadrant dot plot represents autofluorescence of selected unstained cell population (0.66%). **D**, Non specific FITC staining without primary antibody showed nonsignificant signal (2.93%).

SSC-Side light scatter, FSC-Forward light scatter, FL1-H-FITC fluoresecenc intensity (X-axis), FL2-H-Cell count (Y-axis)

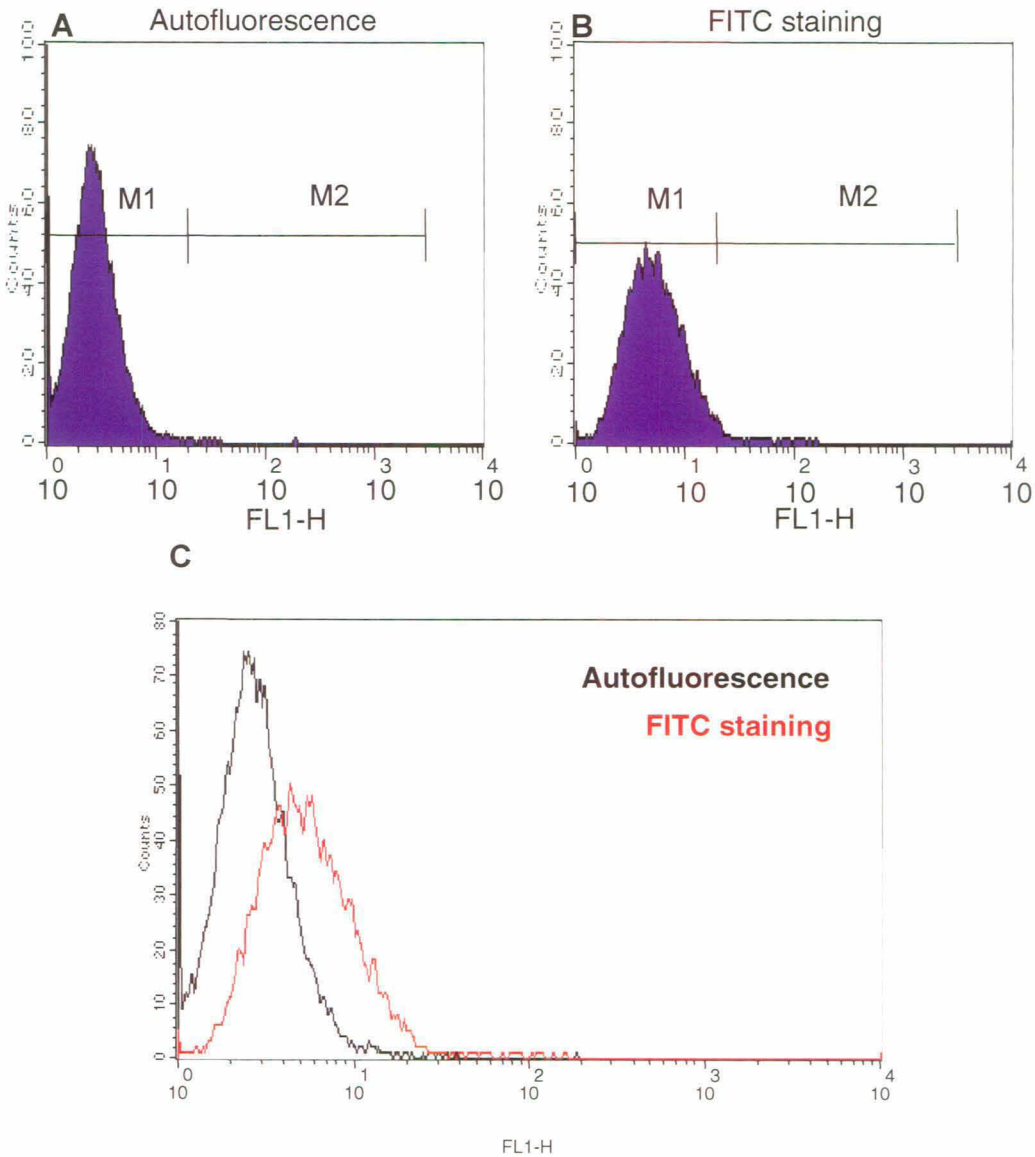


Fig.17: Histogram plot corresponds to autofluorescence and FITC stained MCF-7 cells.
A, Histogram plot of autoflourescence of unstained cells, marker M1 represents selected unstained population whereas, M2 shows cells among population with autofluorescence. **B**, Histogram plot of non specific FITC staining shows slight increase in fluorescence (M2) in comparison to autoflourescence. **C**, Overlay plot of autoflourescence and FITC staining.

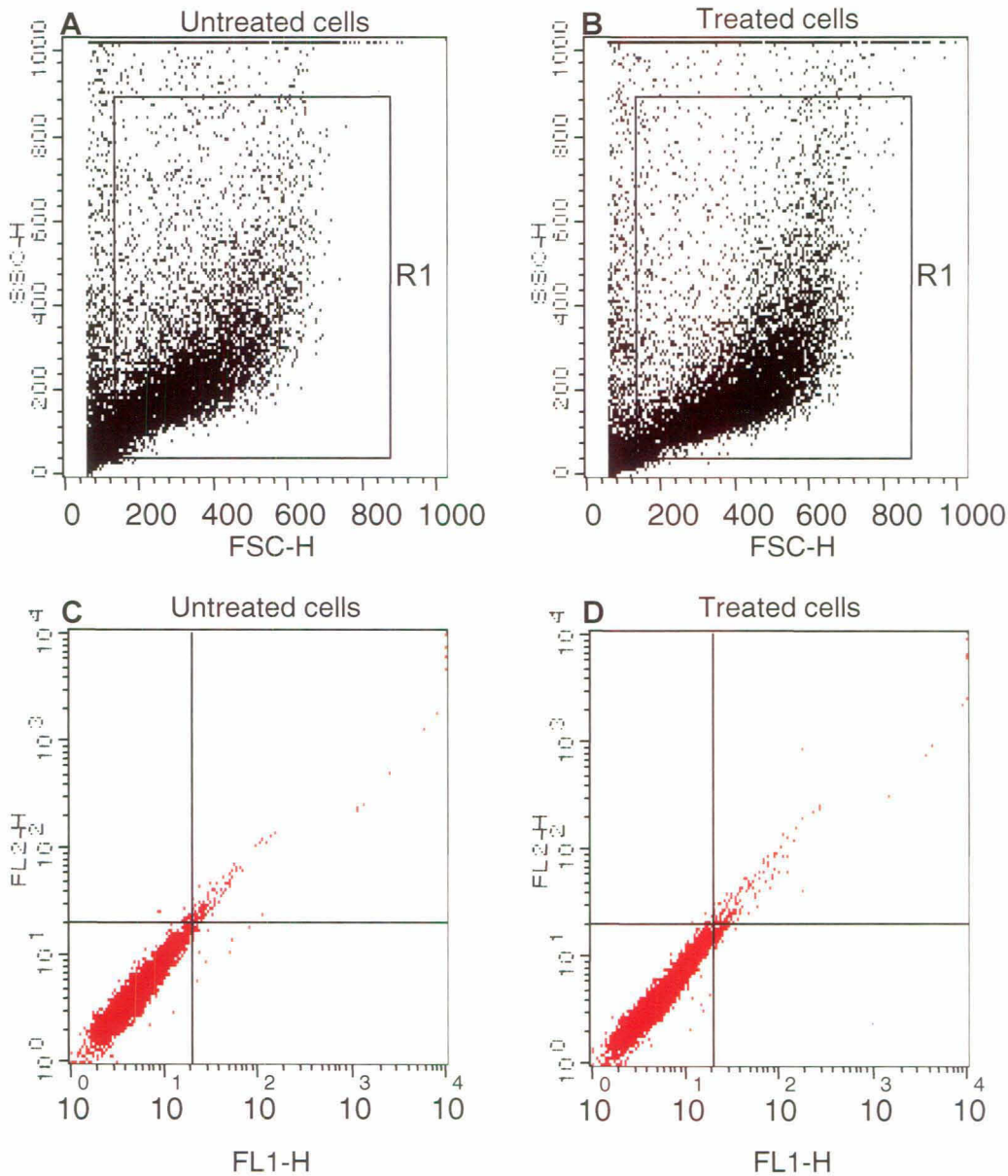


Fig.18: Expression analysis of vimentin in TGF-β1 treated and untreated MCF-7cells. Cells were treated with TGF-β1 (5ng/ml) for 72hrs. Prim.Ab. against vimentin was employed for intracellular probing of vimentin in both untreated and treated cells. FITC-labeled sec. antibody was used against vimentin antibody. **A**, **B**, Represents cell populations selected from gate R1 for dot plot analysis from untreated and treated cells respectively. **C**, **D**, Quadrant plot shows expression of vimentin in terms of fluorescence intensity in untreated cells (2.31%) and treated cells (3.05%).

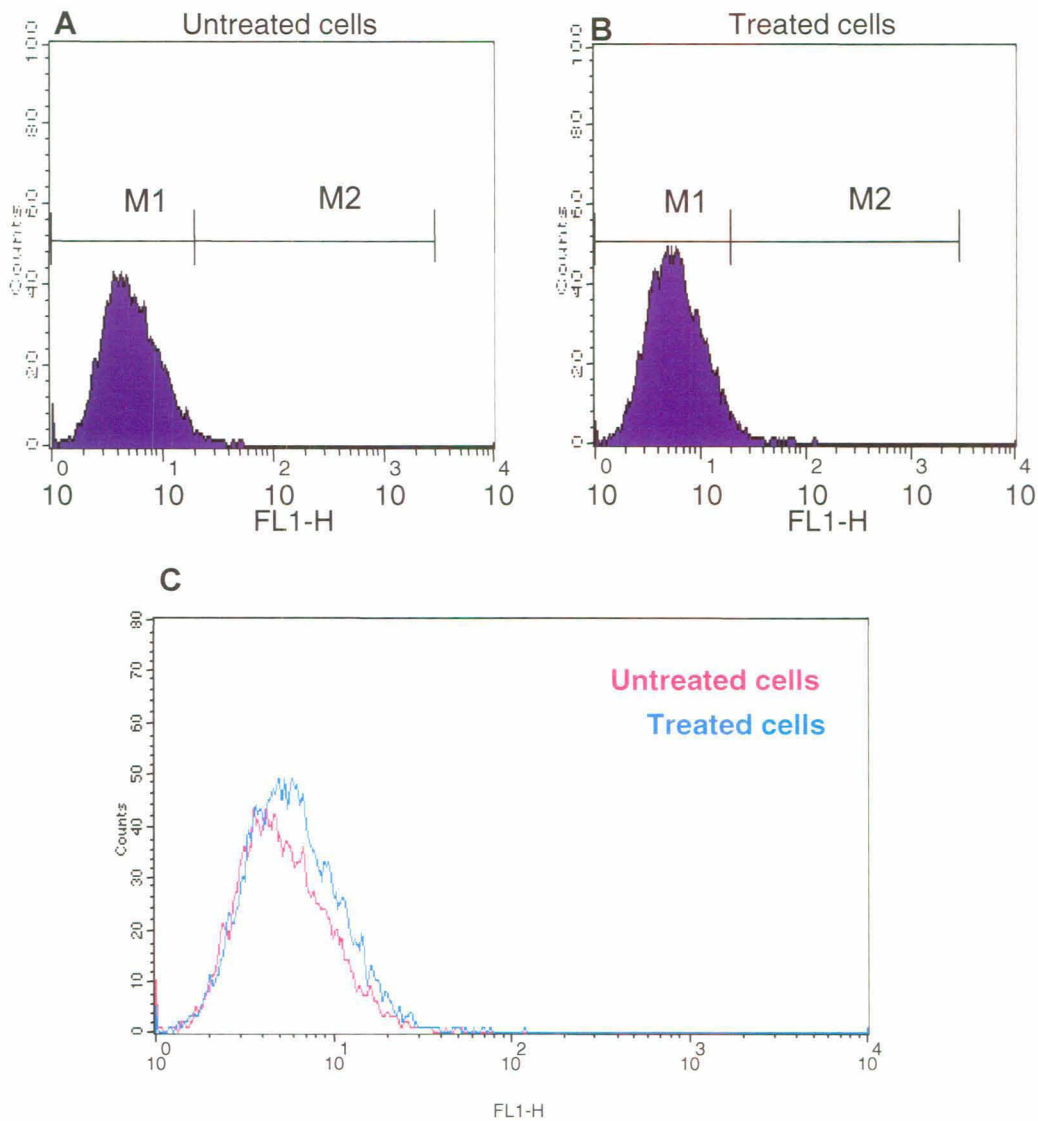


Fig.19: Histogram plot corresponds to vimentin expression in TGF-β1 treated and untreated MCF-7 cells.

A, Histogram plot of untreated cells shows no significant shift of vimentin stained cells in M2 region (2.31%) from unstained M1 region (97.64%). **B**, Treated cells shows slight, but nosignificant shift of vimentin stained cells in M2 region (3.05%). **C**, Overlapping plot shows absence of any significance change in vimentin expression in both treated and untreated cells.

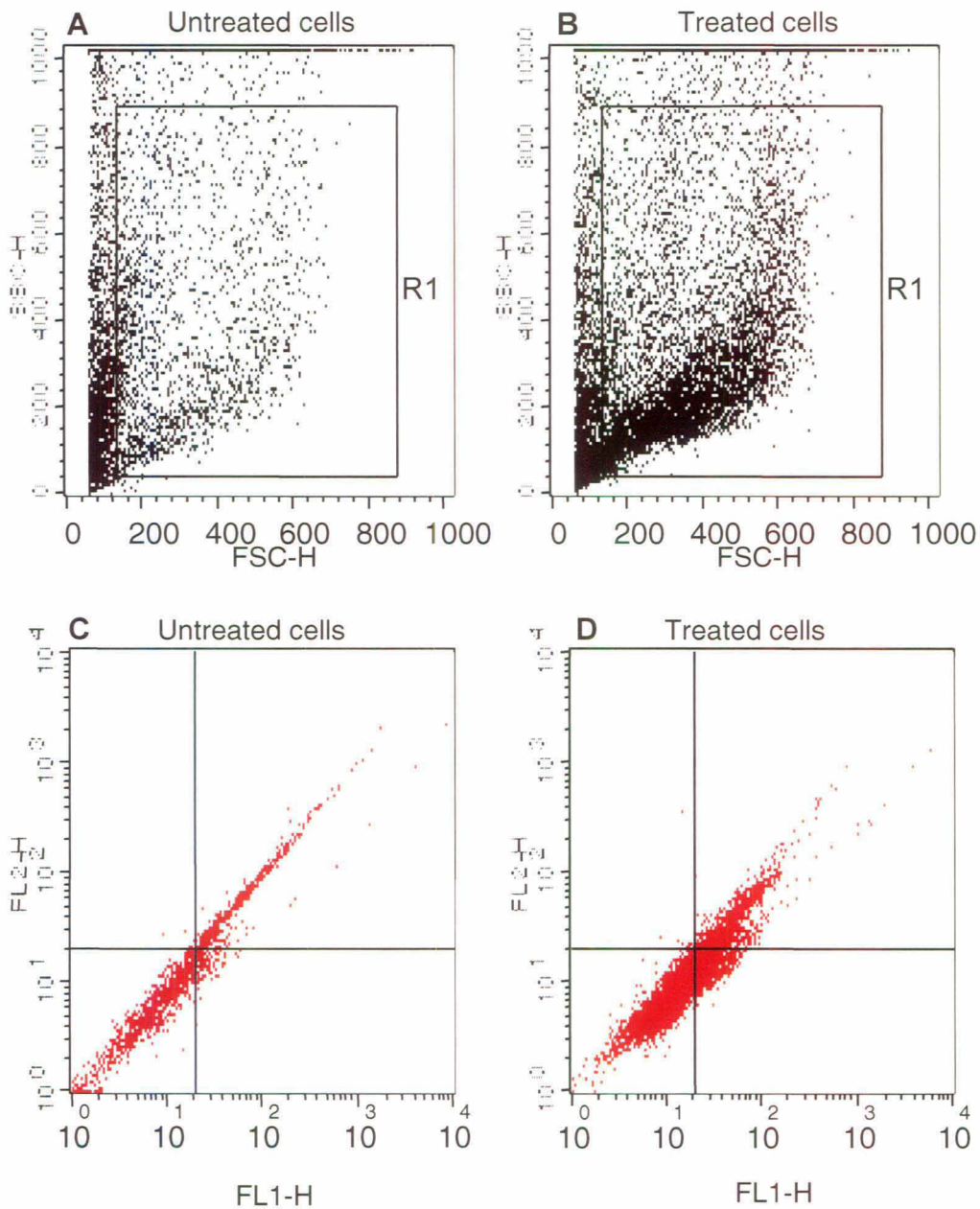


Fig.20: Expression analysis of cytokeratin18 (CK18) in TGF-β1 treated and untreated MCF-7cells.

Cells were treated with TGF-β1 (5ng/ml) for 72hrs. Prim.Ab. against cytokeratin18 was employed for intracellular probing of cytokeratin18 in both untreated and treated cells. FITC-labled sec. antibody was used against cytokeratin18 antibody. **A**, **B**, Represents cell populations selected from gate R1 for dot plot analysis from untreated and treated cells respectively. **C**, **D**, Quadrant plot shows marginal higher expression of CK18 in terms of fluorescence intensity in treated cells (**D**-36.15%) in comparison to untreated cells (**C**-33.26%).

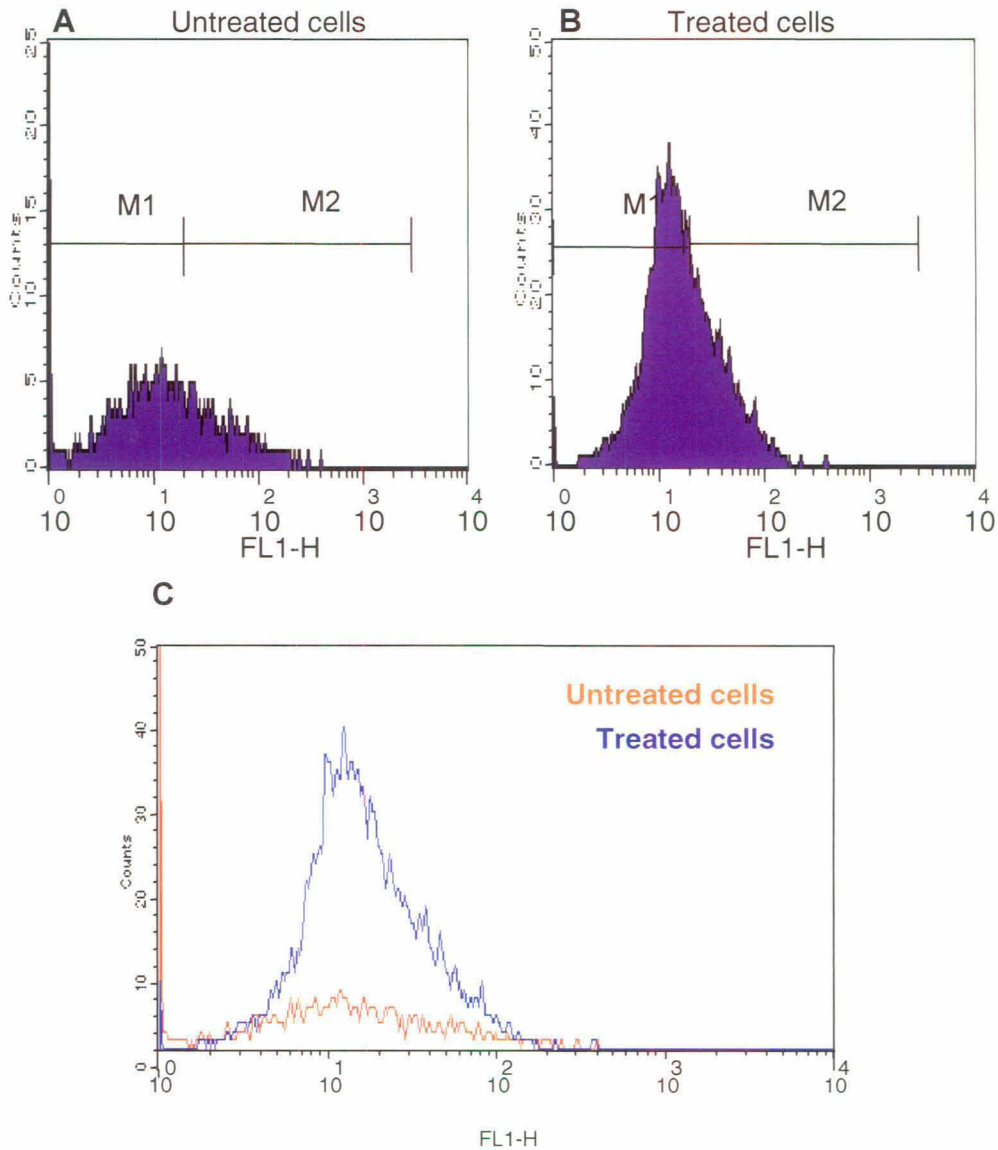


Fig.21:Histogram plot corresponds to cytokeratin18 (CK18) expression in TGF-β1 treated and untreated MCF-7 cells.

A, Histogram plot of untreated cells shows considerable shift of CK18 stained cells in M2 region (33.26%) from unstained M1 region (66.69%). **B**, Treated cells shows slightly marginal shift of CK18 stained cells in M2 region (36.15%) in comparison to CK18 staining in untreated cells. **C**, Overlapping plot shows marginal change in CK18 expression in both treated and untreated cells.

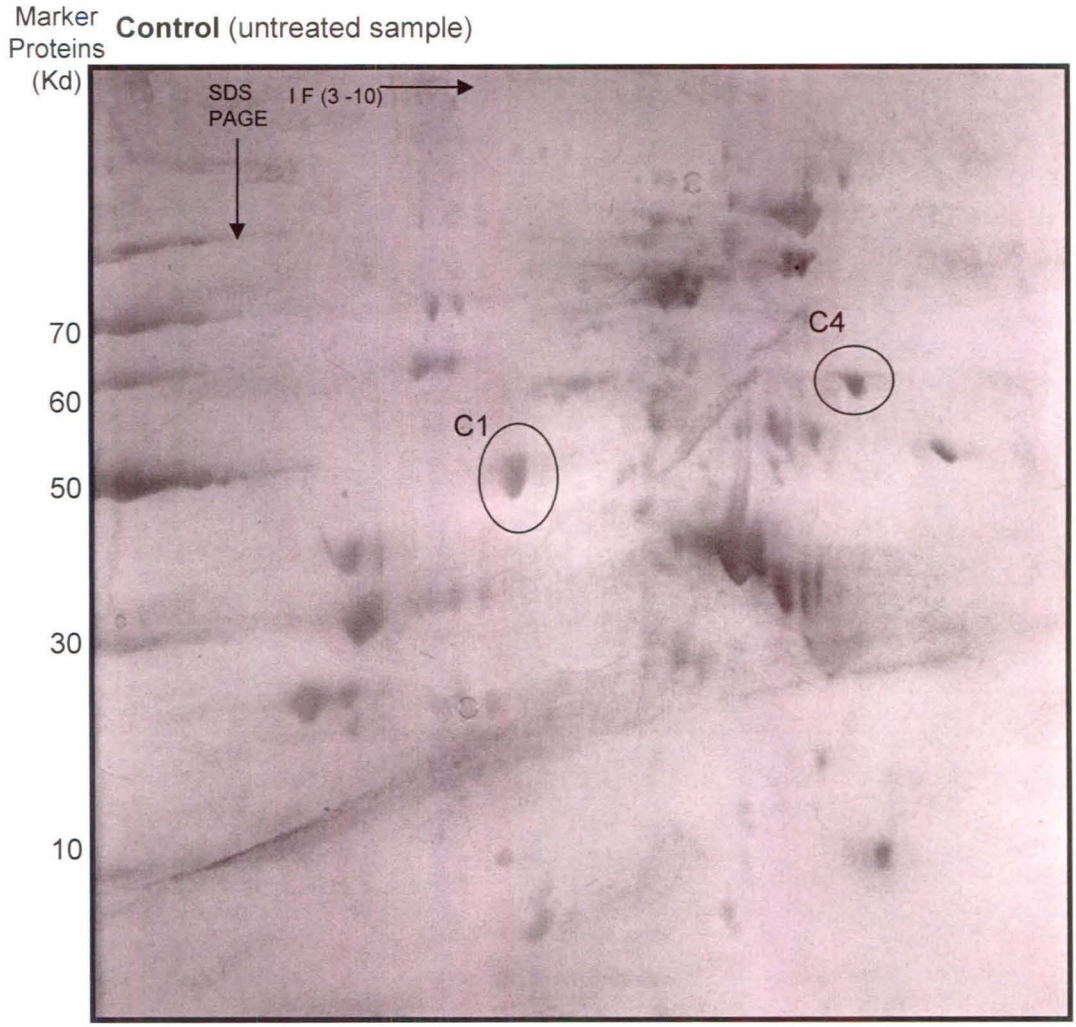


Fig.22: 2D electrophoresis profile of cytosolic proteins of MCF-7 cells. (Focussed on 7cm strip)

Total cytosolic protein was extracted from cells and 60µg of estimated protein was loaded on 7cm, 3-10 linear IEF-strip for electrofocussing. SDS-PAGE (12%) was run for second dimension along with marker proteins.

C-Untreated

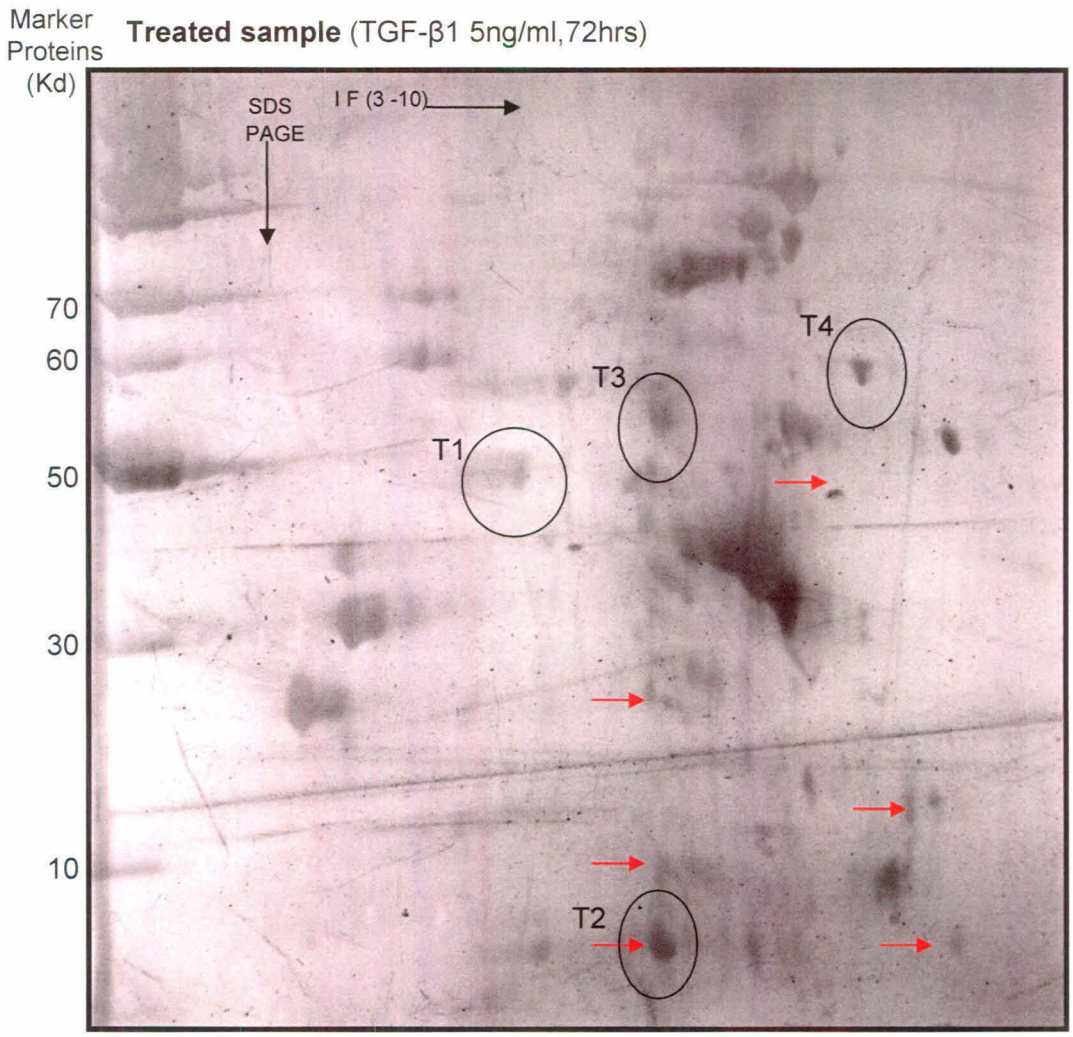


Fig.23: 2D electrophoresis profile of cytosolic proteins of TGF-β1 treated MCF-7 cells. (Focussed on 7cm strip)

Cells were treated with TGF-β1(5ng/ml,72 hrs). Total cytosolic protein was extracted and 60µg of estimated protein was loaded on 7cm, 3-10 linear IEF-strip for electrofocusing. SDS-PAGE (12%) was run for second dimension along with marker proteins.

T-Treated

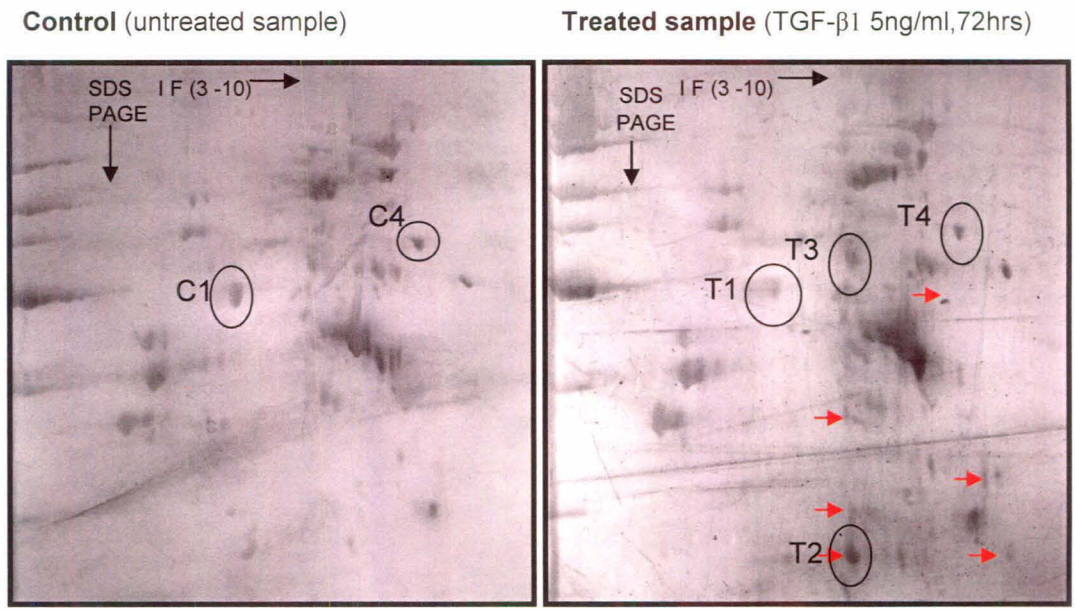


Fig.24: 2D gels of MCF-7 cellular proteins with representative spots corresponds to differential expression and markers of EMT under study. (Focussed on 7cm strip)

Spots with circles were screened for the above mentioned markers. **C1** and **T1** represents Enolase from untreated and TGF-β1 treated cells respectively. **T2** represents HSP-27 with differential expression in treated cells. **C4, T4** and **T3** needs further screening for confirmation of Cytokeratins. Spots shown with arrow represents differential expression in treated cells. Most of the proteins under study fall within 4.5 to 6.0 pH range and need to be separated further by using 4-7 pH gradient strip.

C-Untreated, **T**-Treated

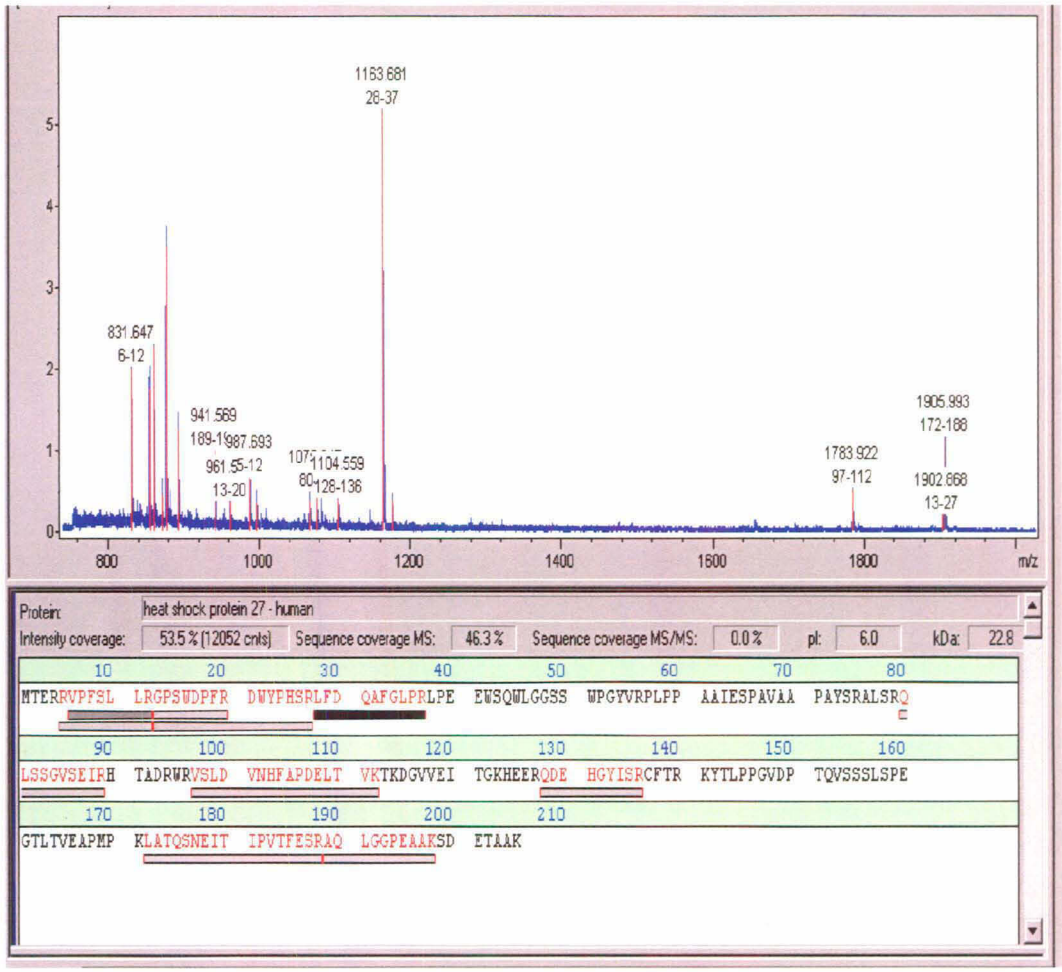
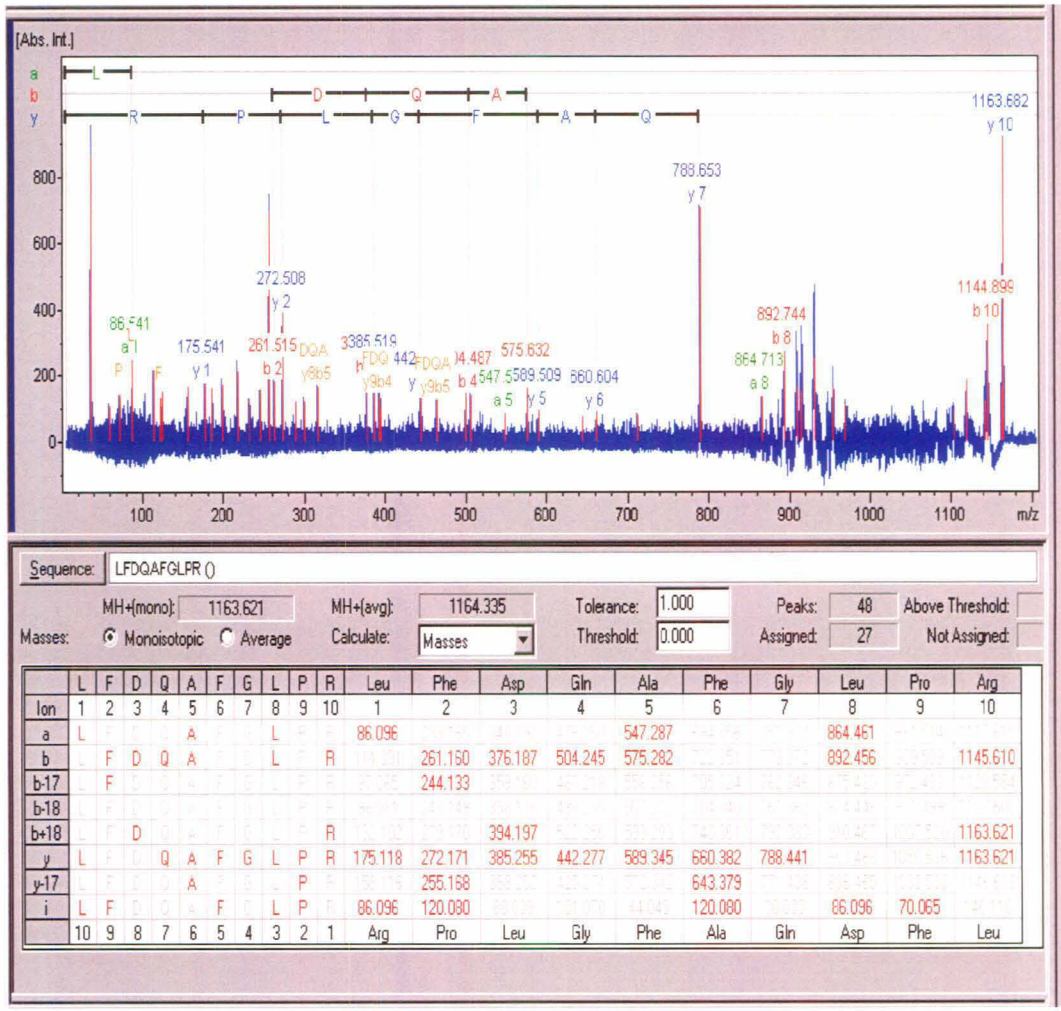


Fig.25: PMF spectrum suggests human Heat shock protein-27.

Peptide peak of mass1163.681corresponds to HSP-27, **LFDQAFGLPR** along with sequence coverage map showing intensity coverage-53.5%, sequence coverage- 46.3%, pI-6.0, kDa-22.8.



	L	F	D	Q	A	F	G	L	P	R	Leu	Phe	Asp	Gln	Ala	Phe	Gly	Leu	Pro	Arg
Ion	1	2	3	4	5	6	7	8	9	10	1	2	3	4	5	6	7	8	9	10
a	L				A			L			86.096				547.287			864.461		
b		F	D	Q	A			L		R		261.150	376.187	504.245	575.282			892.456		1145.610
b-17		F										244.133								
b-18																				
b+18			D							R			394.197							1163.621
y	L			Q	A	F	G	L	P	R	175.118	272.171	385.255	442.277	589.345	660.382	788.441			1163.621
y-17					A				P			255.168				643.379				
i	L	F				F		L	P		86.096	120.080				120.080		86.096	70.065	
	10	9	8	7	6	5	4	3	2	1	Arg	Pro	Leu	Gly	Phe	Ala	Gln	Asp	Phe	Leu

Fig.26: MS/MS spectrum of m/z1163.681 (LFDQAFGLPR) corresponds to HSP-27.

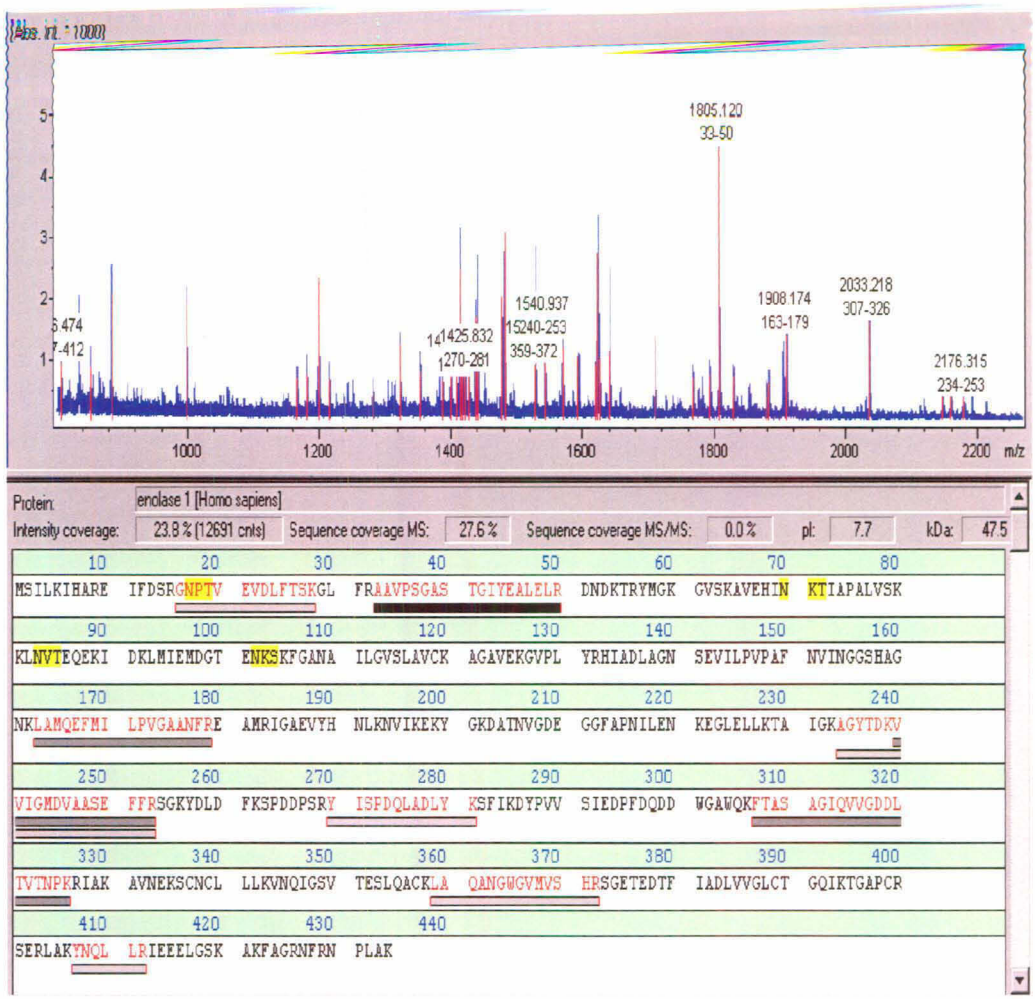


Fig.27: PMF spectrum suggests human Enolase.

Peptide peak of mass1805.120 corresponds to enolase peptide fragment AAVPSGASTGIYEALELR along with sequence coverage map showing intensity coverage-23.8%, sequence coverage- 27.6%, pI-7.7, kDa-47.5.

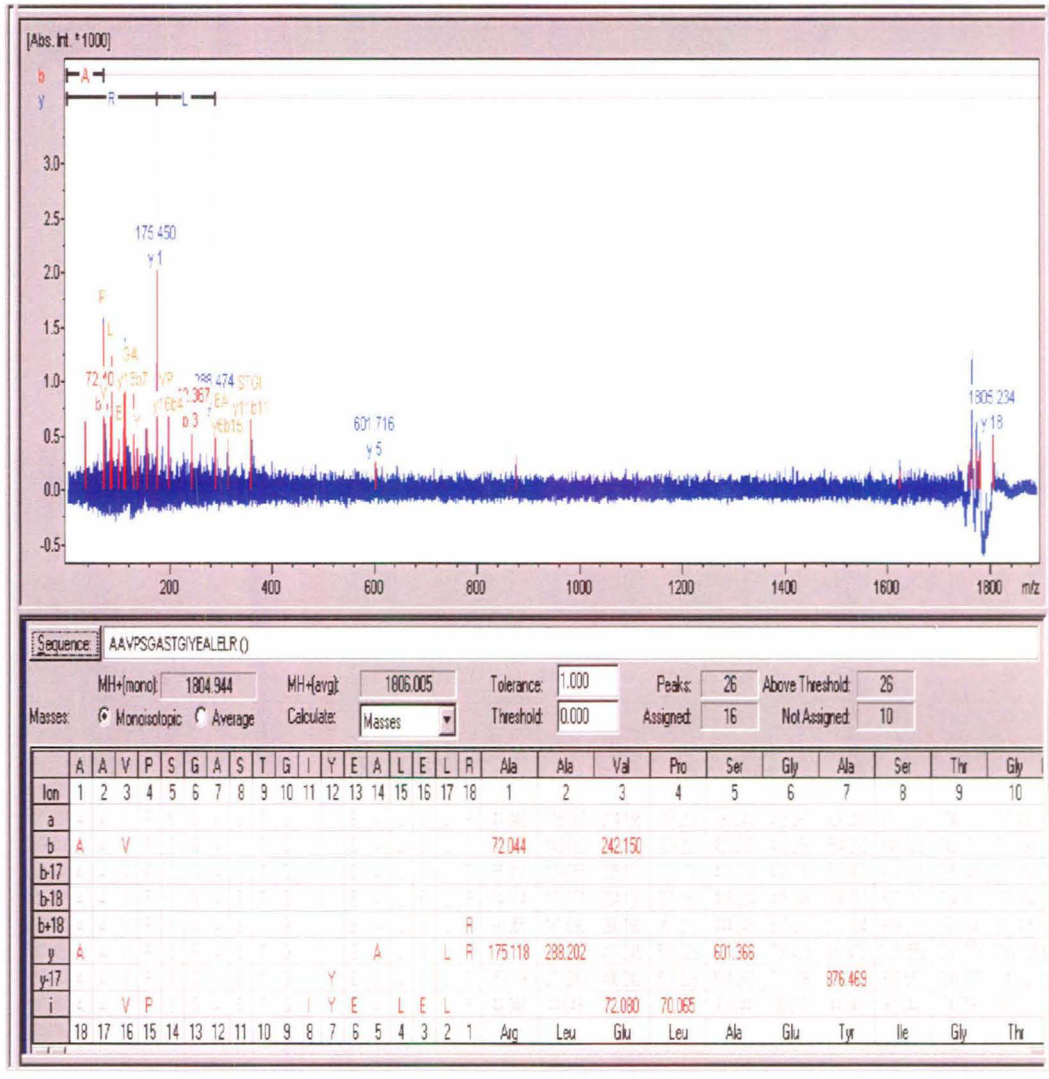


Fig.28: MS/MS spectrum of m/z1805.120 (AAVPSGASTGIYEALELR) corresponds to enolase.

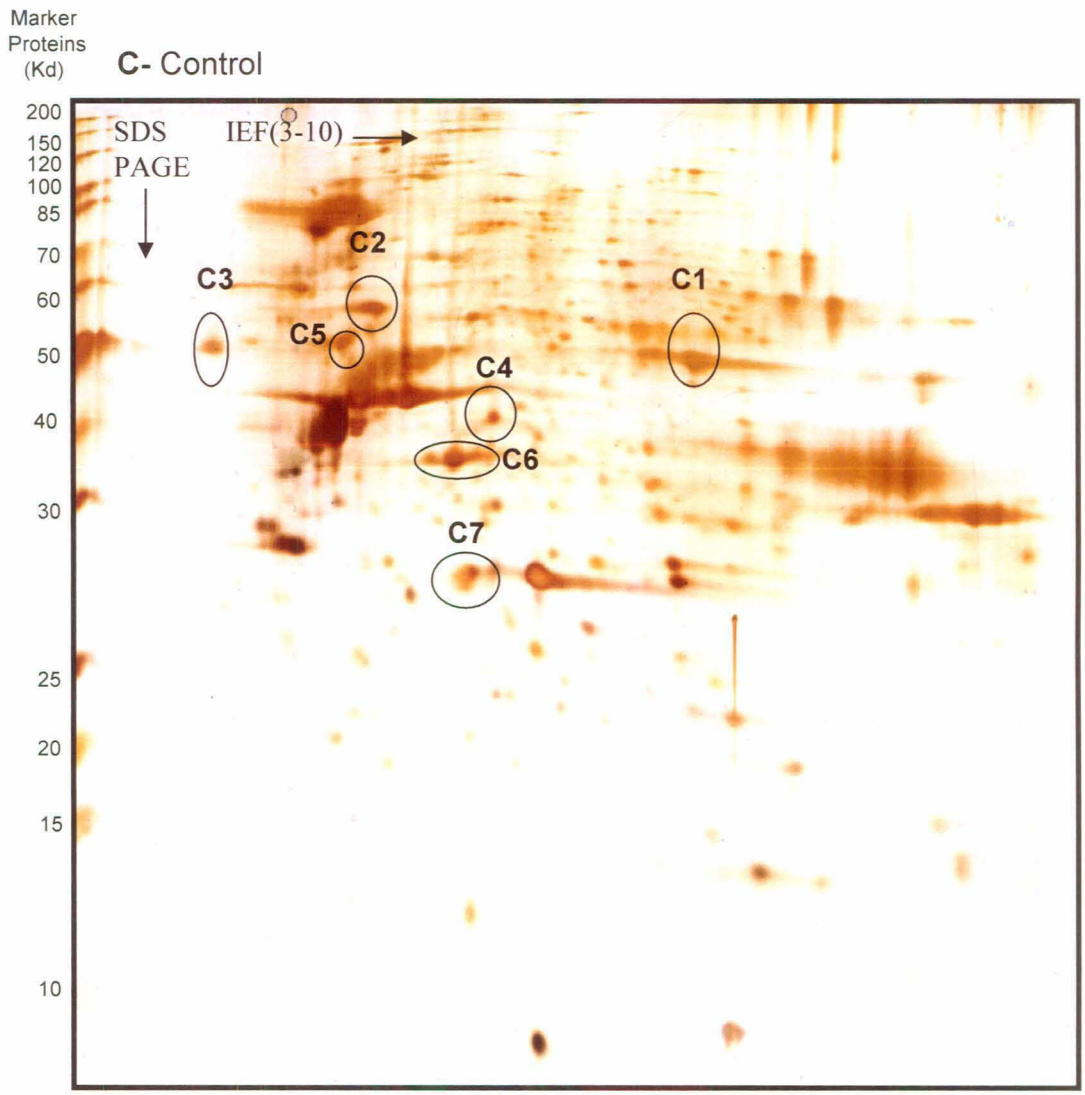


Fig.29: 2D electrophoresis profile of cytosolic proteins of MCF-7 cells.
(Focussed on 7cm strip)

Total cytosolic protein was extracted from cells and 200µg of estimated protein was loaded on 18 cm 3-10 linear IEF-strip for electrofocussing. SDS-PAGE (12%) was run for second dimension along with marker proteins.

Circled spots were given for MALDI-TOF analysis.

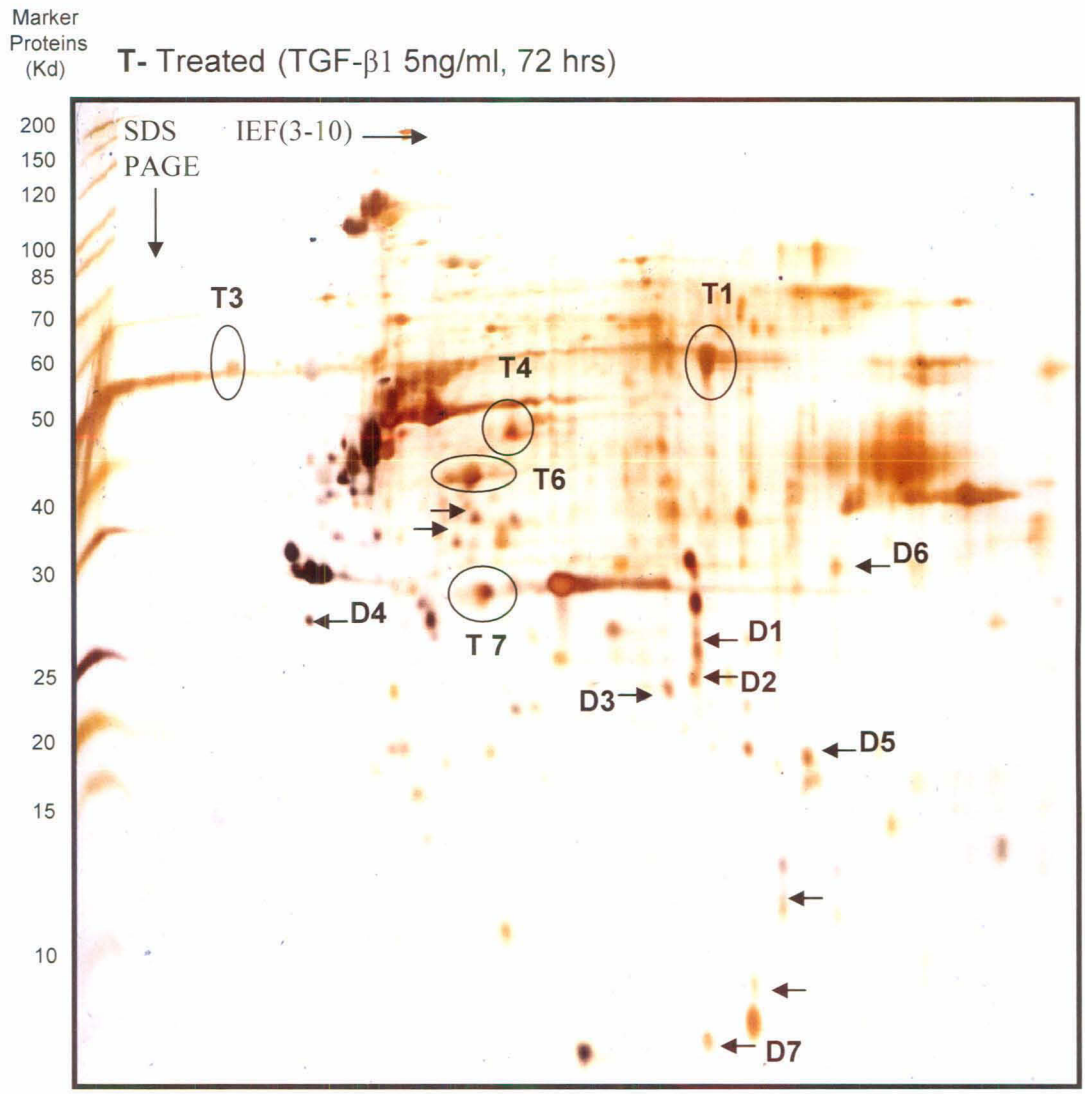


Fig.30: 2D electrophoresis profile of cytosolic proteins of TGF-β1 treated MCF-7 cells. (Focussed on 7cm strip)

Cells were treated with TGF-β1(5ng/ml,72 hrs). Total cytosolic protein was extracted and 200µg of estimated protein was loaded on 18cm, 3-10 linear IEF-strip for electrofocussing. SDS-PAGE (12%) was run for second dimension along with marker proteins. Spots with circle and arrow were given for MALDI-TOF analysis.

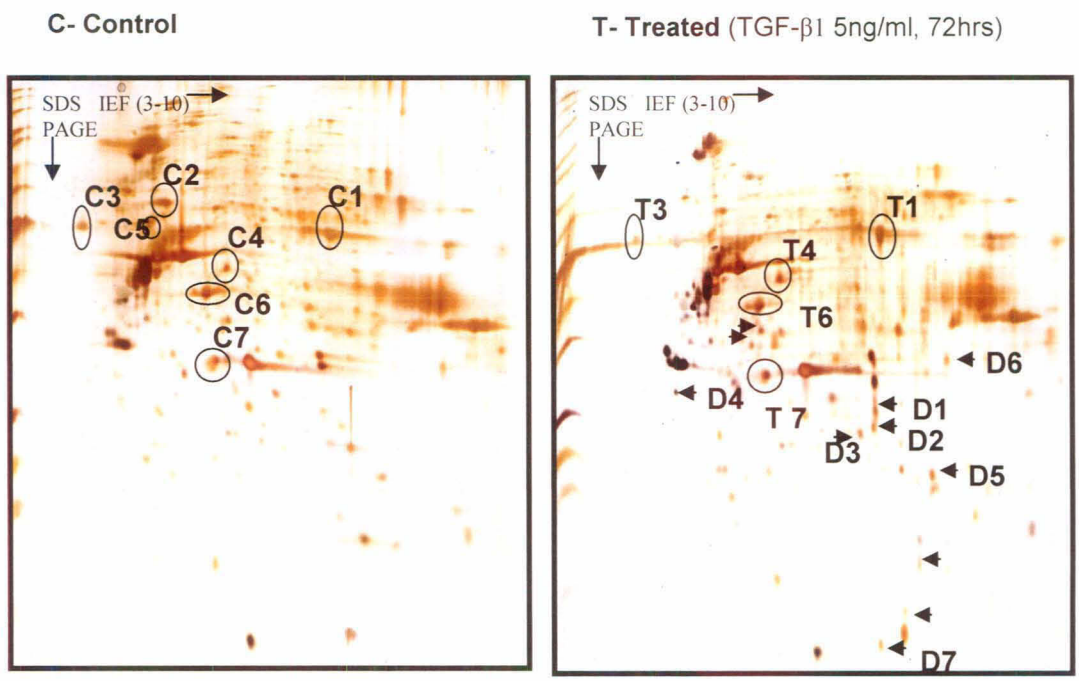


Fig.31: 2D gels of MCF-7 cellular proteins with representative spots corresponds to differential expression and markers of EMT under study. (Focussed on 7cm strip)

Spots with circles and arrows were screened for the EMT related markers. **C1** and **T1** represents Enolase from untreated and TGF-β1 treated cells respectively. **C2, C5** represents Alpha and Beta tubulin respectively. **C3, T3** represents Calreticulin precursor protein. **C4, T4** represents Beta actin protein. **C7, T7** represents TUBB protein. **D1, D2, D3, D4, D5, D6, D7** represents Differential expression. **D1, D2**-Triose phosphate isomerase 1 isoform respectively. **D3**-Heat shock protein-27. **D4**-Monooxygenase activation protein. **D5**-Cytokeratin16. **D6**-Transcription factor Elongin A2. **D7**-Ubiquitin protein.

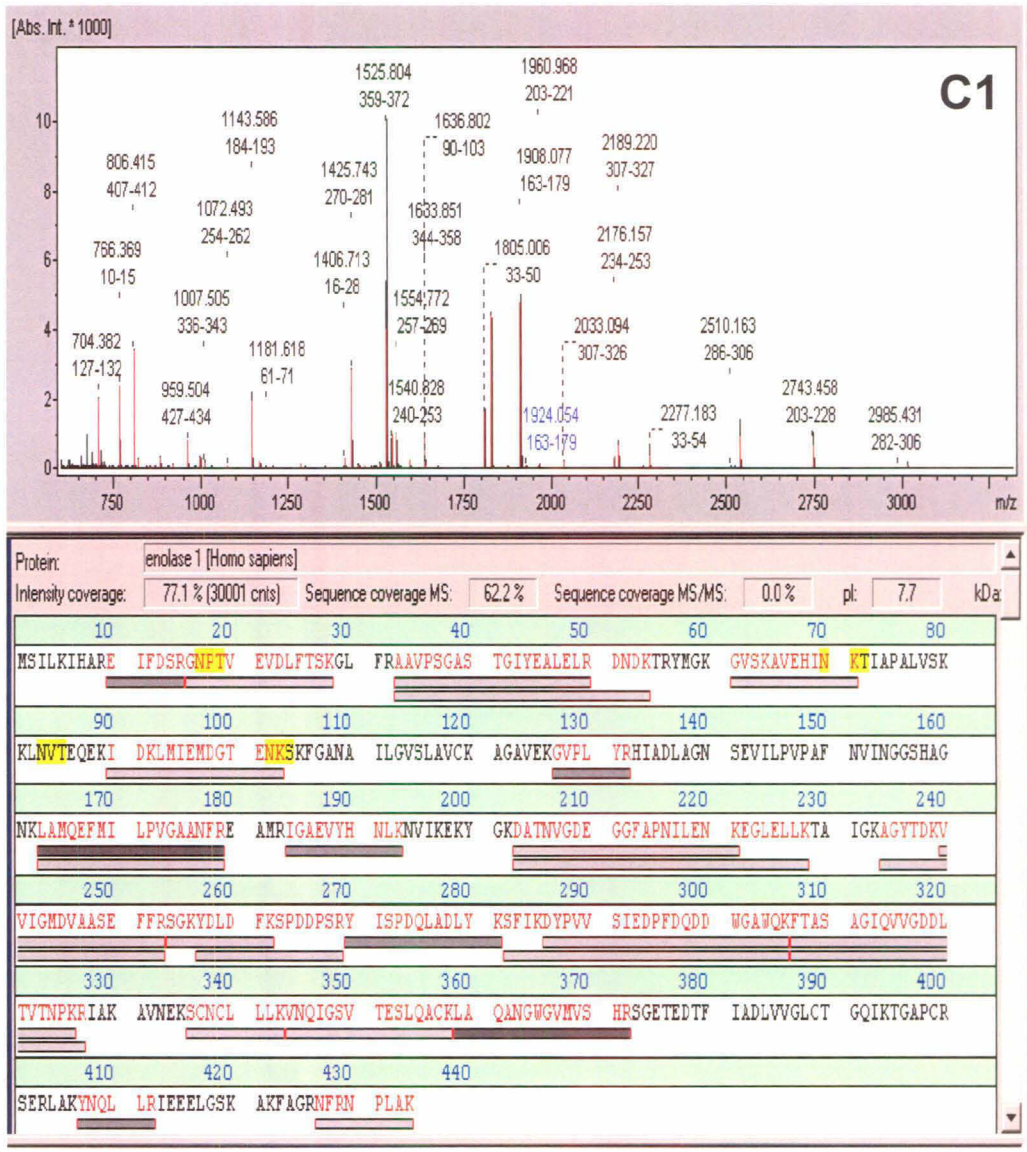


Fig.32: PMF spectrum suggests human Enolase 1.

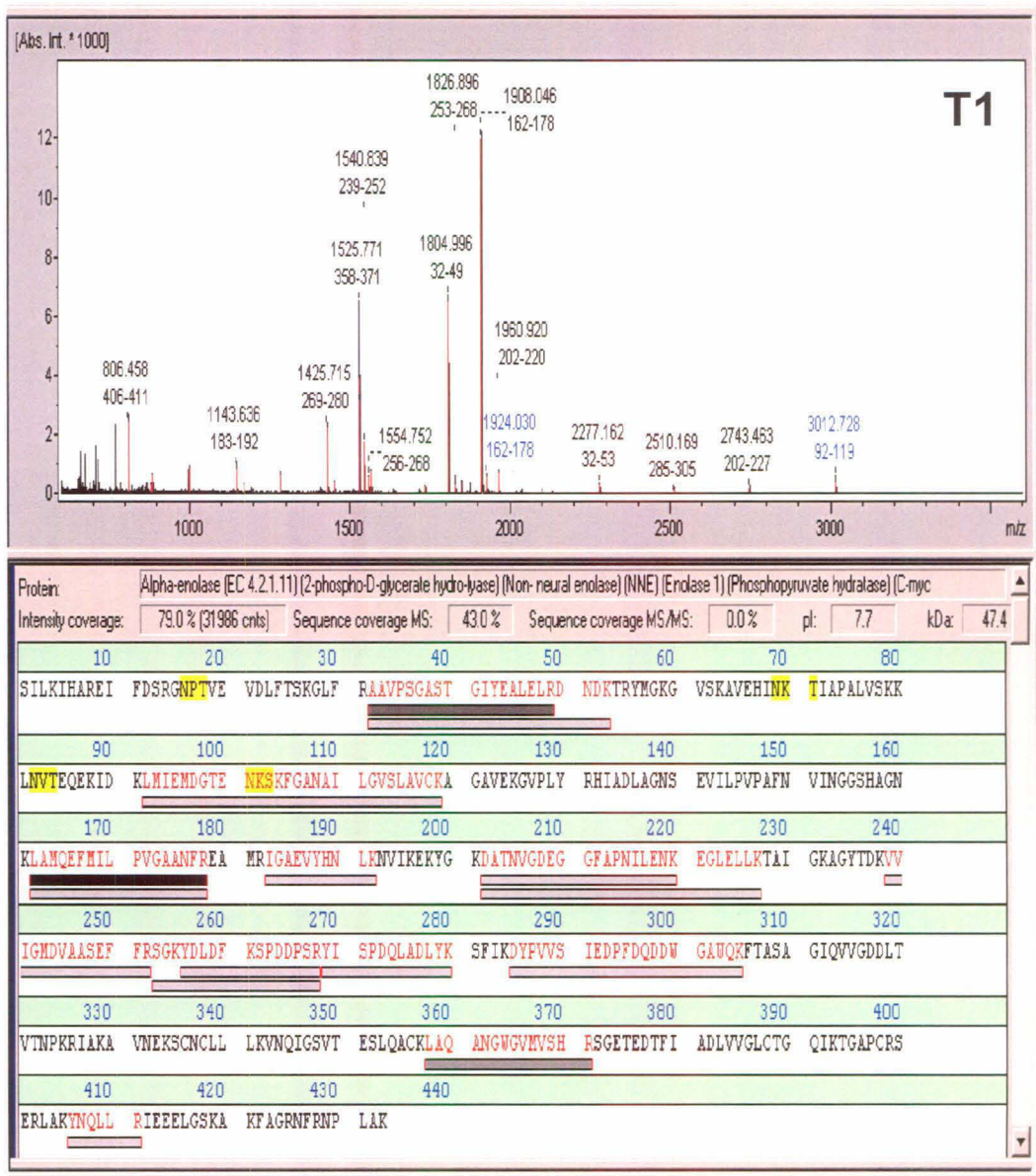


Fig.33: PMF spectrum suggests human αEnolase.

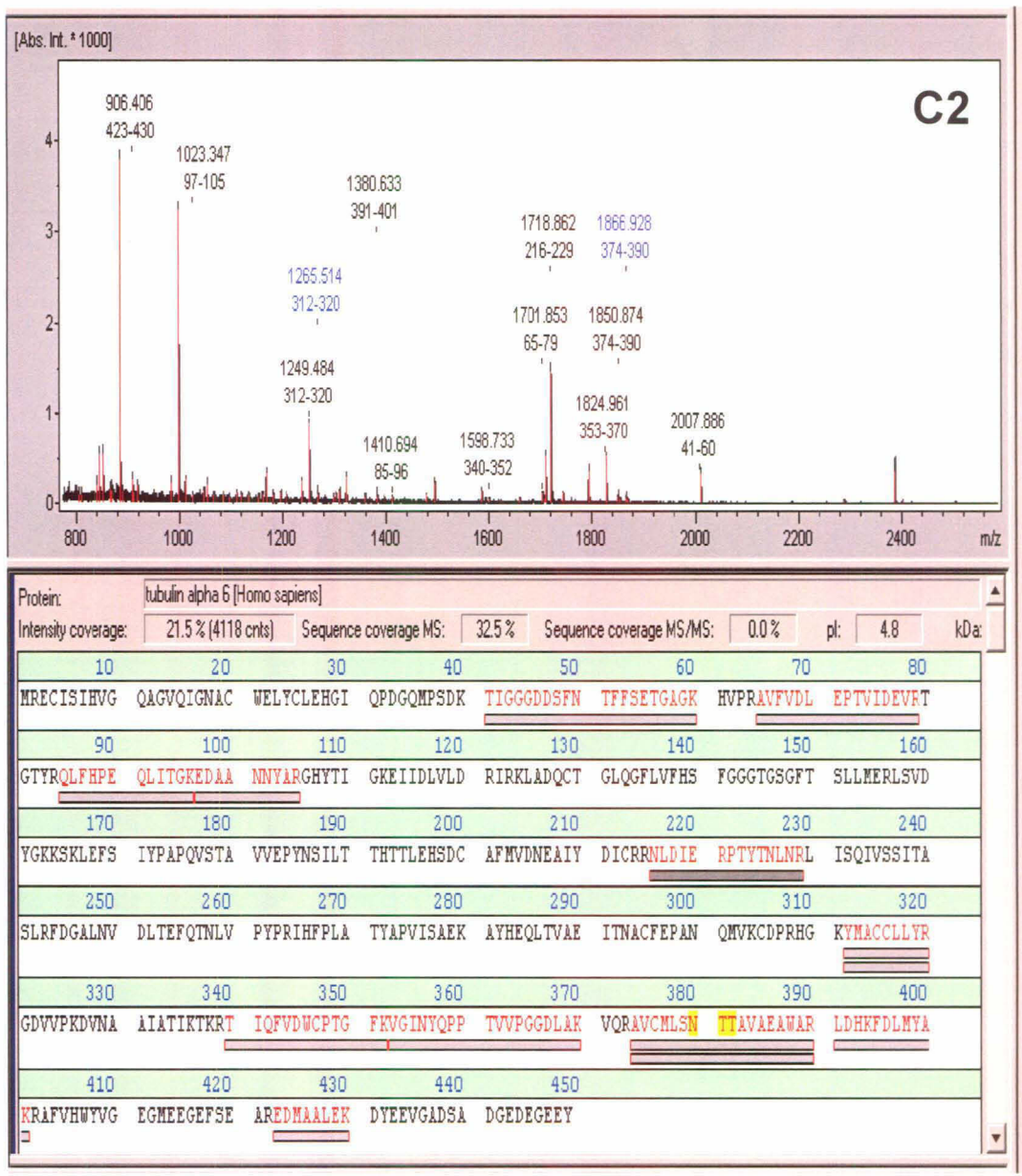


Fig.34: PMF spectrum suggests human α Tubulin.

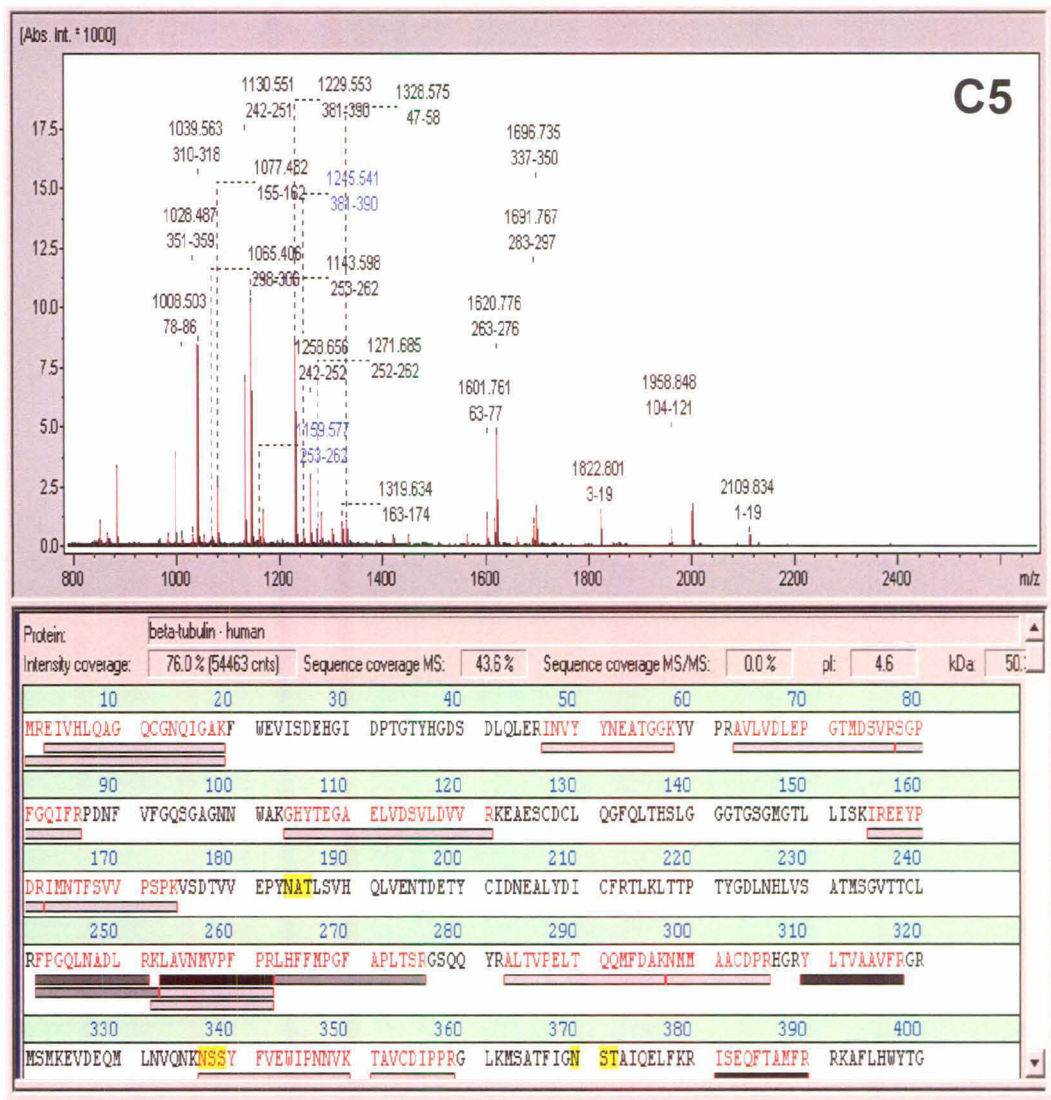


Fig.35: PMF spectrum suggests human β Tubulin.

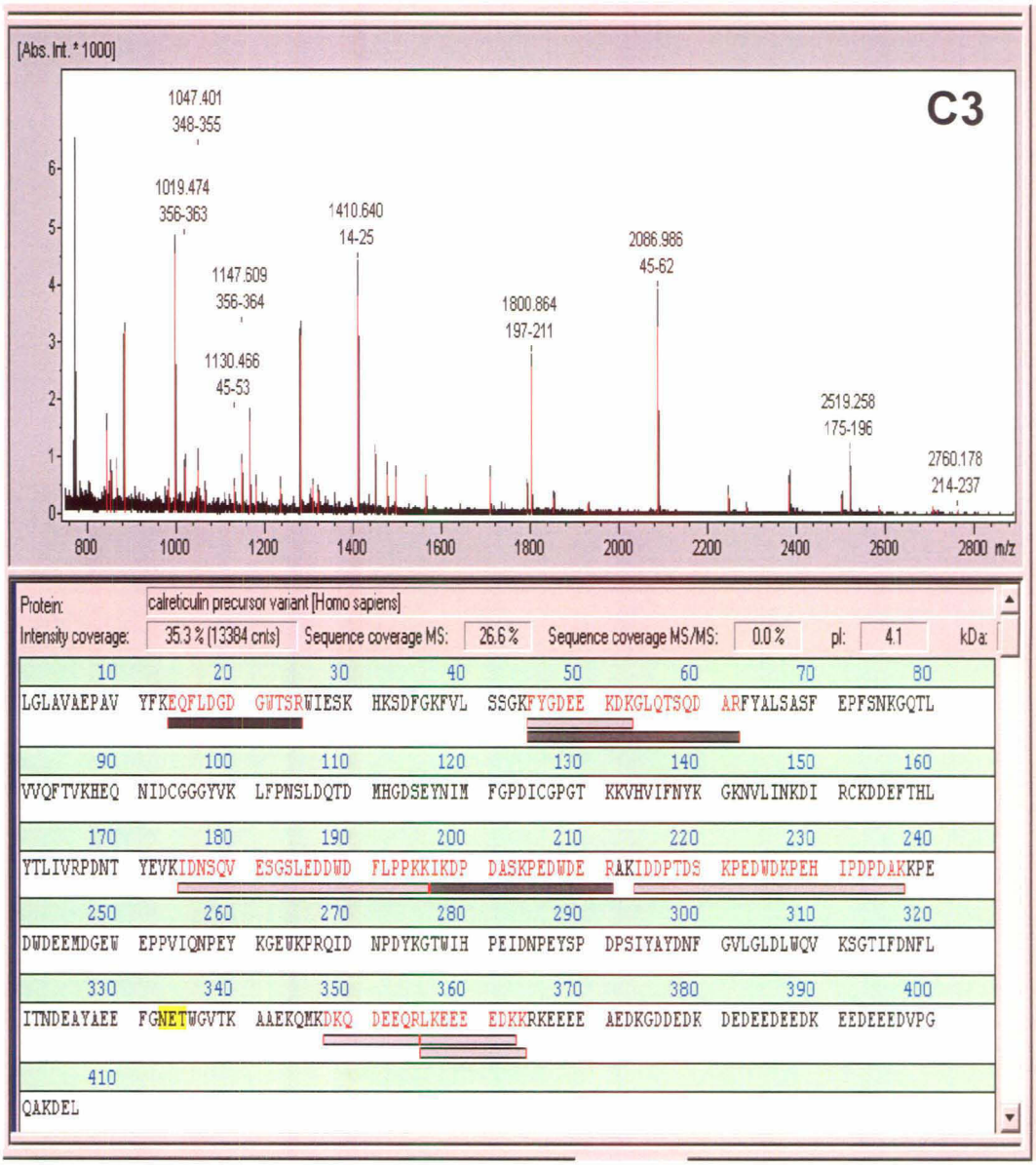


Fig.36: PMF spectrum suggests human Calreticulin precursor protein.

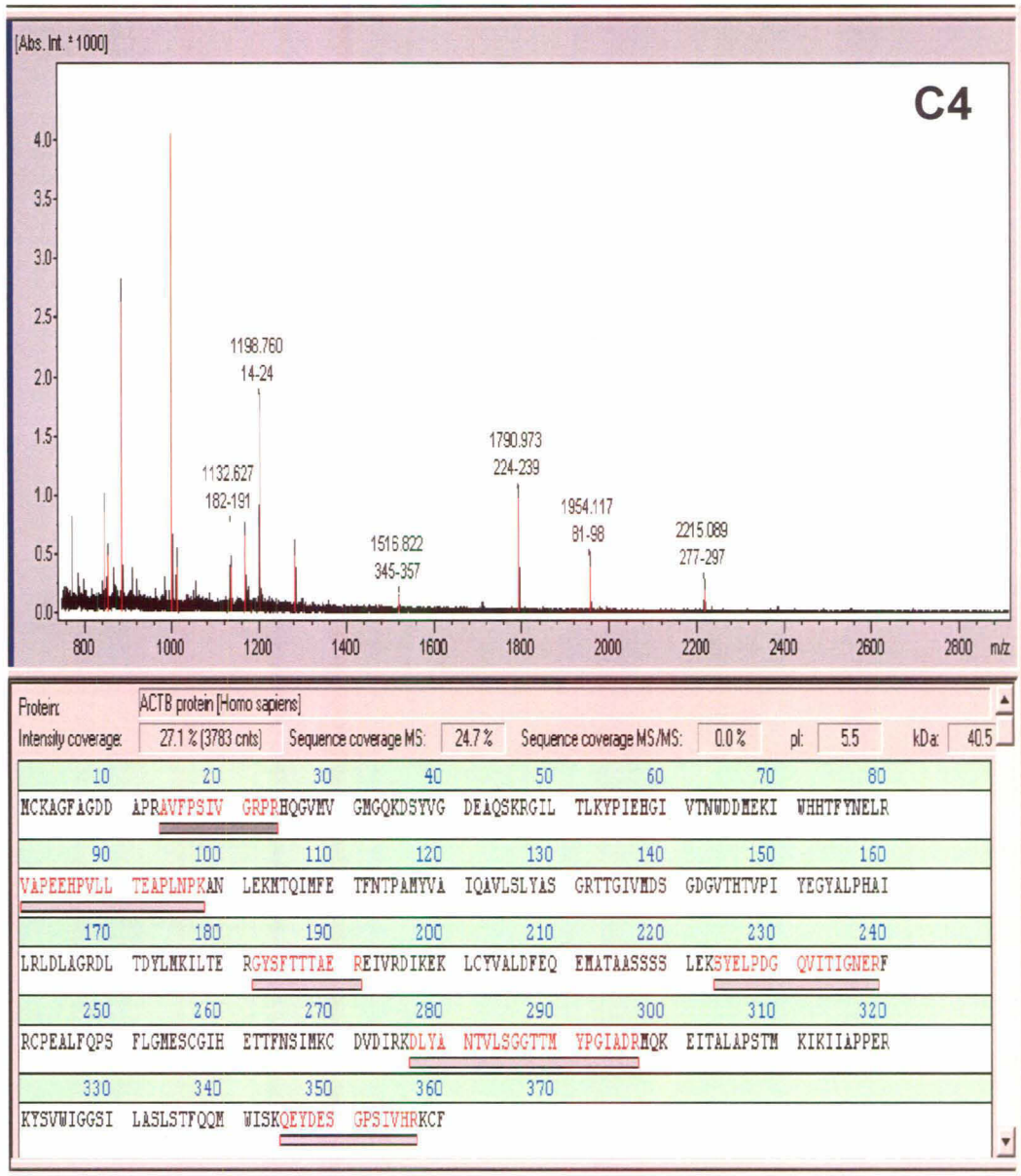


Fig.37: PMF spectrum suggests human β Actin.

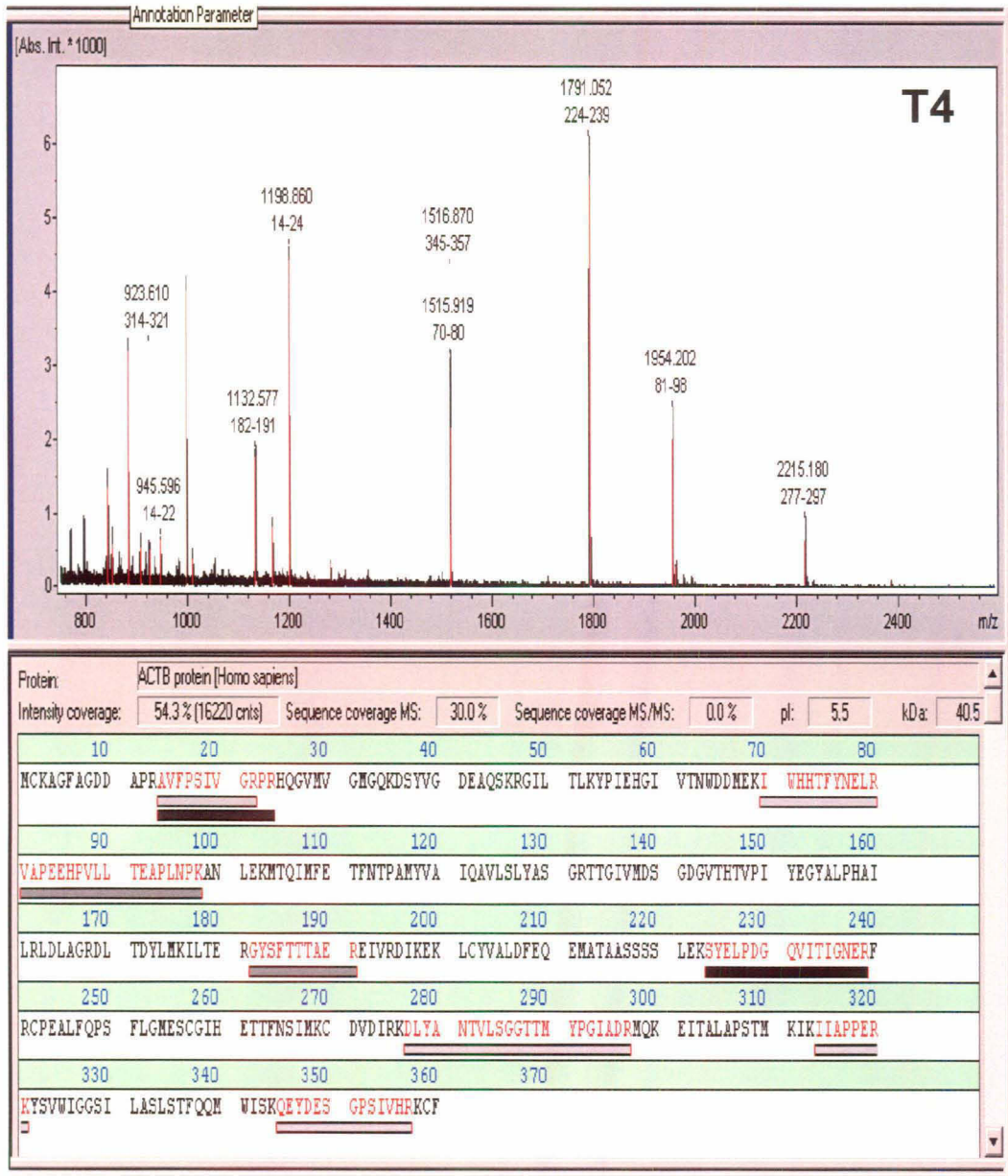


Fig.38: PMF spectrum suggests human β Actin.

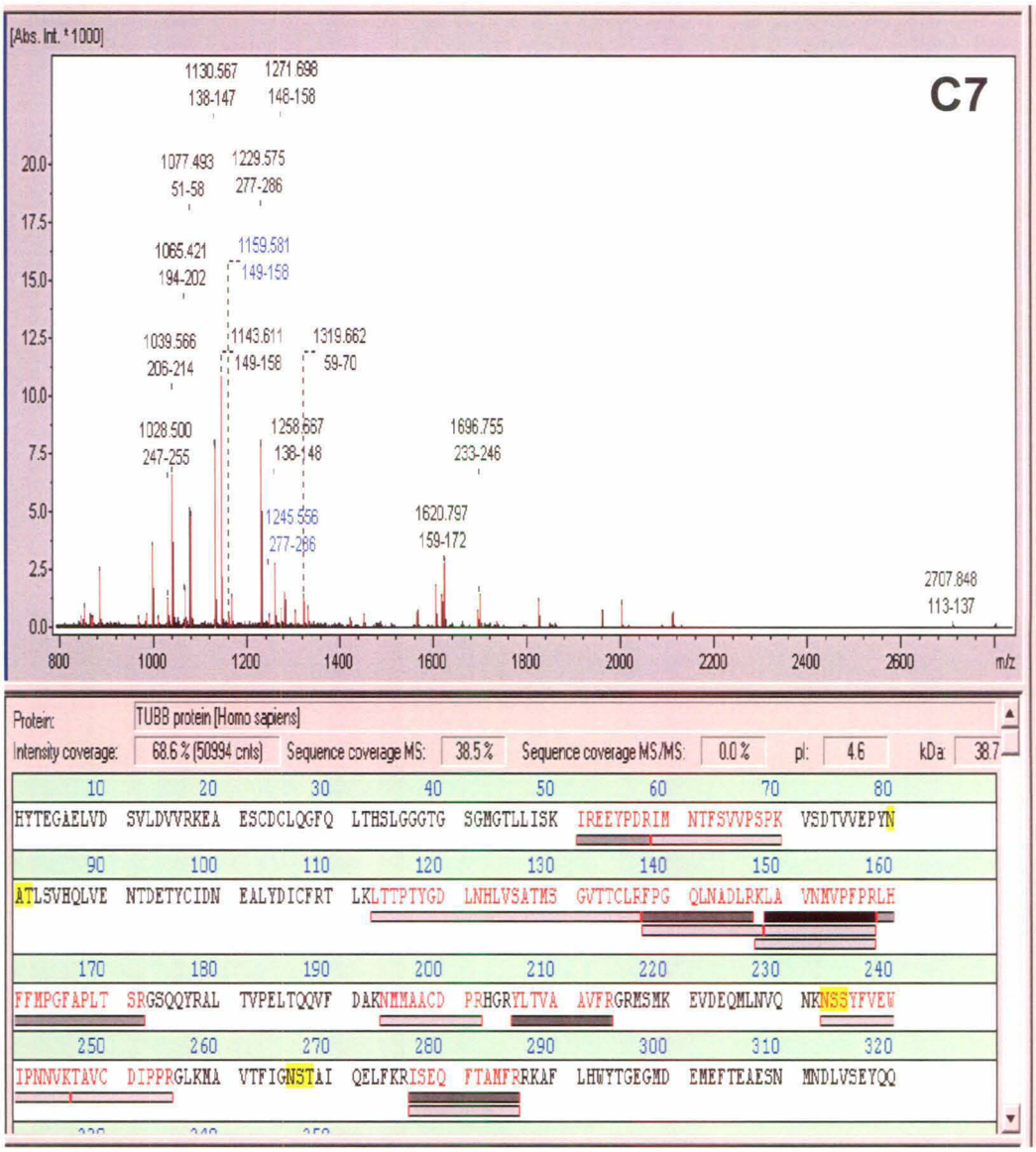


Fig.39: PMF spectrum suggests human TUBB protein.

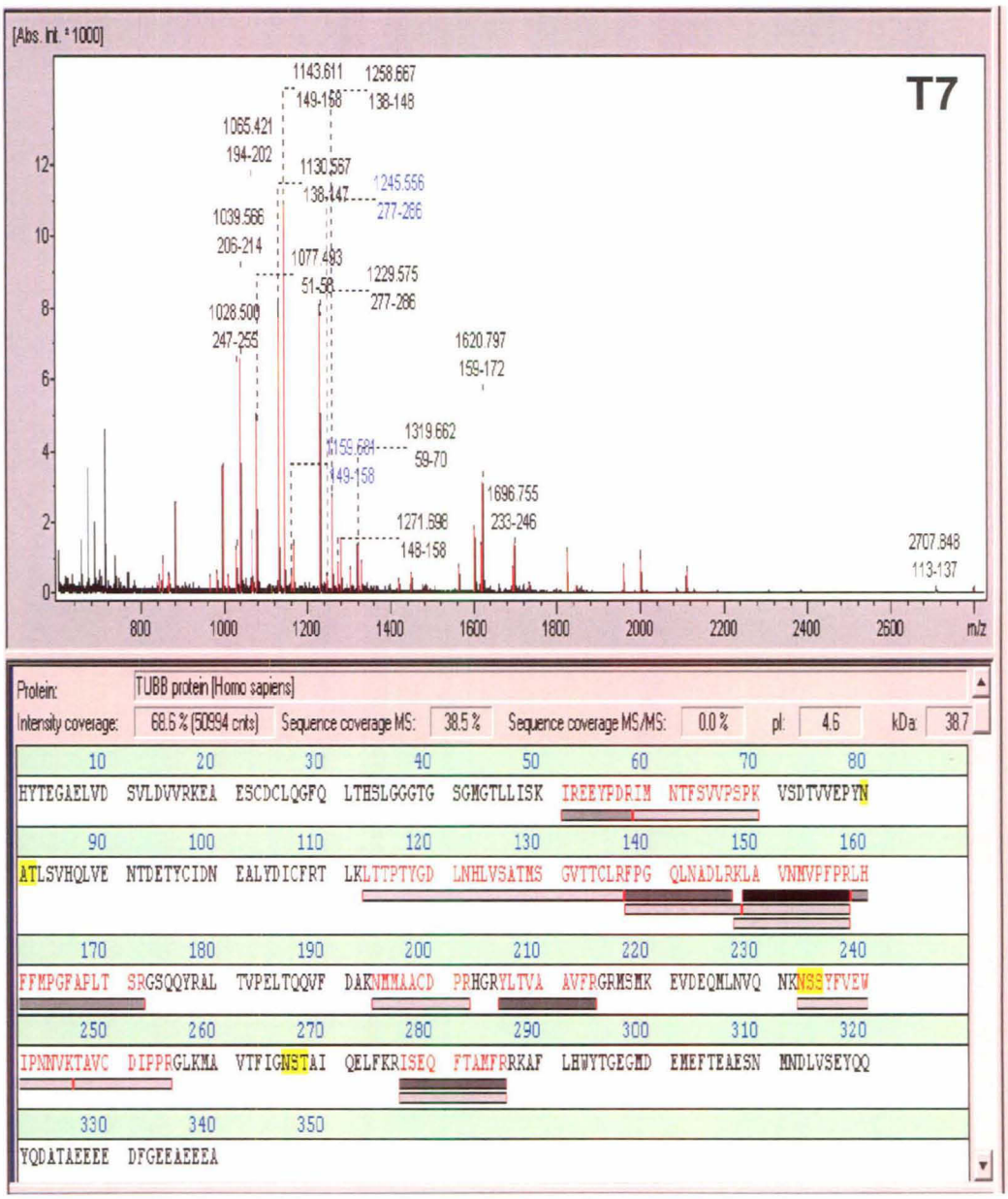


Fig.40: PMF spectrum suggests human TUBB protein.

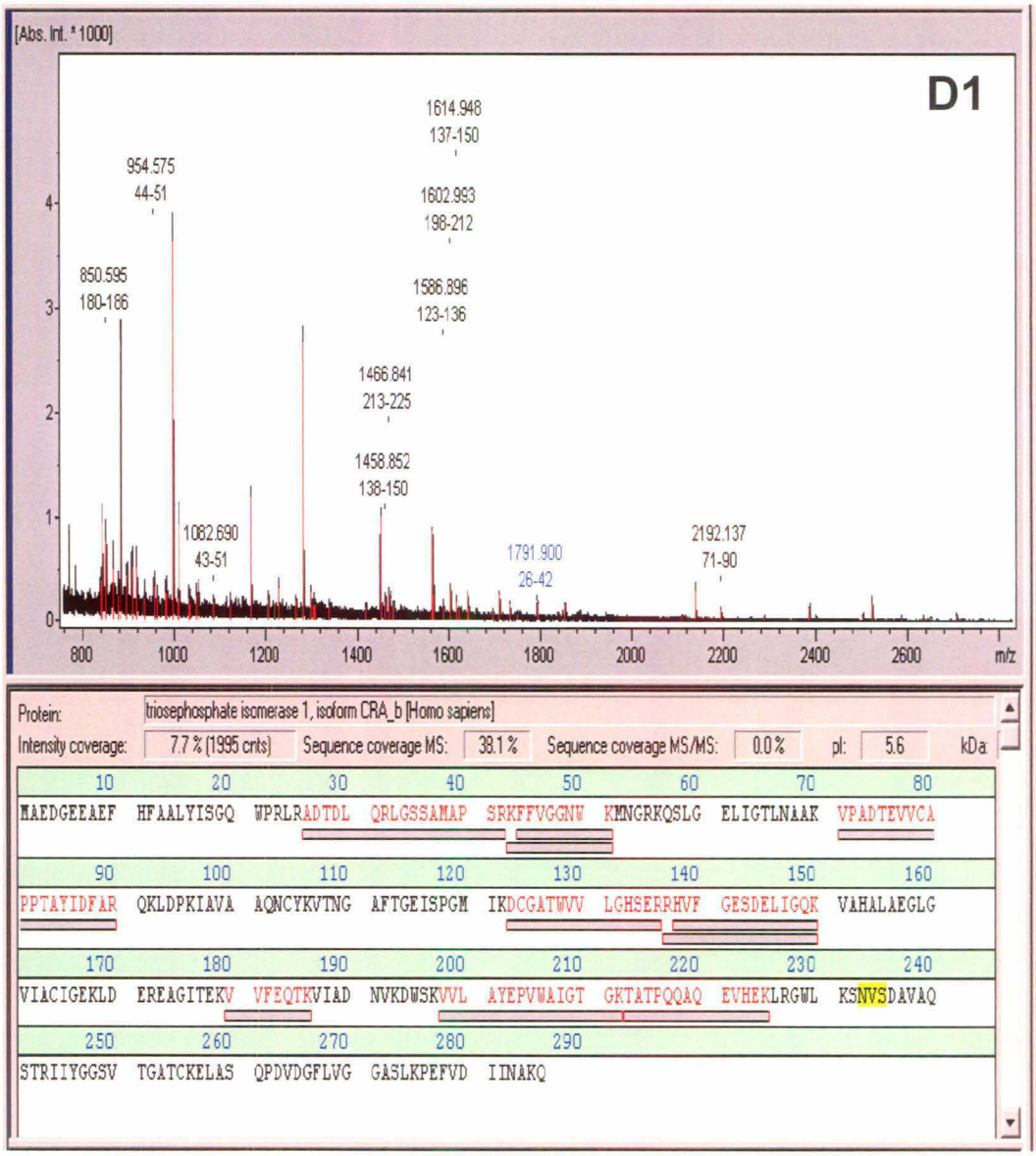


Fig.41: PMF spectrum suggests human Triose phosphate isomerase 1 isoform.

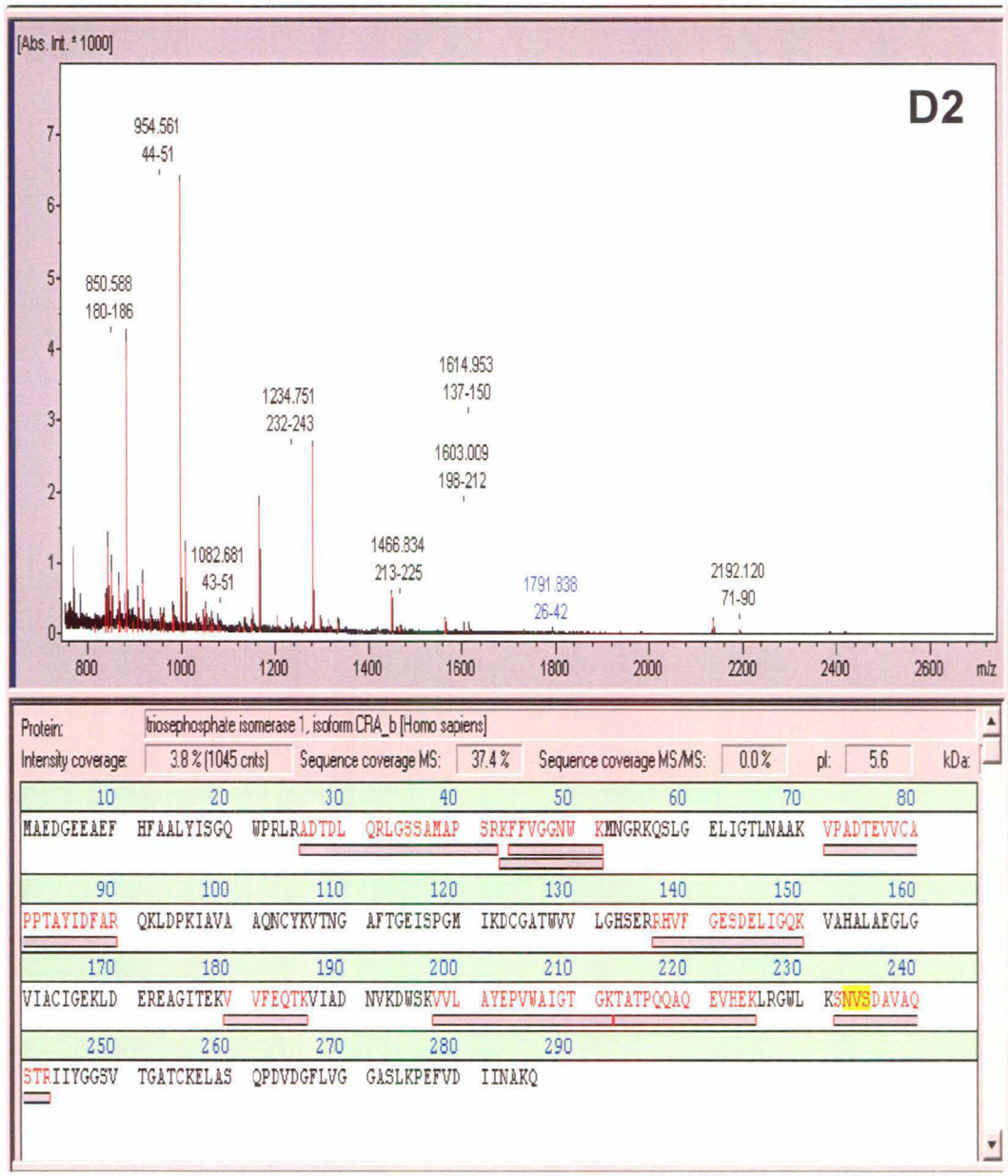


Fig.42: PMF spectrum suggests human Triose phosphate isomerase 1 isoform.

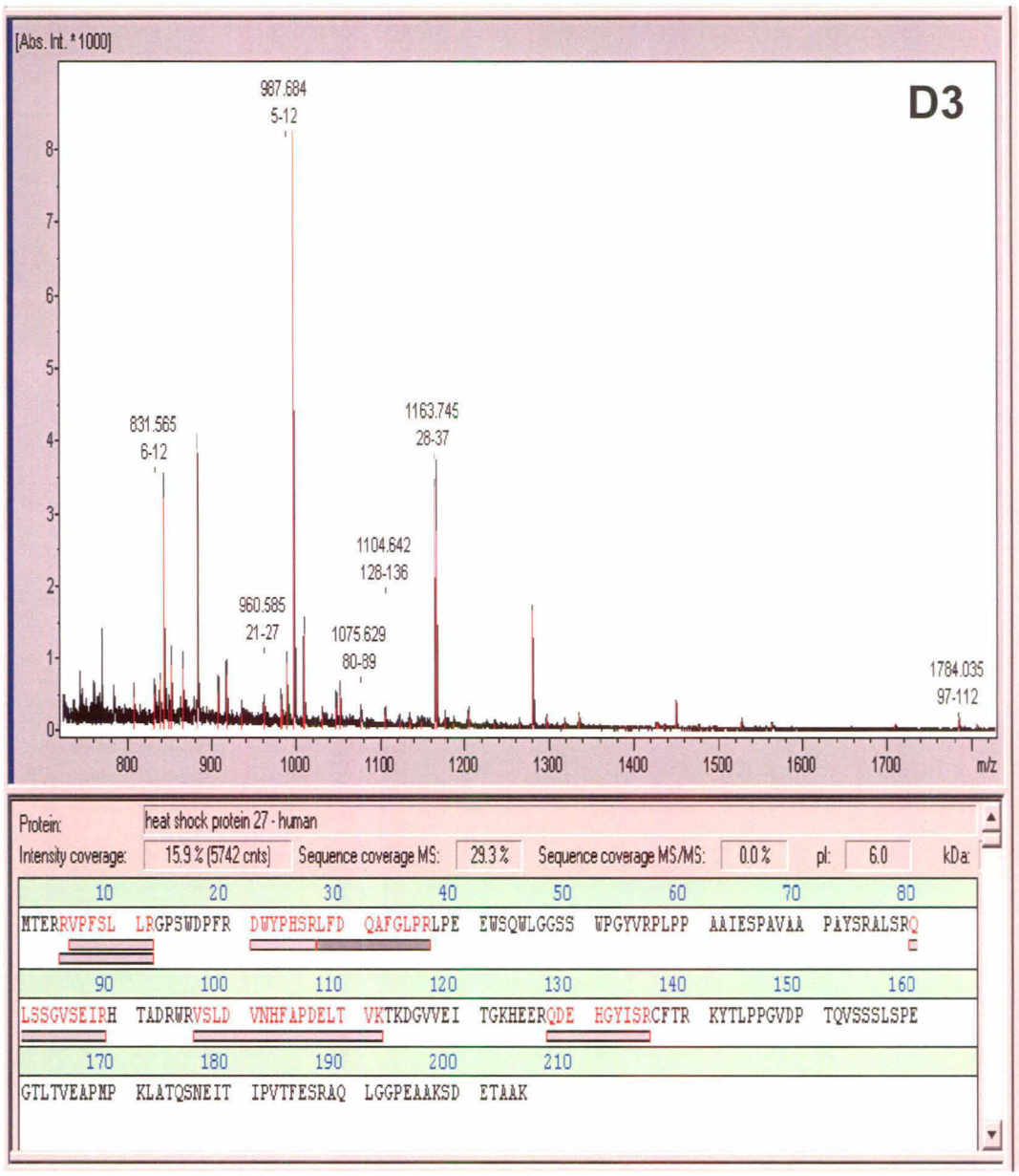


Fig.43: PMF spectrum suggests human Heat shock protein-27.

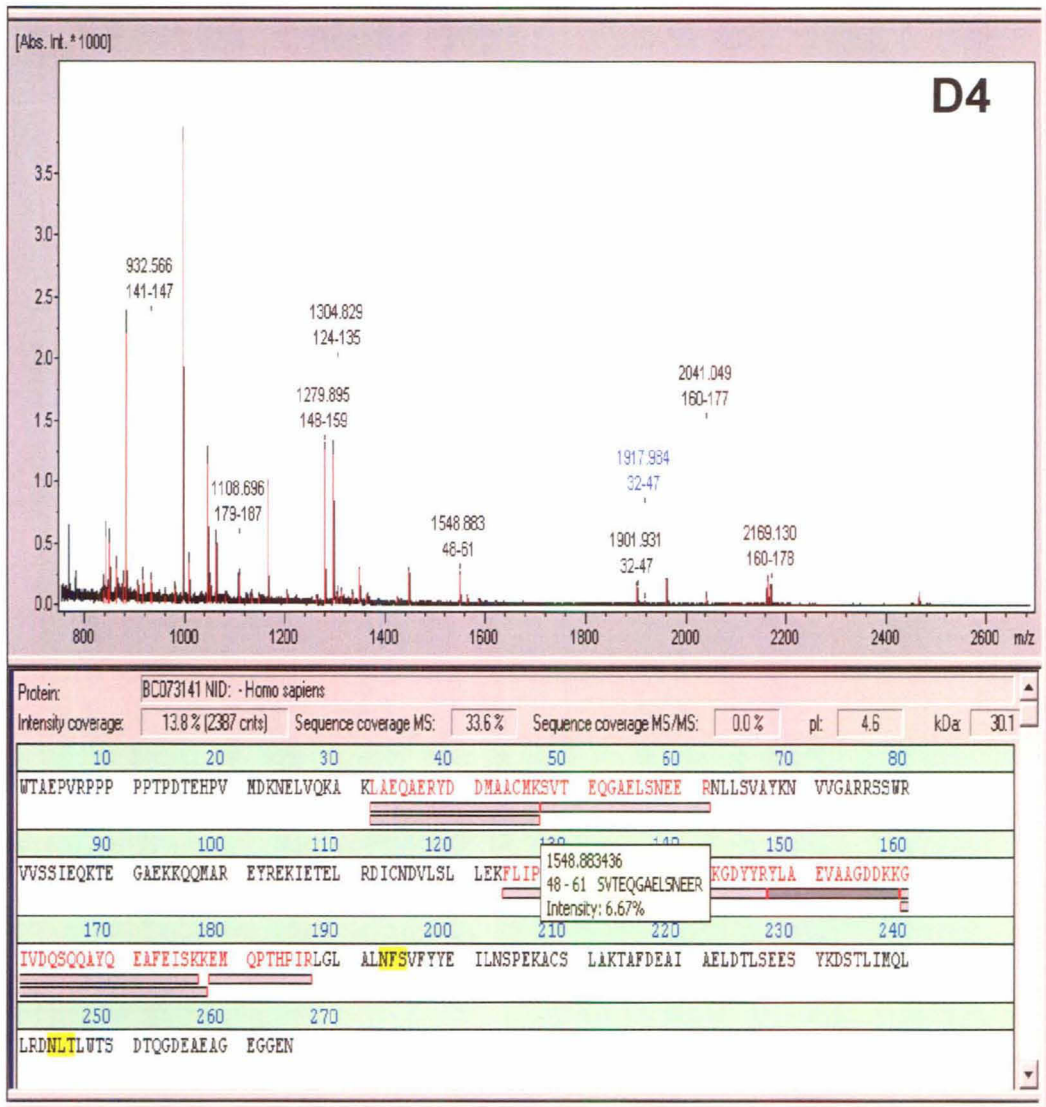


Fig.44: PMF spectrum suggests human 14-3-3ζ (BC-073141) protein.

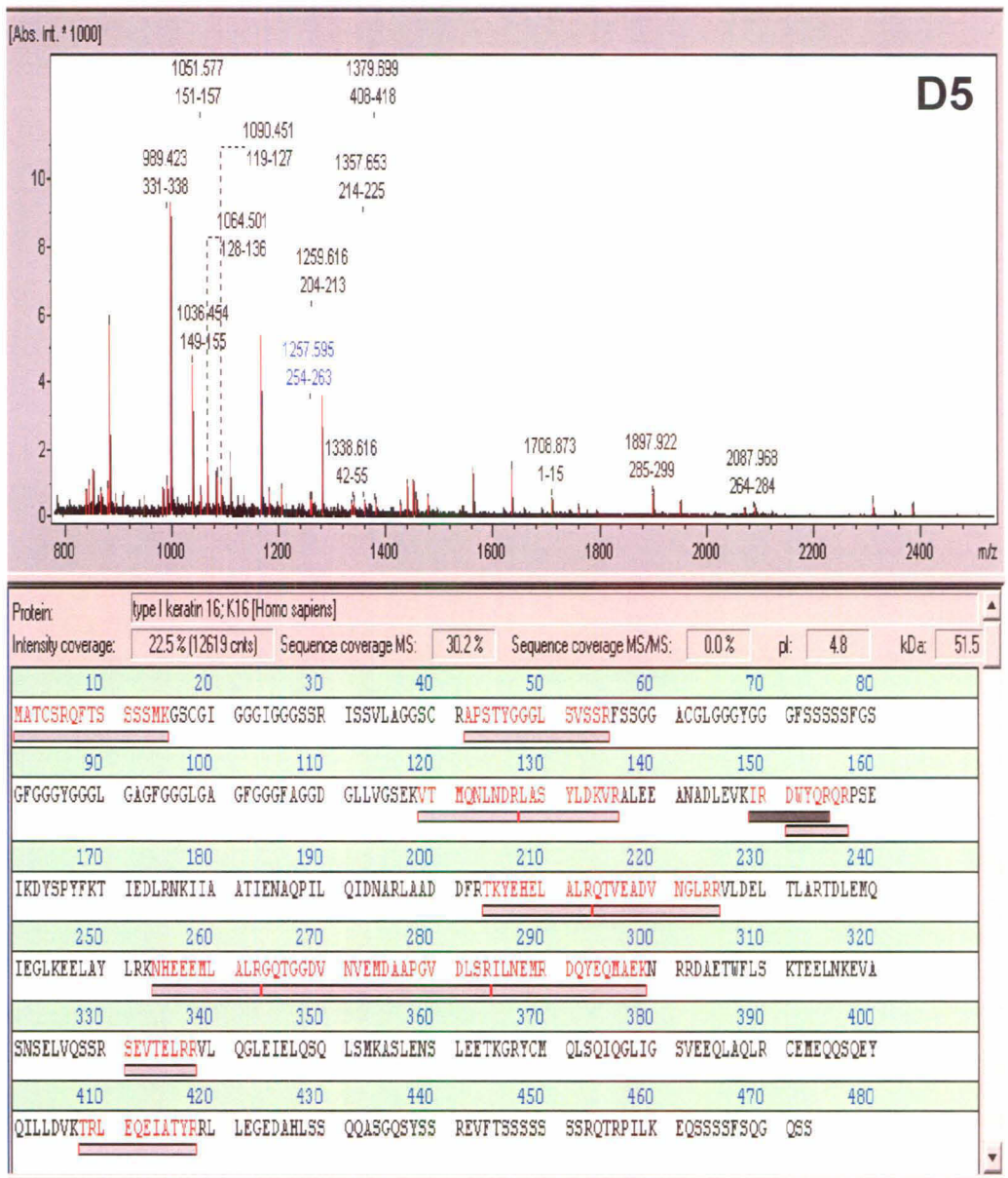


Fig.45: PMF spectrum suggests human Type 1 Keratin 16.

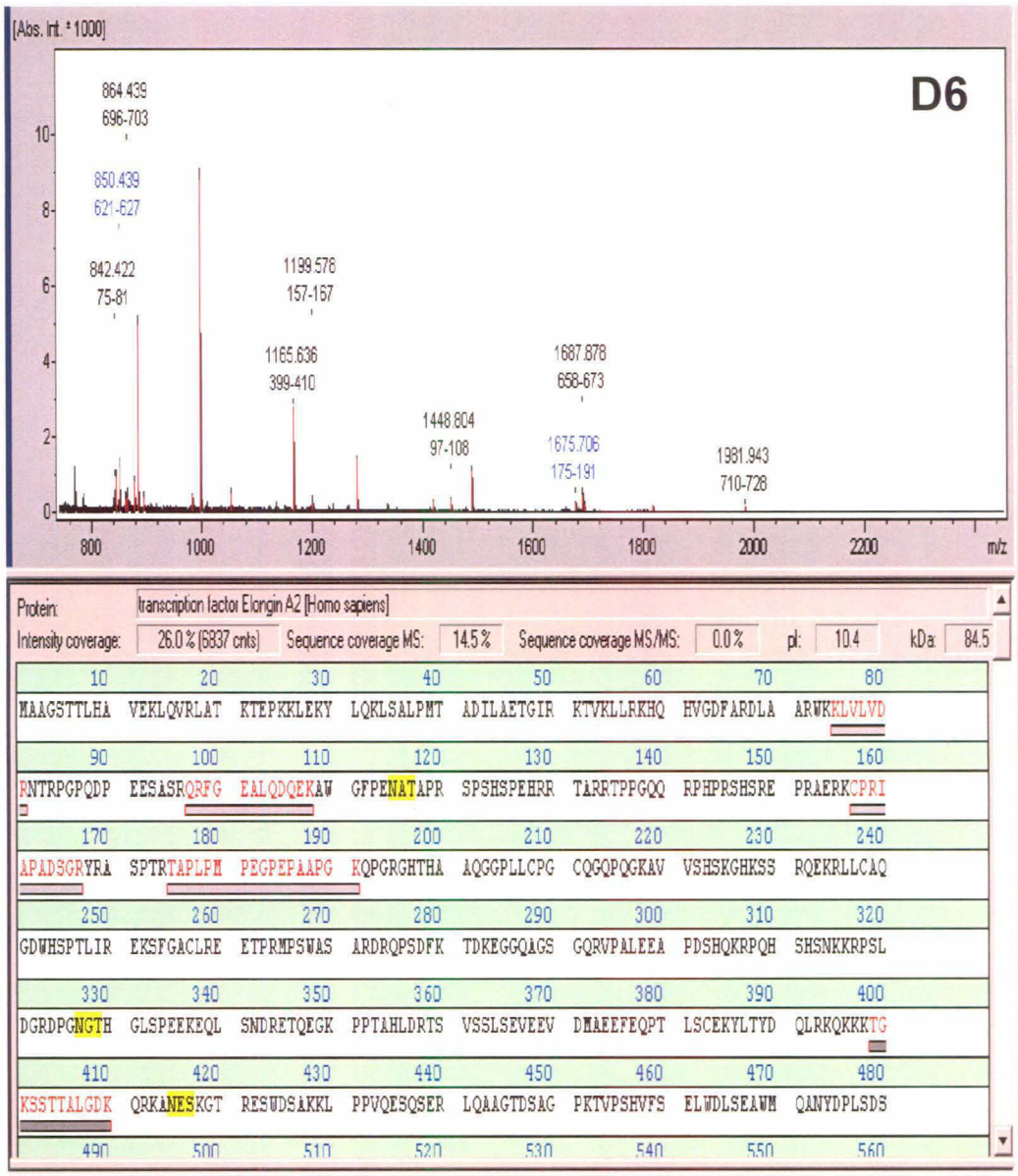


Fig.46: PMF spectrum suggests human transcription factor Elongin A2.

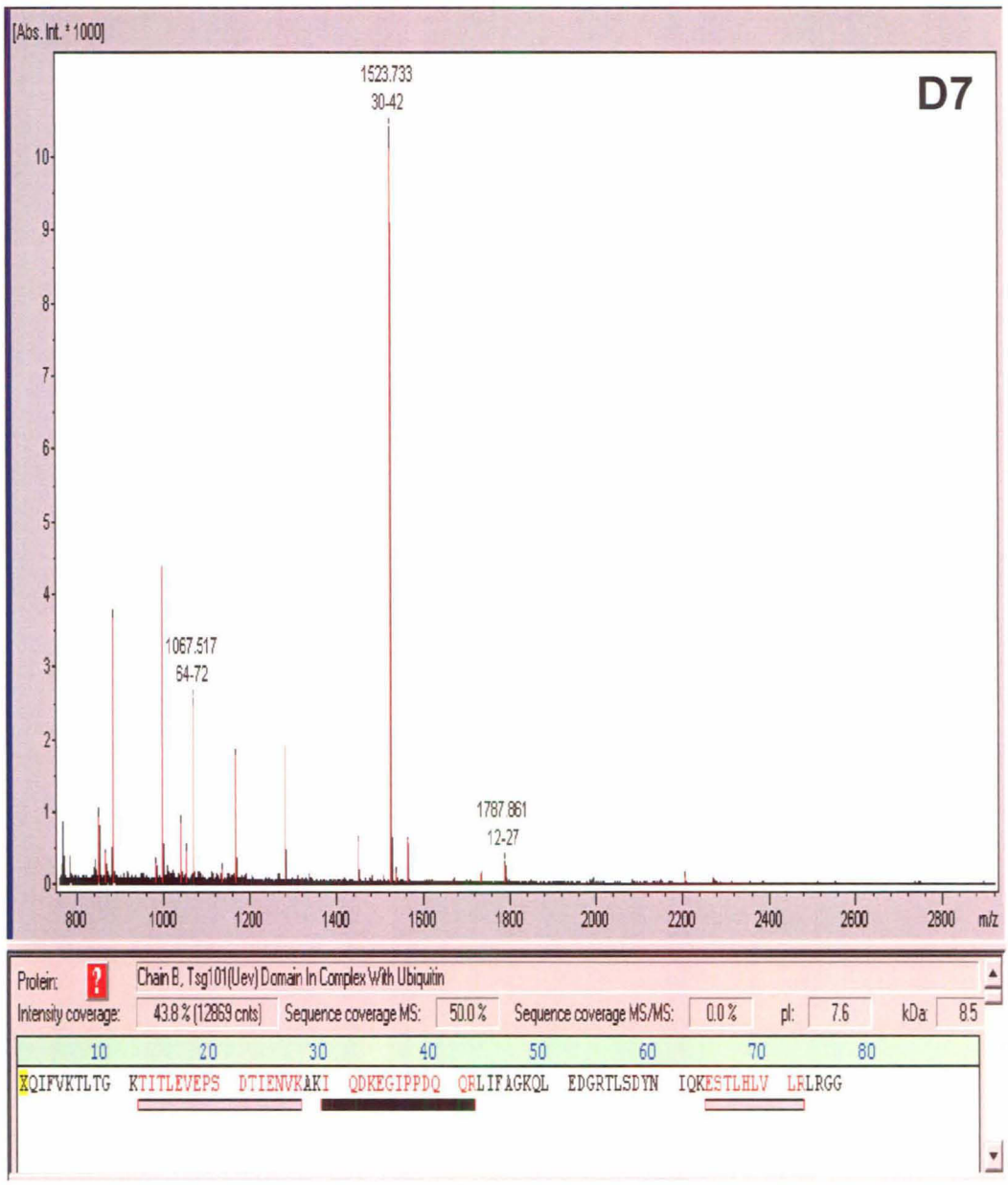


Fig.47: PMF spectrum suggests human Ubiquitin protein.

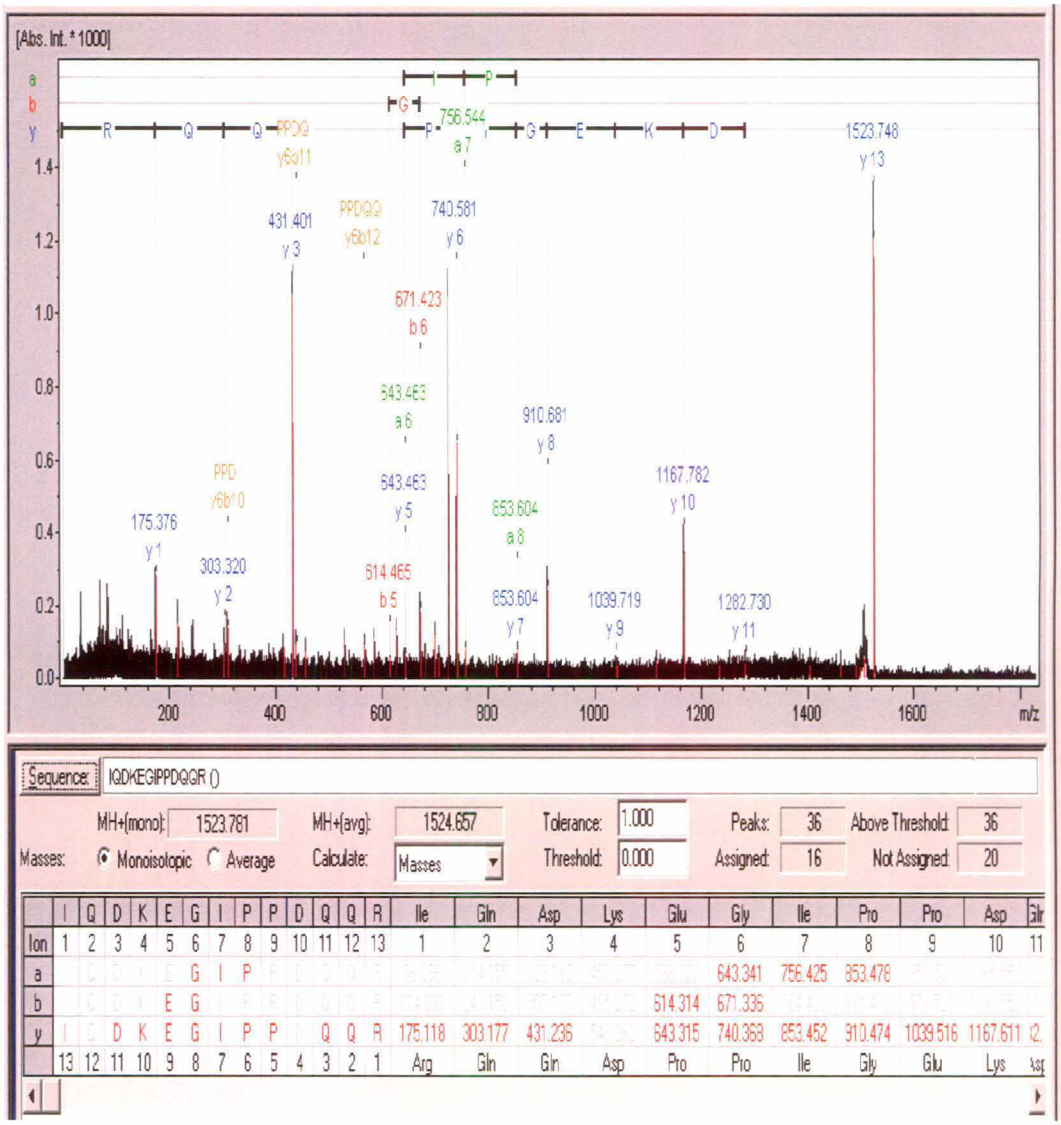


Fig.48: MS/MS spectrum corresponds to Ubiquitin protein.

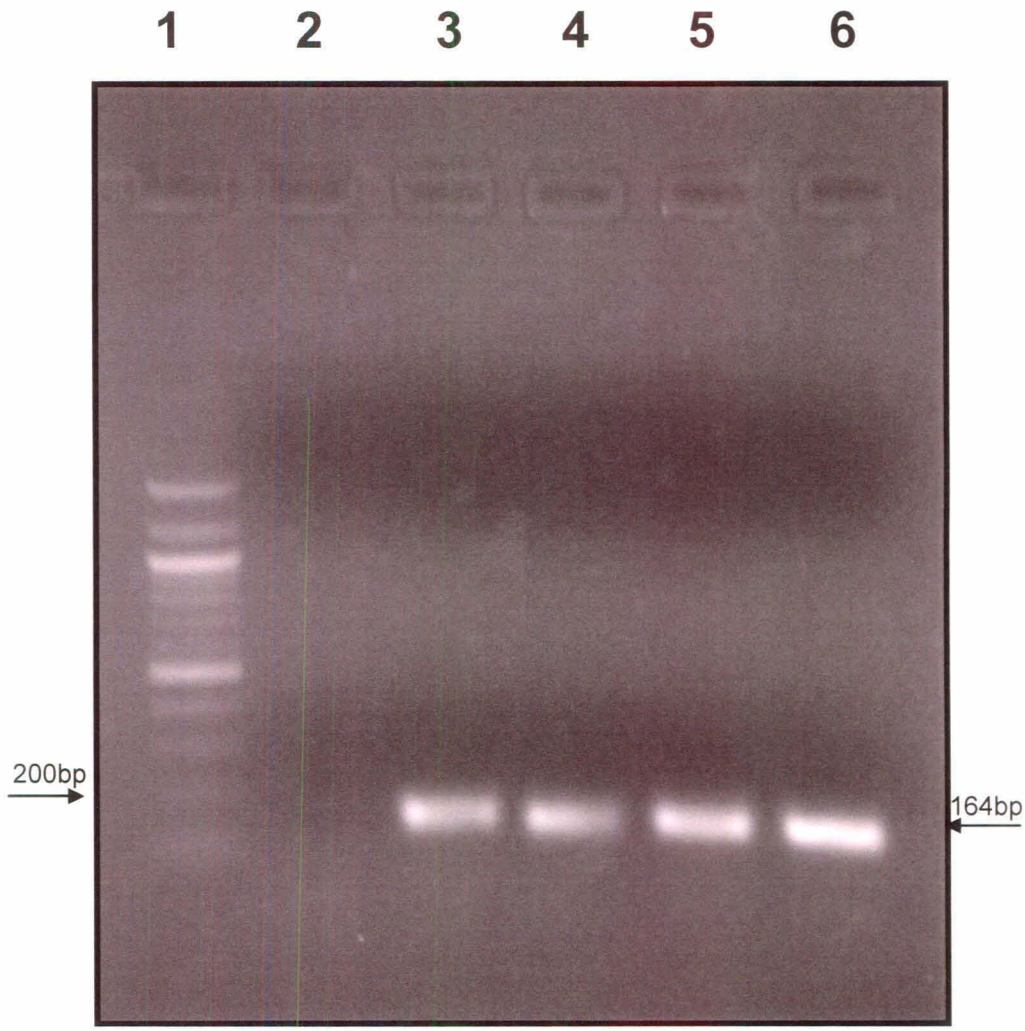


Fig.49: Expression of cytokeratin 18 in MCF-7 cells with time points post TGF-β1 treatment. Expression of cytokeratin18 mRNA was examined by RT-PCR at different time points after TGF-β1 treatment (3ng/ml). **Lane 1,** DNA ladder of 100bp. **Lane 2,** RT-PCR without template. **Lane 3,** Expression of CK18 in untreated cells. **Lane 4,** Expression of CK18 in 24hrs treated cells. **Lane 5,** Expression of CK18 in 48 hrs treated cells. **Lane 6,** Expression of CK18 in 72 hrs treated cells.

Expression of CK18 was found less altered at different time points as well as in untreated cells. No significant difference is observed in CK18 expression.

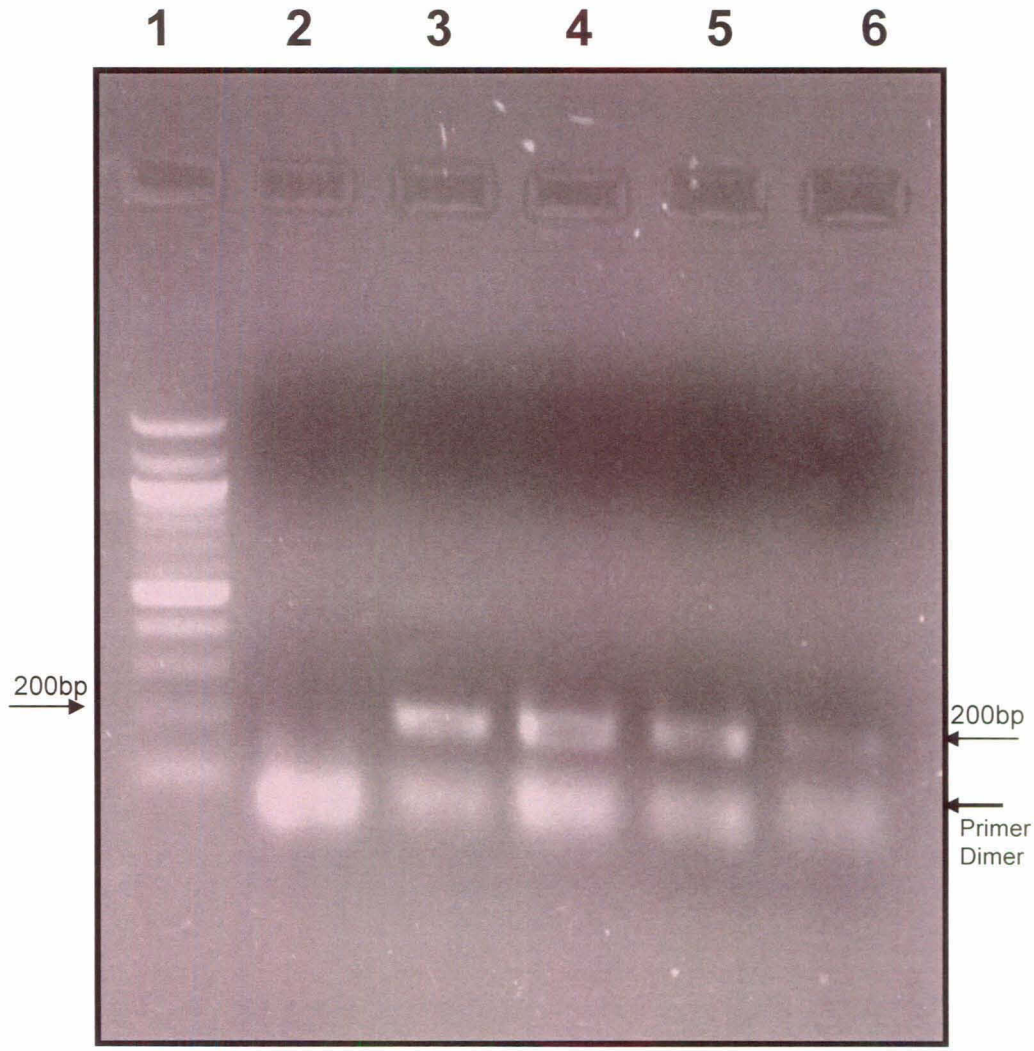


Fig.50: Expression of E-cadherin in MCF-7 cells with time points post TGF-β1 treatment. Expression of E-cadherin mRNA was examined by RT-PCR at different time points after TGF-β1 treatment (3ng/ml). **Lane 1,** DNA ladder of 100bp. **Lane 2,** RT-PCR without template. **Lane 3,** Expression of E-cadherin in untreated cells. **Lane 4,** Expression of E-cadherin in 24hrs treated cells. **Lane 5,** Expression of E-cadherin in 48 hrs treated cells. **Lane 6,** Expression of E-cadherin in 72 hrs treated cells.
Gradual decrease of expression of E-cadherin compare to untreated cells was observed at different time points.

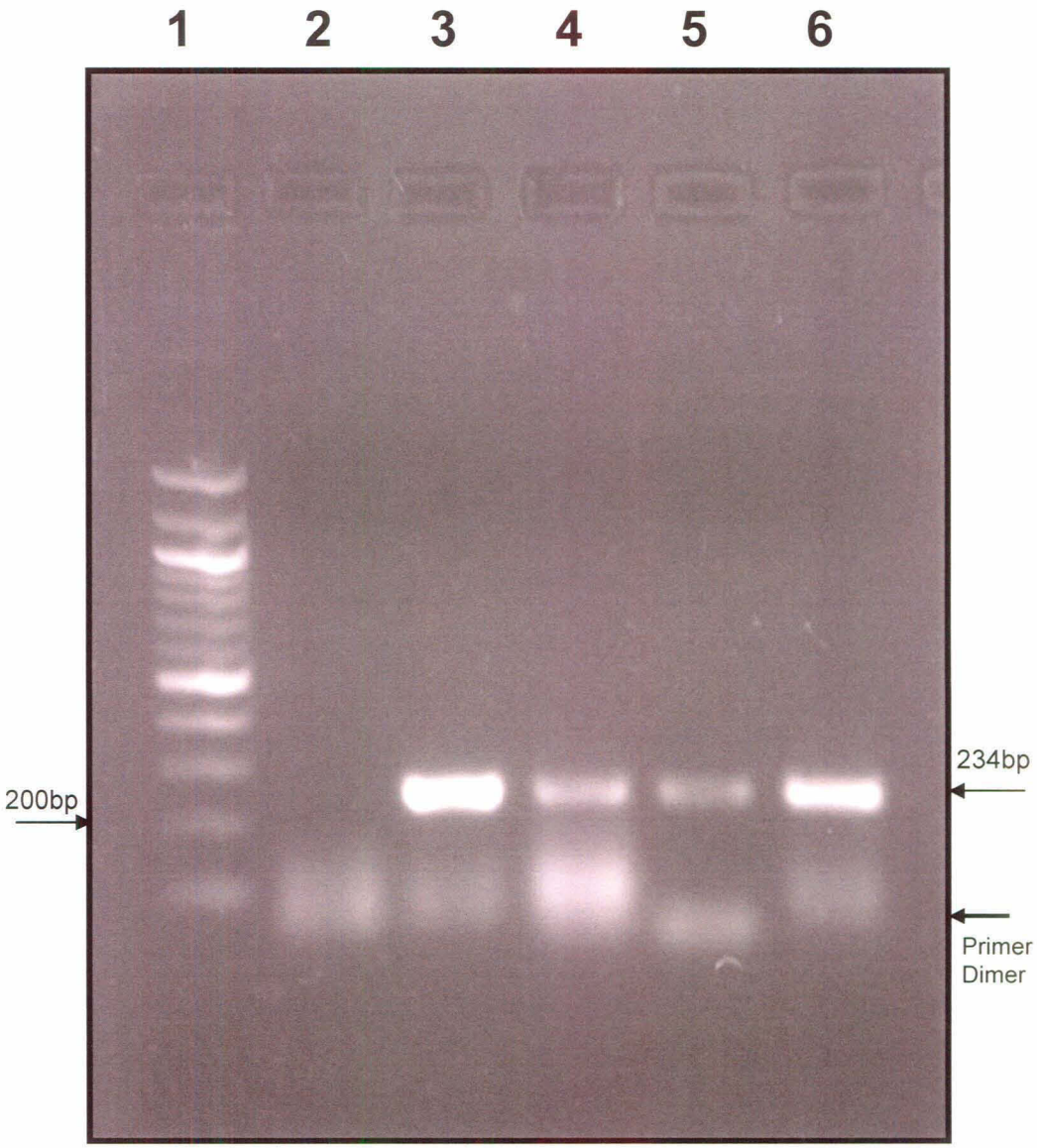


Fig.51: Expression of TGF-β1 in MCF-7 cells with time points post TGF-β1 treatment. Expression of TGF-β1 mRNA was examined by RT-PCR at different time points after TGF-β1 treatment (3ng/ml). **Lane 1,** DNA ladder of 100bp. **Lane 2,** RT-PCR without template. **Lane 3,** Expression of TGF-β1 in untreated cells. **Lane 4,** Expression of TGF-β1 in 24hrs treated cells. **Lane 5,** Expression of TGF-β1 in 48 hrs treated cells. **Lane 6,** Expression of TGF-β1 in 72 hrs treated cells.

Untreated cells showed prominent expression whereas gradual decrease was found at 24hrs and 48hrs time points. Regain of expression was observed at 72 hrs time point.

Discussion

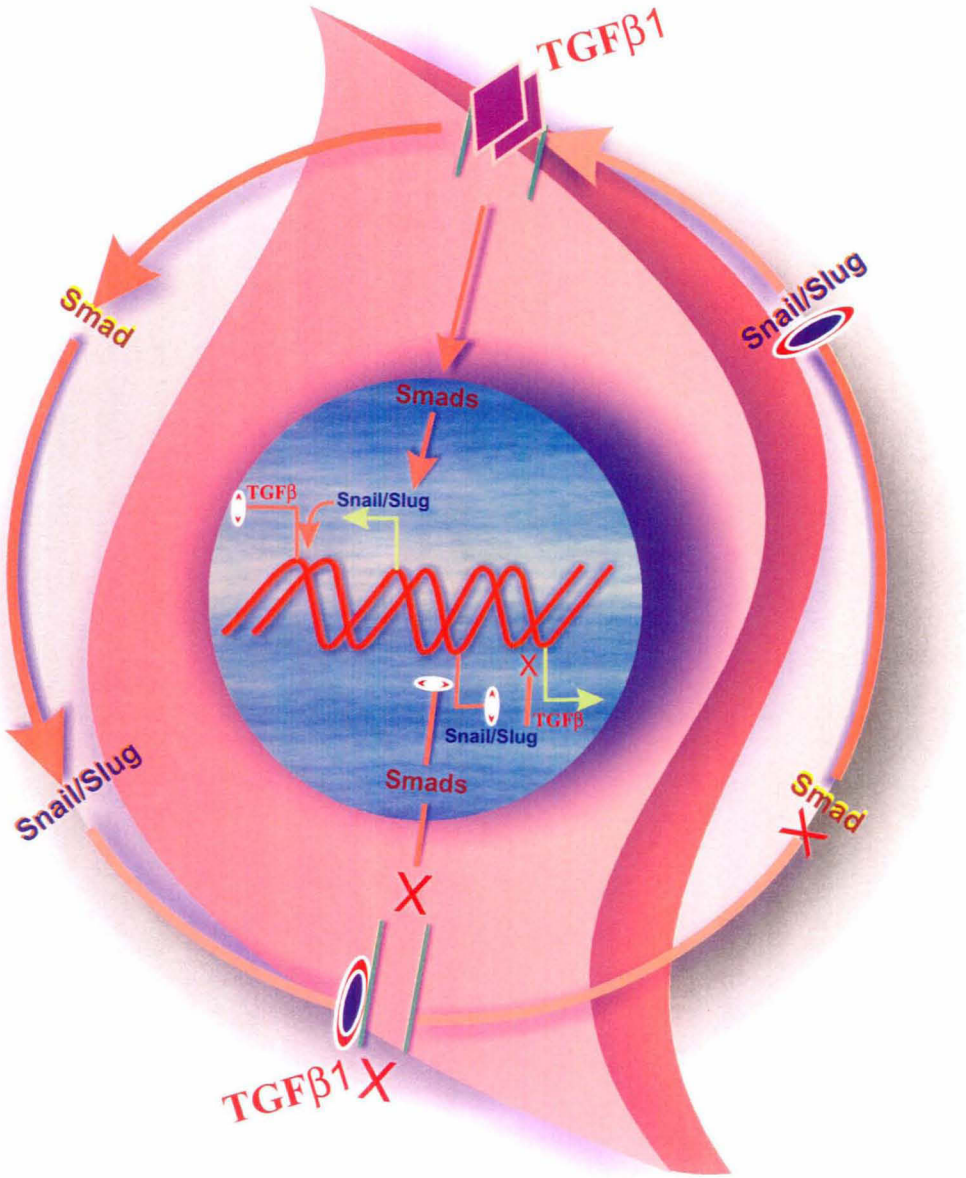


Plate IX: Feed-back regulation of TGF-β1 in cancerous cell.
The projected model of feed-back regulation may apply to the cancerous cell. TGF-β1 induces expression of Snail/Slug through Smads. Presence of Snail/Slug downregulates TGF-β1. Absence of TGF-β1 lowers the presence of Snail/Slug. Absence of Snail/Slug promotes the self-expression of TGF-β1 through Smads/AP-1 (not shown in fig).

5 DISCUSSION

5.1 Background

Epithelial to mesenchymal transition (EMT) is a key process in normal embryonic development where epithelial cells acquire a migratory morphology accompanied by the loss of epithelial markers (Hay, 1995; Thiery & Chopin, 1999). In recent years, epithelial to mesenchymal transition has been depicted and recognized as a potential mechanism for the onset and progression of cancer (Thiery, 2002, 2003; Gotzmann et al., 2004). Most of the cancers of epithelial cell origin have shown loss of E-cadherin mediated cell-cell junctions during progression of aggressive form of carcinoma (Cavallaro et al., 2004; Hanahan et al., 2000). Findings of various studies showed several ways of E-cadherin loss e.g. inactivation of E-cadherin gene due to mutations in gastric and breast carcinoma (Hajra et al., 2002; Nollet et al.,1999) and hypermethylation of the DNA encoding E-cadherin and repression by transcription factors, mediated through two E-boxes, a region in the E-cadherin promoter (Van et al., 2001). Characerization of E-cadherin promoter in human, murine and canine genes revealed several E-boxes at different positions. In human promoter three E-boxes at –79, –30 and +22 positions are located (Hennig et al., 1995; Giroldi et al., 1997; Batlle et al., 2000). In mouse promoter two adjacent E-boxes at –86 and –80, inside the E-pal element and the proximal E-box at –31 are located but unlike in human it showed lack of the downstream E-box at +22 (Behrens et al., 1991; Rodrigo et al., 1999), whereas the canine promoter shares similar locations of E-boxes as in the human promoter at the –79 and –30 positions (Comijn et al., 2001). Prominently, two members of the Snail family of transcription factors namely Snail and Slug have been found to be involved in downregulation of E-cadherin transcription (Thiery et al., 2002; De Craene et al., 2005a, 2005b). Snail has been implicated in downregulation of E-cadherin in melanoma (Poser et al., 2001), hepatocellular (Jiao et al., 2002), gastric (Rosivatz et al., 2002) and oral carcinoma (Yokoyama et al., 2001). Slug has been shown to downregulate E-cadherin in breast cancer (Hajra et al., 2002; Come et al., 2006). SIP1 and E-47 are the other regulatory factors that are able to repress E-cadherin in various cell lines (Thiery et al., 2002).

Among so many transcriptional repressors, Snail was the first proposed candidate involved in transcriptional repression of E-cadherin (Cano et al., 2000; Batlle et al., 2000; Grooteclaes & Frisch, 2000; Perez-Moreno et al., 2001, 2003; Comijn et al., 2001). Apart from programmed repression of E-cadherin, Snail has other target

molecules involved in onset and progression of EMT. Snail upregulates the expression of mesenchymal molecules such as vimentin, fibronectin, matrix proteases, Wnt5α factor, transcriptional factors ZEB1 and LEF1 and downregulates the epithelial markers viz. cytokeratin 18, desmoplakin as well as claudin, occludin and MUC1 essential for maintenance of epithelial cell polarity (Nieto et al., 2002; Guaita et al., 2002; Ikenouchi et al., 2003; Taki et al., 2003; Miyoshi et al., 2004). Expression of Snail has been detected in biopsies or resected tissue samples from patients with breast cancer (Blanco et al., 2002), gastric cancer (Rosivatz et al., 2002), hepatocellular carcinomas (Jiao et al., 2002), oral squamous cell carcinoma (Yokoyama et al., 2001) and ovarian carcinoma (Elloul et al., 2006; Rosivatz et al., 2006; Blechschmidt et al., 2007). Several studies demonstrated that expression of Snail triggers the manifestation of EMT under various experimental conditions (Spagnoli et al., 2000; Tan et al., 2001; Gotzmann et al., 2002; Yanez-Mo et al., 2003; Peinado et al., 2003; Grille et al., 2003; Barbera et al., 2004). Reciprocal relationship between Snail and E-cadherin in the transition from adenoma to carcinoma is very well established with invasive properties of tumours (Vleminckx et al., 1991; Perl et al., 1998). Snail blocks E-cadherin expression by binding to specific CACCTG boxes in its promoter (Battle et al., 2000; Cano et al., 2000). In addition to in vitro studies, biopsies of human breast cancer showed strong correlation between Snail expression and E-cadherin repression with respect to invasive grade of the tumours (Blanco et al., 2002). Overall, this strongly suggests a role for Snail as an E-cadherin repressor consequently leading to progression of breast carcinomas in vivo (Bolos et al., 2002).

Moreno-Bueno et al, (2006) have shown that the transcription factors Snail, Slug and bHLH E-47 a very well described direct repressors of E-cadherin in contexts with EMT can induce common and specific genetic programs, supporting a differential role of these factors in tumor progression and invasion (Moreno-Bueno et al., 2006; Elloul et al., 2005). Applying short hairpin RNA against Snail showed marked decrease in markers of mesenchymal form such as ID1, MMP-9 and SPARC in MDA-MB-231 cells with increased sensitivity to chemotherapeutics e.g. gemcitabine and docetaxel relevant in breast cancer treatments (Olmeda et al., 2007). Proposal of Snail to be considered as a marker for malignancy has come up with the hope of opening up new avenues for the design of drugs against advancing carcinoma (Cano et al., 2000).

So far, studies on characterization and regulation of Snail protein have shown that it has two well defined domains that interact with each other, the C-terminal domain is responsible for binding to the DNA with defined specificity for core sequence-CACCTG. The C-terminus of Snail1 protein can be phosphorylated by PAK-1 kinase to maintain the protein in the nucleus (Yang et al., 2005) whereas, its transcription and promoter activity is controlled by PI3 kinase (Peinado et al., 2003). However, these pathways are active in epithelial cells and do not entirely explain the specificity of expression of Snail in mesenchymal cells. Peiro et al, (2006) described presence of 50-CACCTG-30 E-box that Snail binds to this element and therefore creates a negative loop controlling its own expression with a possibility of oscillatory pattern of expression of this gene during somatogenesis (Freeman et al., 2000; Dale et al., 2006). RNA interference experiments also indicated that the existence of this self-repression is significant for controlling Snail expression in epithelial cells. Therefore, this loop would be responsible for controlling the stability of Snail expression. This capability of Snail protein to bind its own promoter has also been detected in cells with a mesenchymal phenotype. Mutation of the E-box in Snail promoter increased the activity of this promoter in these cells, indicating that the feedback control is also active (Peiro et al., 2006). This self inhibitory loop has been suggested to be responsible for controlling the stability of Snail1 expression in context with EMT (Peiro et al., 2006).

TGF-β is known as an inducer of EMT in epithelial cells which is necessary for acquisition of invasive phenotype in carcinomas (Oft et al., 1998; Akhurst et al., 1999). TGF-β modulates epithelial cell plasticity during morphogenesis, wound healing and tumor formation in context with EMT (Zavadil et al., 2001). It has been reported that the cytokine TGF-β which is often regulated in human tumors enhances SIP1 expression as well as Snail expression (Hemavathy et al., 2000). Targets of these repressors include genes for constituents of the intermediate filaments (e.g, cytokeratin and desmoplakin) and cell adhesion molecules such as E-cadherin that share E-boxes present in promoter region (**Plate VIII,** see, introductory page of results). Promoter regions of Slug, Snail, SIP1 and TGF-β1 also show presence of E-boxes and other binding motifs which may keep these under auto-regulatory control.

Promoter DNA sequence analysis of TGF-β1 showed absence of "TATA" box or "CAAT" box whereas, it showed highly G+C rich regions, 11 CCGCCC repeats and seven putative transcription factor SP1 binding sites (Kim et al.,1989). Sequence covering 1400 to 300 base pairs upstream of transcription initiation site include most of

the positive and negative regulatory sites. A fragment of 130-base pair located between -453 and -323 base pairs showed positive regulatory activity in vitro (Kim et al., 1989). These studies suggested that the 5'-flanking sequences of the human TGF-β1 gene contains two negative regulatory regions (-1362 to -1132 and -731 to -453) which repress the strong transcription unit very efficiently with the possibility of counter action by enhancer-like elements located between -1132 and -731. Interestingly, the region of +1 to +271 has been implicated as a second promoter and actively involved in the expression and regulation of the major human TGF-β 1 mRNAs independently (Kim et al., 1989).

Another study by Ventura et al, (2003) implicated role of JNK in regulation of autocrine expression of TGF-β1. Promoter analysis studies in JNK deficient cells showed that AP-1 may be critically involved in TGF-β1 gene regulation in mouse and human cells. The mouse TGF-β1 promoter contains a distal negative regulatory region with an AP-1 site (TGAGTCA; -1403/-1397 bp) and a proximal positive regulatory region with a second AP-1 site (TGACACT; -379/-373). Similarly, deletion analysis of the TGF-β1 promoter in JNK deficient cells demonstrated that the distal negative regulatory region (-1799/-1027) was required for transcription regulation activity through AP-1. Interestingly, these AP-1 sites in the TGF-β1 promoter are located close to tandem SBE elements (CAGAC). It is likely that these SBE sites act as positive regulatory elements since TGF-β1 promoter is activated by Smad transcription factor complexes (Ashcroft et al., 1999). JNK deficient cells compared to wild-type cells showed increased Smad binding and decreased cJun binding to the TGF-β1 promoter (Ventura et al., 2004). Recruitment of HDAC3 to promoter complexes is also involved in negative transcriptional regulation (Weiss et al., 2003). Negative regulatory role of cJun binding to the distal regulatory region of the TGF-β1 promoter has been suggested to be mediated by recruitment of HDAC3 to the promoter complex (Ventura et al., 2004). Overall, these observations point out that suppression of TGF-β1 expression by JNK mediated pathway permits increased AP-1 binding to the distal (negative) regulatory region in the TGF-β1 promoter as well as inhibits Smad pathway that increases TGF-β1 expression (Ventura et al., 2003). The autoinduction of TGF-β1 is considered to be associated with increased Smad binding to the TGF-β1 promoter. The results of this study (Ventura et al., 2003), indicated that the JNK signaling pathway represents an additional regulatory mechanism that can contribute to autocrine TGF-β1expression (Dennler et al., 2000; Verrecchia et al., 2000, 2001, 2002).

Our study proposed that TGF-β1 mediated up regulation of various repressors, Slug, Snail and SIP1 may be responsible for the loss of cyotkeratins and other intermediate filaments e.g. desmoplakin in breast cancer. In addition to the loss of cytokeratins and E-cadherin, we further hypothesize that Slug, Snail and SIP1 may be the key regulators of TGF-β1 as well as their own expression through TGF-β1-mediated regulatory cycle in the progression of EMT. We thought this may be one of the TGF-β1 mediated circuits responsible for the progression of EMT.

We found EMT like phenomena in MCF-7 cells treated with TGF-β1. The transition manifests itself as morphological changes, specific markers of transition probed by employing specific antibodies. Mass analysis of proteins expressed in TGF-β1-treated cells indicates special roles for cytoskeletal rearrangement and structural stability -associated proteins in EMT. Further, we have observed the DNA-protein interaction of TGF-β1 and Snail probes that contains binding motif for proposed candidate molecules (Snail, Slug and SIP1) under study. With the findings obtained in DNA-protein interaction studies, we suggest that Snail may involved in regulation of TGF-β1 as well as its own expression through autoregulatory loop proposed in the feedback regulatory module (**Plate IX**). It is further supported with striking match with time kinetic analysis of TGF-β1 expression with gradual decrease and regain of expression with time after TGF-β1 treatment. Super shift assay using specific antibody against candidate repressor molecules would further resolve the possible interplay and multiplicity of regulatory molecules in the above situation. These results support the hypothesis of regulation of TGF-β1 and proposed transcription of repressors through a regulatory feedback mechanism.

5.2 EMT in response to TGF-β1 confirms the transition in MCF-7 cells under normal proliferation conditions over certain passages

Adherence of epithelial cells with each other through specialized junctions are essential organizations to maintain a framework of epithelial morphology. A structural network linked through cytokeratins seems to be strongest among all (Demlehner et al., 1995; Garrod et al., 1993). In vitro studies show desmoplakin interacts directly with cytokeratin filaments and appears to enhance desmosome stability (Stappenbeck et al., 1993, 1994; Kouklis et al., 1994). Cell-cell junctions formed by cadherins show remarkable dynamism as it is required for the reorganization and migration of cells during EMT accompanying development and metastasis. Cytoskeletal rearrangement is an essential

event in the manifestation of EMT. Our study begins with the observation in normal culture conditions of proliferative MCF-7 cells (**Fig 2**), representative of aggressive adenocarcinoma of mammary gland that showed striking morphological changes with loss of typical epithelial pattern suggestive of epithelial to mesenchymal transition over certain passages. This event of detachment of cell-cell junctions was initiated with a small numbers of cells. This observation suggest that without any treatment, endogenous expression of various molecules involved in molecular interplay required for onset and progression of EMT may sufficient to induce morphological changes like EMT in MCF-7 cells. To confirm the primary observations we followed TGF-β1 treatment and found epithelial to mesenchymal transition in MCF-7 cells (**Fig 8**). Cells in culture do respond to various stimuli to undergo EMT. Loss of cell-cell junctions e.g. adherin junctions and desmosomal junctions are striking features of EMT. TGF-β1 is well known as an inducer of EMT (Bhowmick et al 2001; Oft et al., 1996, 2002). TGF-β1 induces epithelial to mesenchymal transition in breast cancer cells. It also stimulates growth of metastatic cancers and the increased migration and invasion that contribute to the metastatic phenotype (Reiss et al., 1999; Jakowlew et al., 2006; Zavadil et al., 2005; Yates et al., 2007). We observed increased dissociation of cell-cell connections in TGF-β1 treated cells in comparison to untreated MCF-7 cells that showed intact epitheloid pattern characteristic of epithelial cells. At various time points TGF-β1-treated cells showed loss of various junctions and significant dissociation of cells (24 hrs) followed by further increase in dissociation and spread in culture (36 hrs).

5.3 Presence of vimentin in cytosol suffices for EMT whereas presence in nucleus irrespective of EMT suggests multiple roles for vimentin

MCF-7 cells undergoing transitions were further confirmed for the expression of vimentin known as a marker of mesenchymal cells. Vimentin is a type-III intermediate filament normally expressed in cells of mesenchymal origin. In addition to reorganization of E-cadherin-based adhesive junctions, expression of vimentin is another very essential event critically associated with EMT and with the metastatic phenotype of epithelial cells (Steinert & Roop, 1988). In support of primary observation of EMT like changes under normal culture condition over certain passages, we probed for mesenchymal specific marker vimentin in these cells. We observed localization of vimentin in MCF-7 cells (**Fig 3**) prominently in dispersing cells from main population appearing as a migratory front suggesting EMT. Our results are in agreement with other

studies that have also demonstrated expression of vimentin in epithelial cells in context with physiological or pathological processes involving epithelial cell migration (Ramaekers et al., 1983; Guarino, 1995; Gilles et al., 1999, 2003). Vimentin-specific antisense cDNA or oligonucleotide transfection in vimentin-expressing cell lines is known to reduce in vitro invasiveness or migration, strongly emphasizing a functional contribution of vimentin to epithelial cell invasion/migration during EMT (Hendrix et al., 1997; Gilles et al., 1999; Singh et al., 2003).

In addition to involvement in cytoskeletal rearrangement during EMT, vimentin is found to be associated with mechanical transduction of signals from the cell surface to the nucleus (Gilles et al., 1999; Eckes et al., 2000; Helfand et al., 2004). Apart from cytosolic existence, we observed nuclear localization of vimentin in MCF-7 cells (**Fig 13**). Immunofluorescence studies in MCF-7 cells showed cytosolic localization consistent with our observation of EMT in normal culture without TGF-β1 treatment (**Fig 2, 3**). Cells prominently showed nuclear localization in addition to its presence in cytosol after treatment with TGF-β1 with the exposure of 48 hrs. It has been shown that intermediate filament (IF) proteins exhibit considerable in vitro affinity for nuclear constituents such as DNA, RNA and histones. They are structurally related to nuclear matrix proteins and transcription factors, suggesting their participation in DNA-based nuclear events like DNA replication, transcription, recombination and repair (Traub & Shoeman, 1994). Microinjected DNA/vimentin complex have clearly shown immunolocalization of vimentin in nucleus of PtK2 and MCF-7 cells suggesting the nuclear import of vimentin. DNA-mediated entry of vimentin into nucleus opens the possibility of its functional role in genomic activities (Hartig et al., 1998). Whereas, interactions of vimentin with Erk prevents dephosphorylation of Erk ensuring Erk activation as long as vimentin stays bound, this suggests a role of vimentin as a chaperon like molecule (Perlson et al., 2006) with the proposal of these interactions for enabling long distance transport of phosphorylated Erk within the cell (Perlson et al., 2005; Helfand et al., 2005; Hanz& Fainzilber, 2006).

Immunofluorescence study of vimentin expression in both cytosol and nucleus was further examined by western blotting (**Fig 14**) showing nuclear localization of vimentin irrespective of TGF-β1 treatment along with increased cytosolic expression in response to TGF-β1. Nuclear fraction of treated cells prominently showed expression of vimentin suggesting shuttling or trafficking of vimentin in nucleus. Further investigation of the finding of nuclear localization of vimentin in subsequent western blot (**Fig 15-A)**

clearly showed increased cytosolic expression of vimentin compared to untreated cells in response to TGF-β1 and nuclear expression of vimentin independent of TGF-β1 treatment (72 hrs). Our results of increased cytosolic vimentin in response to TGF-β1 are quite consistent with the findings of Medici et al, (2006), as they observed significant increase of vimentin in immunostaining post TGF-β1 treatment supporting TGF-β1 promoted EMT in MCF-7 cells. Presence of vimentin in the nucleus of control and TGF -β1 treated cells are consistent with the findings of various studies suggesting its functional role in genomic activities (Hartig et al., 1998) and as a vehicle in the tansport of various kinases to the nucleus and within the cell (Perlson et al., 2005; Helfand et al., 2005; Hanz& Fainzilber, 2006).

Overall, vimentin shows a multidimensional role in cellular as well as nuclear processes. In context with EMT, expression of vimentin is inevitable to confer cytoskeletal rearrangement that offers motility to a migratory cell whereas, nuclear function irrespective of EMT points towards its involvement in transcriptional regulation mechanism in epithelial and in mesenchymal cells. Till now transcriptional regulation of vimentin during the EMT is poorly understood. Yates et al, (2007) developed and studied a series of transformed mouse mammary epithelial cell lines undergoing EMT in response to TGF-β1 in vitro (Chen et al., 2003). The regulatory elements for vimentin were not limited to well-characterized proximal promoter in ras-transformed cells in response to TGF-β1. In epithelial cells, vimentin expression is higher in migratory cells and may contribute to the migratory and invasive phenotype of metastatic cells. Thus, it is important to determine the role of TGF-β1 in regulation of vimentin gene expression during cell differentiation and in metastasis (Bindels et al., 2006; Wu et al., 2007).

5.4 DNA-protein interaction studies support probable interplay of TGF-β1 and TGF-β1-induced transcription repressors through a feedback loop

To date, regulation of TGF-β1 at transcriptional level in contexts to EMT have not been explored with respect to repressor proteins Snail, Slug and SIP1. Human TGF-β1 promoter shows several binding sites recognized by these proteins. Studies on regulation of TGF-β1 by Kim et al, (1989, 1990) support the identification of several regulatory elements in TGF-β1 promoter region. They showed that the upstream sequences of the TGF-β1 gene contain five distinct regulatory regions including two different promoter regions. Among these regions, two are involved in negative regulatory activity and one has enhancer-like activity (Kim et al., 1989). The negative regulatory regions (-1362 to

-1132 and -731 to -453) strongly repress the activity of the transcriptional unit; however, enhancer-like sequences at nucleotides -1132 to -731 efficiently overcome the activity of the more downstream (-731 to -453) negative regulatory region. Further, a region with positive regulatory activity located between nucleotides -453 to -323 is required for the transcriptional ability of the upstream TGF-β1 promoter. In our study with the established system of EMT in MCF-7 cells, for a further understanding of EMT at the level of regulation of TGF-β1 through TGF-β1-induced transcription repressors such as Snail, Slug and SIP1 in feedback manner, we explored the possibility of probable role of these repressors in regulation of TGF-β1 and their own regulation through E-box present in their promoter region. Electrophoretic mobility shift assay (EMSA) with promoter region specific probes containing the E-box were used in these studies.

5.4.1 Electrophoretic mobility shift for TGF-β1 DNA with nuclear extract from MCF-7 cells indicate probable role of Snail in TGF-β1 regulation

An evidence towards regulation of TGF-β1 by transcription repressors through E-box in context with EMT was found in untreated MCF-7cells undergoing EMT and in MDA-MB-231 cells showing no signs of EMT (**Fig 6**) We did obtain prominent electrophoretic mobility shift in MCF-7cells for TGF-β1 probe suggesting the probable interactions of Snail, Slug and SIP1 with TGF-β1 promoter as they are very well known for their interactions with E-box in context with EMT. We used a probe sequence (-519 to -491) containing CAGGTG motif from TGF-β1 promoter. Our probe sequence represents a part of the distinct negative regulatory region (-731 to -453) as suggested by Kim et al, (1989). In addition to this, deletion analysis studies of the TGF-β1 promoter have also shown a distal negative regulatory region located at -1799 to -1027 attributed with regulatory activity through AP-1 in JNK deficient cells (Ventura et al., 2003, 2004). Negative transcriptional regulation of TGF-β1 has been suggested with the binding of cJun to the distal regulatory region mediated by recruitment of HDAC3 to the promoter complex (Weiss et al., 2003; Ventura et al., 2004). Tandem SBE elements (CAGAC) are suggested to be acting as positive regulatory elements located close to AP-1 sites in the TGF-β1 promoter (Ashcroft et al., 1999). Our findings of DNA-protein interaction with TGF-β1 probe indicate for another potential site for negative regulation of TGF-β1 through E-box present in -519 to -419 region through interaction of regulatory factors (Snail, Slug and SIP1) in context with EMT (**Plate X**).

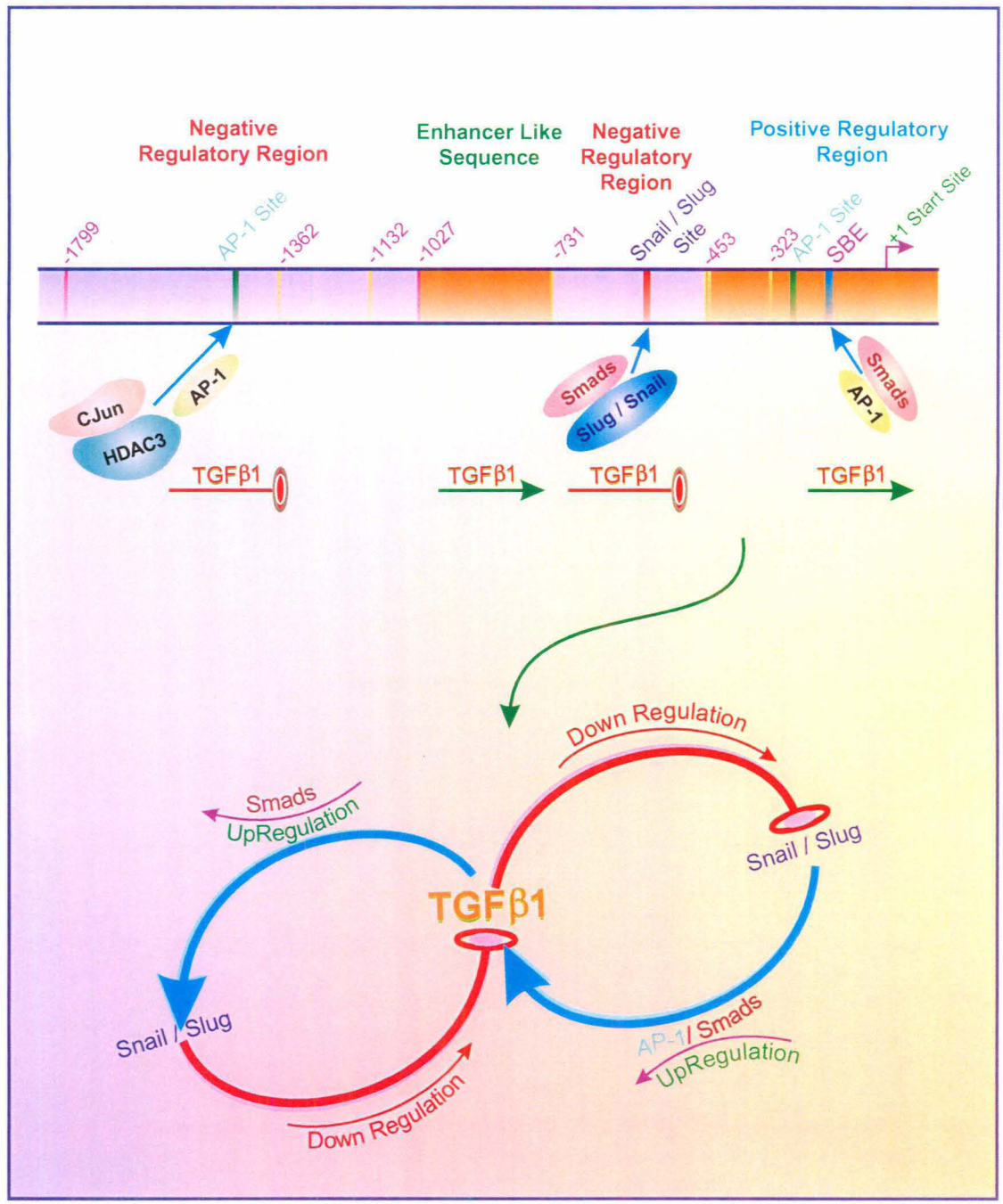


Plate X : Analysis of TGF-β1 promoter with proposed feed-back regulatory model. Distant negative regulatory region (-1799 to -1027, -1362 to -1132) shows cJun and HDAC3-mediated repression. The enhancer like region (-1027 to -731) may be involved in the positive regulation of the TGF-β1. Another negative regulatory region (-731 to -453) shows Snail/Slug-mediated repression with proximal positive regulatory region mediated through Smads/Ap-1. The proposed model of feed-back regulatory loop represents feed-back regulation of TGF-β1 through Snail/Slug.

5.4.2 Auto-regulation of Snail through inhibitory loop by binding with its own promoter through E-box

In recent years, extensive studies pointed out programmed repression of E-cadherin and upregulation of vimentin in the manifestation of EMT during early embryo development. Onset of metastasis inevitably required the presence of Snail. Therefore, the study of the mechanism that control Snail expression become important (Peiro et al., 2006). Studies on Snail regulation have shown that Snail gene expression requires the activity of Erk2 and PI3K signaling pathways (Peinado et al., 2003; Barbera et al., 2004). However, involvement of these two pathways is not specific to mesenchymal cells in context with EMT. Therefore, we propose additional mechanism of self regulation through TGF-β1-mediated feedback regulation of Snail. Our results support Snail expression through self inhibitory loop by binding to an E-box sequence present in its promoter.

In addition to probable interactions of Snail, Slug and SIP1 with TGF-β1 promoter, these regulatory factors might be auto-regulated by interaction with their own promoters containing E-box. With this speculation of auto-regulation, gel shift assay was performed for Snail probe containing CACCTG motif in promoter region (-151 to -122) in MCF-7 cells undergoing EMT (**Fig 7**). We observed prominent shift with the Snail probe containing E-box sequence (Peiro et al., 2006).

5.4.3 MCF-7 cells with intact junctions show less or no factor-binding with TGF-β1 and Snail probe compared to those undergoing EMT

In another EMSA experiment (**Fig 12**), two sets of MCF-7 cells were used. Among these sets one set was kept untreated ensuring intact cell-cell junctions and typical epitheloid morphology and another one was treated with TGF-β1. Marked dissociation was observed in TGF-β1 treated cells after 24 hrs and significantly high after 48 hrs with the manifestation of EMT. On the other hand, significantly fewer changes were observed in untreated cells with no visible alteration in cellular morphology and a much smaller number of cells undergoing dissociation. TGF-β1 probe showed no prominent shift with extract from untreated cells whereas prominent shift was observed in case of treated cells. Similarly, Snail probe showed no prominent shift in untreated cells whereas more than one shift was observed in treated cells. Multiple shifts are suggestive of more than one transcription factor/repressor interaction with Snail probe.

Molecular interplay involved in case of EMT in MCF-7 cells under influence of TGF-β is most probably triggered in case of aggressive phenotype achieved over certain

passages under normal proliferative conditions. Cells that do not show any sign of spread indicative of onset of EMT do not show these molecular events required for onset of EMT. Binding of these regulatory factors (Snail, Slug and SIP1) to TGF-β1 probe and Snail probe may suggest that MCF-7 cells a representative form of aggressive carcinoma, express EMT inducing growth factors and transcription repressors essential for EMT over certain passages (**Fig 6, 7**).

In case of Snail, our findings are similar to the evidences obtained by Peiro et al, (2006). The idea of self-regulation of their own promoters may be a general property of the Snail family. Existing single E-box in the -600 bp region of the Snail promoter would be the probable reason of modest repression of self promoter by Snail in comparison with those previously documented on other promoters (Peiro et al., 2006). Our results support the self-regulation of Snail with proposed feed-back regulatory pathway. The regulatory module may provide the avenue to understand molecular play involved in onset and progression of EMT during developmental processes and tumor invasion.

5.4.4 Electrophoretic mobility shift for E-cadherin probe support binding of Snail with TGF-β1 probe and Snail probe

Promoter analysis of human and mouse E-cadherin have widely supported the idea that transcriptional repression of E-cadherin is an essential event involved in the onset and progression of EMT (Henning et al., 1996; Giroldi et al., 1997; Faraldo et al., 1997; Hajra et al., 1999; Rodrigo et al., 1999). Studies on tumor cell lines and fibroblasts have shown the importance of the two E-boxes present in a proximal region of the E-cadherin promoter for the regulatory function observed for certain genes. These boxes contain core sequence CACCTG. This sequence is identical to the DNA-binding motif recognized by the transcriptional repressor Snail (Battle et al., 2000; Giroldi et al., 1997; Leptin et al., 1990; Smith et al., 1992).

Probe sequence containing CACCTG motif in E-cadherin promoter region was used for assay (**Fig 9**) and taken as a reference probe (Battle et al., 2000). An extensive analysis of various E-boxes by Battle et al, (2000) for Snail-binding ability in E-cadherin promoter performing reporter assays revealed that three boxes do respond to Snail-mediated E-cadherin repression with strongest repression activity in third E-box located farthest downstream at +22 to +27 position. These three oligonucleotide probes from different E-boxes having an identical CACCTG core sequence, showed different patterns of binding and migration, indicating specific interactions of the repressor with the

different sequences flanking each core binding site (Battle et al., 2000). Our results showed prominent shifts of E-cadherin probe in untreated cells as well as in TGF-β1 treated cells. Binding of Snail to E-cadherin probe is a well studied and established fact in context with EMT (Cano et al., 2000; Battle et al., 2000; Giroldi et al., 1997). Among all candidate repressors (Snail, Slug and SIP1) our EMSA results with reference to mobility shift of E-cadherin probe and RT-PCR results showing modulation of TGF-β1 expression with time, may suggest probable role of Snail as a repressor factor involved in the regulation of TGF-β1 and auto-regulation of its own expression (Peiro et al., 2006) through proposed feedback regulatory model. These findings are in conformity with another study of Bolos et al, (2002) on binding affinities of Slug, Snail and E-47 fusion proteins to the E-box element that showed that Snail has the highest affinity for E-box binding followed by E-47 and Slug. Besides, different combinations of the three factors similarly showed higher affinity of Snail for the E-box element compared with the other two factors. Presence of all three factors in stoichiometric amount could also show that Snail complex is predominant over the E-47 and Slug complexes (Bolos et al., 2002). In view of the above said, our observation suggests binding of Snail to TGF-β1 probe and its own probe.

5.4.5 Normal sequence and mutant sequence probes show different binding affinities

It has been shown that various combinations of mutations especially triple-mutant construct and point mutations generated in E-boxes show partial repression activity suggesting that point mutations are not enough to block Snail-mediated repression and may retain some affinity for the mutated E-boxes. Moreover, in cells with high expression of E-cadherin severe mutations or full deletions of E-boxes could show a loss of promoter activity with further suggestion of overlapping of E-boxes with sequences that are likely to be involved in positive regulation of the E-cadherin promoter (Battle et al., 2000).

Binding to a consensus motif was examined under varied stringency with a probe containing four times CACCTG repeat (**Fig 10**). Another EMSA experiment was performed with sequence mutant probe containing four times AACCTA repeat (**Fig 11**) under similar conditions as for the reference probe. Shift was observed with extract from both treated and untreated cells. Mutant sequence probe showed stronger binding compared to normal sequence probe. The binding stringency and affinity towards probe

might involve a space between two motifs and orientation of sequence with core binding nucleotides. Our results with normal and mutant E-box sequence support the findings of Battle et al, (2000) related to combinations of mutated Snail-binding sites in the E-boxes.

E-boxes with core consensus sequence are also present in the promoter regions of other genes such as desmoplakin, cytokeratin 18 and α6 integrin (Sorokin et al., 1990). This core sequence is also present in TGF-β1 as well as in Snail promoter. Theses genes are involved in epithelial morphogenesis. The involvement of these Snail binding motifs itself signifies a profound role of Snail in repression as an essential part of EMT during embryogenesis and in advancement of carcinoma. We propose Snail mediated regulation of TGF-β1 and auto-regulation of its own expression through regulatory feedback mechanism.

5.5 Proteomic study reveals roles of markers of EMT and differentially expressed proteins

Epithelial to mesenchymal transition underlies versatile molecular interplay that essentially begins with downregulation of cell adhesion molecules such as E-cadherin and upregulation of vimentin, remodeling of cytoskeletal structure and rearrangement of extra-cellular matrix. Many of the epithelial specific structural proteins of the intermediate filaments e.g. various cytokeratins, desmoplakins and structural stability associated proteins such as α and β actins are influenced by transcriptional reprogramming. TGF-β1 recruits key regulatory factors that enforce structural alterations essential for EMT. Proteomics provides complementary tool to genomic research and has proven very useful to examine cancer related changes in protein expression profile in order to achieve insight into functional correlation of these proteins with disease manifestation as well as to identifying the markers or potential therapeutic targets. To understand the interplay of various molecules involved in manifestation of EMT at proteomic level, 2D gel electrophoresis was performed and the spots showing signs of deferential expression were given for MALDI-TOF analysis.

5.5.1 MALDI-TOF analysis point out cytoskeletal rearrangement in aggressive form of MCF-7 cells undergoing EMT in response to TGF-β1 treatment

During EMT, loss of adherent junctions is further evidenced with cytoskeletal rearrangements of actin and vimentin with the partial replacement of various cytokeratins that offers increased cell motility, prerequisite to invasiveness and metastasis (Bhowmick

et al., 2001; Maeda et al., 2005). TGF-β promotes actin reorganization and cell motility through ID1 that has been shown to be involved in tumorigenesis and suggested to be a determining factor for TGF-β switch from an inhibitor to tumor promoting factor during carcinigenesis of prostate epithelial cells (Di et al., 2007). It has been found that overexpression of ID1 activates HSP-27 and upregulation of HSP-27 is reported to facilitate stress fiber formation, which in turn enhances cell motility in response to TGF-β (Guayet al., 1997; Gerthoffer et al., 2001; Di et al., 2007).

MCF-7 cells represent aggressive form of breast carcinoma and respond very well to TGF-β1-mediated EMT accompanied with differential expression of HSP-27 justifying aggressive behavior of cells in response to TGF-β1 treatment (**Fig 22, 23, 24**). PMF spectrum of HSP-27 (**Fig 25**) along with sequence coverage map showed intensity coverage-53.5% and sequence coverage- 46.3%. MS/MS spectrum (**Fig 26**) further confirmed the matched sequences: LFDQAFGLPR of m/z1163.681 corresponding to HSP-27. Also it has been seen in MCF-7 cells that microtubule interfering agent Vincristine, markedly induces phosphorylation of HSP-27 at Ser78, suggesting involvement of HSP-27 in resisting the interference to microtubule dynamics by drugs and thus enhancing cell survival, consistent with the HSP-27 regulated cell invasion and migration (Shin et al.,2005; Xu et al., 2006). Recently, HSP-27 has been shown to be markedly expressed in HER2/neu positive tumors and cell lines. The enhanced level of HSP-27 may be one of the factors contributing to its aggressive tumor behavior and poor prognosis in HER2/neu positive tumors. The functional correlation of HSP-27 with HER2/neu status and other clinicopathological implications are objectives of further studies (Zang et al., 2005, 2007).

Cells do contain functionally distinct subsets of stable and dynamic microtubules that are regulated by extra-cellular stimuli (Gundersen et al., 1984). Serum starvation or loss of cell-cell contact triggers loss of microtubule stabilization. Conversely, treatment of cells with lysophosphatidic acid or TGF-β induces microtubule stability (Cook et al., 1998; Palazzo et al., 2004; Gundersen et al., 1994). Consistent with this notion PMF spectrum of the spots **C2** and **C5** (**Fig 34, 35**) suggested α and β tubulin respectively whereas, those of spots **C7**and **T7** (**Fig 39, 40**) suggested TUBB protein signifying an attempt to restore the stability lost during EMT. Besides, a recent study of Ahmed et al, (2007) showed that TGF-β induces microtubule stability and modulates Paclitaxel sensitivity in vitro and in patients receiving Paclitaxel therapy suggesting TGF-β to be probable biomarker for selecting patients with respect to Taxane therapy.

With the feedback of cytoskeletal rearrangement in MCF-7 cells undergoing EMT in response to TGF-β1, we further explored markers of EMT and differentially expressed proteins (**Fig 29, 30, 31**). The spots **D1**and **D2** (**Fig 41, 42**) gave a peptide mass profile of triosephosphate isomerase 1 isoform (TPI). Glycolytic enzymes TPI, PGK1 and enolase have been shown to be significantly overexpressed in the HER2/neu-positive breast tumors. Overexpression of these enzymes is very well justified with the increased requirement of energy for the rapidly growing tumors (Zang et al., 2005). PMF spectrum of the spot **D4** (**Fig 44**) corresponds to tyrosine3/tryptophan5-monooxygenase activation protein, ζ polypeptide, also known as 14-3-3ζ is basically a member of the 14-3-3 family of proteins spanning seven isotypes in mammals. It has been found that 14-3-3 ζ protein is overexpressed in various types of carcinomas. The 14-3-3 ζ protein has prominently found to be involved in promotion of EMT in response to TGF-β (Keshamouni et al., 2006). Recent knockdown study on 14-3-3 ζ protein showed induction of two isoform-specific phenotypes with potent oncogenic activity comprising activation of JNK/p38 pathway and stress induced apoptosis as well as expression of cell adhesion proteins in absence of 14-3-3 ζ protein during tumorigenesis (Niemantsverdriet et al., 2008). Another study on patients of non small cell lung cancer (NSCLC) and animal model showed correlation between elevated expression of 14-3-3 ζ protein with advanced pathologic stage in non small cell lung cancer suggesting potential target for developing a prognostic biomarker and therapeutics against NSCLC (Fan et al., 2007). PMF spectrum of the spot **D6** (**Fig 46**) yields peptide fragments indicating transcription factor elongin A2. We further need to explore the relevance of this protein in context with TGF-β mediated EMT. The suggested isoelectric point (pI-10.4) for elongin A2 by MALDI-analysis and Mascot search have shown variation with actual isoelectric point (pI </~ 8.0) located in 2D gel. The alteration in isoelectric point may suggest some modifications such as phosphorylation or complex formation that need to be resolved with further research. PMF spectrum of the spot **D7** (**Fig 47**) corresponds to ubiquitin which was further confirmed with matched sequence IQDKEGIPPDQQR of m/z1523.73 corresponding to ubiquitin by MS/MS analysis (**Fig 48**). Regulation through ubiquitination seems to be an integral part of the Smads-mediated TGF-β signaling pathway. It provides a further level of regulation through ubiquitin-mediated targeting and proteasomal degradation of down-stream components of the TGF-β signaling pathway. Primarily, E3 ubiquitin ligases are known to be responsible for the recognition of specific target proteins and downregulation of E3 ubiquitin ligases are implicated in

development of cancer (Nakayama & Nakayama 2006). Several E3 ligases such as Cbl-b, a single U-box type ligase, carboxyl terminus of Hsc70 interacting protein (CHIP), RING-finger type E3 ligases, Skp1/Culin/F-box protein (SCF)-Skp2, SCF-b-TrCP1, Roc1-SCF-b-TrCP1, anaphase promoting complex (APC), Ectodermin and PRAJA have significant roles in ubiquitination of various Smads involved in BMP and TGF-β signaling in many cancers (Zhu et al., 1999; Zang et al., 2001; Glasgow & Mishra, 2008).

Consistent with 2D profile in Figure-24, differential expression of HSP-27 (**Fig 43-D3**) in cells undergoing EMT in response to TGF-β1, suggests its role in offering structural stability by maintaining unaltered conformation of actin in these cells. Further, activation of HSP-27 associated with F-actin stress fiber formation ensures increased cell-substrate adhesion and increased rate of cell migration in TGF-β1 treated cells. It has been reported that the TGF-β induces activation of HSP-27 during cell invasion (Xu et al., 2006). During EMT, the active form of HSP-27 stimulates cytoskeletal reorganization and stress fiber formation as an essential framework for cell migration and invasion (Guayet al., 1997; Rousseau et al., 2006; Gerthoffer et al., 2001; Shin et al., 2005). Upregulation of HSP-27 has been reported in several types of tumors (Cornford et al., 2000; Rocchi et al., 2004). Elevated level of HSP-27 has also been observed in prostate carcinoma (Cornford et al., 2000; Tomita et al., 2000; Rocchi et al., 2004; Jaggi et al., 2006).

Expression of cytokeratin16 (**Fig 45-D5**) along with β actin (**Fig 37-C4, 38- T4**), α and β tubulin collectively signifies their importance in structural stability and loss of the same in context with EMT. HSP-27, Isoforms of triose phosphate isomerase 1 and ubiquitin strongly indicates aggressive form of carcinoma that MCF-7 cells become in response to TGF-β1 treatment. A further investigation with regard to the role of the ubiquitins in modulation of TGF-β signaling may provide an insight into the mechanism of many cancers, which eventually help in providing precise therapies in treating the disease. It has been seen that 14-3-3 proteins can interact with more than 100 cellular proteins at their phosphorylation sites including various protein kinases, receptor proteins, structural and cytoskeletal proteins, enzymes and proteins involved in cell cycle regulation and apoptosis (van et al., 2001; Rosenquist et al., 2003; Fu et al., 2000). Apart from 14-3-3ζ protein another isoform 14-3-3 α, formerly characterized as a human mammary epithelium specific marker-1 (HME1) have shown increased expression during epithelial differentiation (Prasad et al., 1992). It has a versatile role in intracellular

signaling (Muslin et al., 2000; van et al., 2001) and it is thought to be tumor suppressor protein since it has been found decreased in many types of human carcinomas (Liu et al., 2004, 2006; Ferguson et al., 2000; Iwata et al., 2000; Gasco et al., 2002; Gasco et al., 2002; Yatabe et al., 2002; Cheng et al., 2004).

Overall, mass analyses of proteins indicated special roles for cytoskeletal rearrangement and structural stability associated proteins in MCF-7 cells undergoing EMT in response to TGF-β1 treatment (**Plate XI**). Furthermore, our results agree with a direct or indirect interaction of intermediate filament (IF) such as vimentin with microfilaments and microtubules and more particularly with molecules such as actin, plectin or integrins essentially required for cytoskeletal dynamics associated with structural reorganization and gain of motility offered to a cell undergoing EMT (Svitkina et al., 1996; Maniotis et al., 1997; Homan et al., 1998; Goldman et al., 1999; Wu et al., 1999; Gonzales et al., 2001; Tsuruta & Jones, 2003; Helfand et al., 2004; Kreis et al., 2005).

5.6 Time course of expression of EMT markers

Manifestation of EMT involves rearrangement of several membrane-associated proteins such as E-cadherin, ZO-1 and changes in the cytoskeletal organization such as modulation of cytokeratins and upregulation of vimentin. Expression of vimentin in human epithelial cancers is frequently observed (Singh et al., 2003; Lang et al., 2002) and has been suggested as a prognostic marker (Hu et al., 2004; Dandachi et al., 2001). RT-PCR analysis of expression of cytokeratin 18, E-cadherin and TGF-β1 at different time points (24 hrs, 48 hrs and 72 hrs) following TGB-β1 treatment showed less altered expression of cytokeratin 18 (**Fig 49**). Consistent with the observation of Yates et al, (2007) in a series of transformed cell lines treated with TGF-β1 with a marginal change in cytokeratin expression. Our results of flowcytometry (**Fig 16-21**) also suggested marginal difference in cytokeratin 18 in both control and TGF-β1 treated MCF-7 cells (**Fig 20, 21**). On the contrary, in case of vimentin expression (**Fig 18, 19**), no significant change was observed in control and treated cells. We speculate that cells showing no sign of morphological changes like EMT might not respond to a cytokine such as TGF-β1 to induce expression of mesenchymal markers like vimentin and consequently EMT.

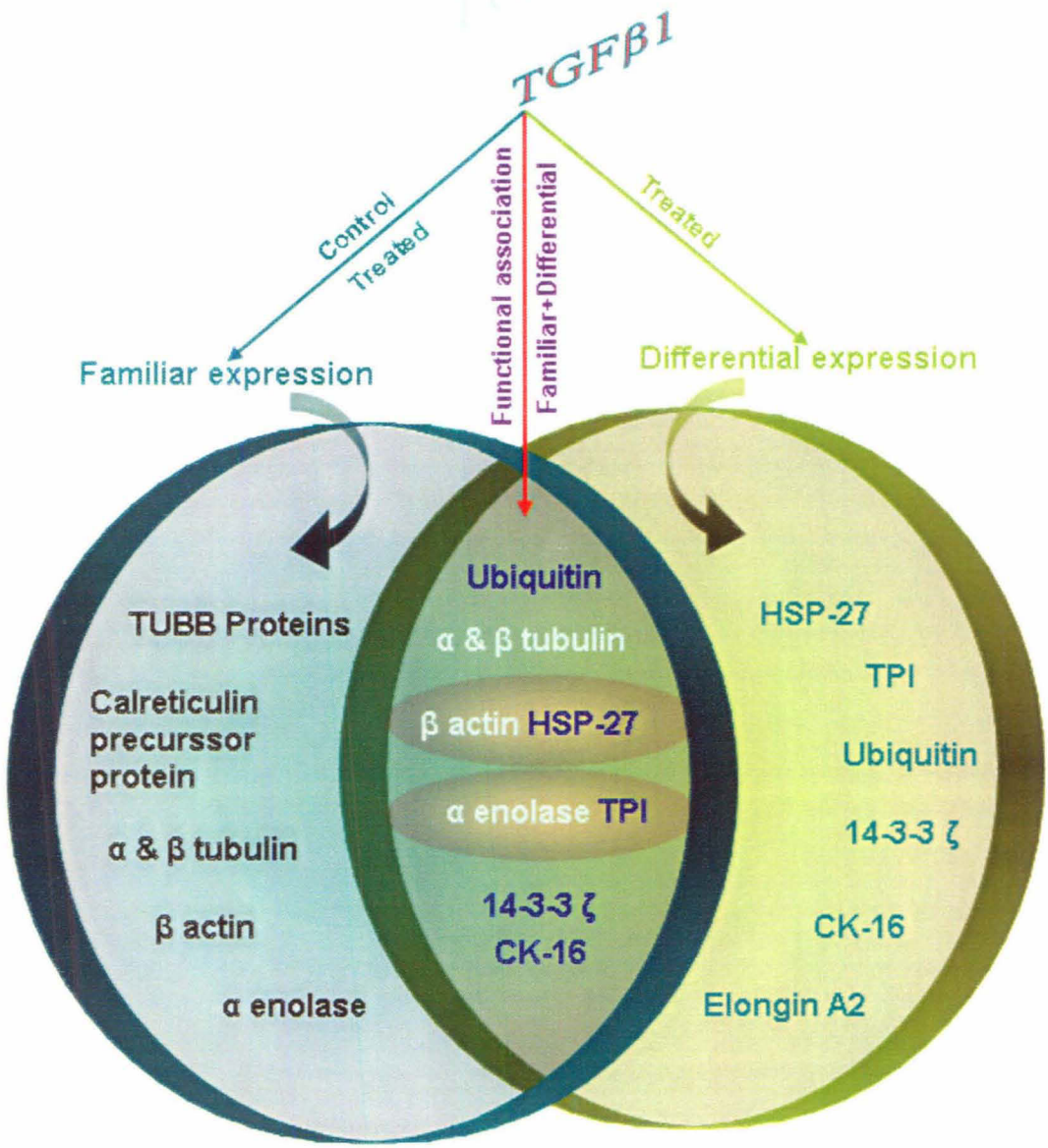


Plate XI: Protein expression profile points out cytoskeletal rearrangement in MCF-7 cells in response to TGF-β1.

Differential expression of HSP-27, CK-16, 14-3-3ζ indicate cytoskeletal reorganization with respect to loss of stability of cytoskeletal proteins in cancerous cells undergoing EMT. Microtubule dynamics are sensitive to TGF-β1 response. TPI along with commonly expressed α enolase are required to met energy expenditure from metastatic cells. Ubiquitins show further layer of regulation of TGF-β1 signaling. Expression of elongin A2 with respect to TGF-β1 treatment need to be explored with further study. Familiar expression represents general proteins from both control and treated cells. Differential expression represents differential proteins expressed in treated cells. Functional association represents functional interactions of TGF-β1 with familiar and differential proteins.

This is consistent with the continued expression of vimentin and cytokeratin in metastatic lesions thought to have undergone EMT in response to TGF-β1 suggesting that vimentin and cytokeratin 18 are not reciprocally regulated (Yates et al., 2007).

We further observed gradual decrease of E-cadherin expression in RT-PCR analysis with time (**Fig 50**). Lowering of E-cadherin is a hallmark of onset and progression of EMT. TGF-β modulates E-cadherin expression in context with EMT. Consistent with established behavior of E-cadherin in EMT, MCF-7 cells showed downregulation of E-cadherin with time following TGF-β1 treatment. The observed downfall in E-cadherin expression with increase in time in response to TGF-β1 may correlate with the proposed interaction of regulatory factor Snail with its own probe and TGF-β1 probe observed in EMSA studies.

5.6.1 Expression profile of TGF-β1 correlates with presumed interplay with Snail in auto-regulation

TGF-β1 has an auto-regulatory mechanism through transcription factor AP-1 (Kim et al., 1990). Another level of TGF-β1 regulation is suggested to be added through the interaction of Snail with E-box motif present in TGF-β1 promoter on the basis of EMSA studies. Consistent with the auto-regulatory loop model, TGF-β1 showed gradual decrease and regain of expression in MCF-7 cells (**Fig 51**) with time after TGF-β1 treatment. Expression of TGF-β1 was examined by RT-PCR at different time points. Visibly significant intensity corresponding to 234bp amplicon was observed in untreated sample, whereas, the intensity and size was drastically decreased in cells at 24 hrs and 48 hrs after TGF-β1 treatment. At 72 hrs, regain of expression in terms of increased intensity of the band was observed. We suggest the involvement of Snail in downregulation of TGF-β1 at different time points (24 hrs, 48 hrs) and regain of expression (72 hrs) through TGF-β1 mediated auto-regulatory mechanism in absence of Snail protein. These observations in TGF-β1 expression with varied time strikingly match with presumed regulatory interplay of TGF-β1 and Snail in feedback manner in context with EMT and support the hypothesis of regulation of TGF-β1 and TGF-β1-mediated transcription repressors through a regulatory feedback mechanism involving auto-regulatory loop.

Summary & Conclusions

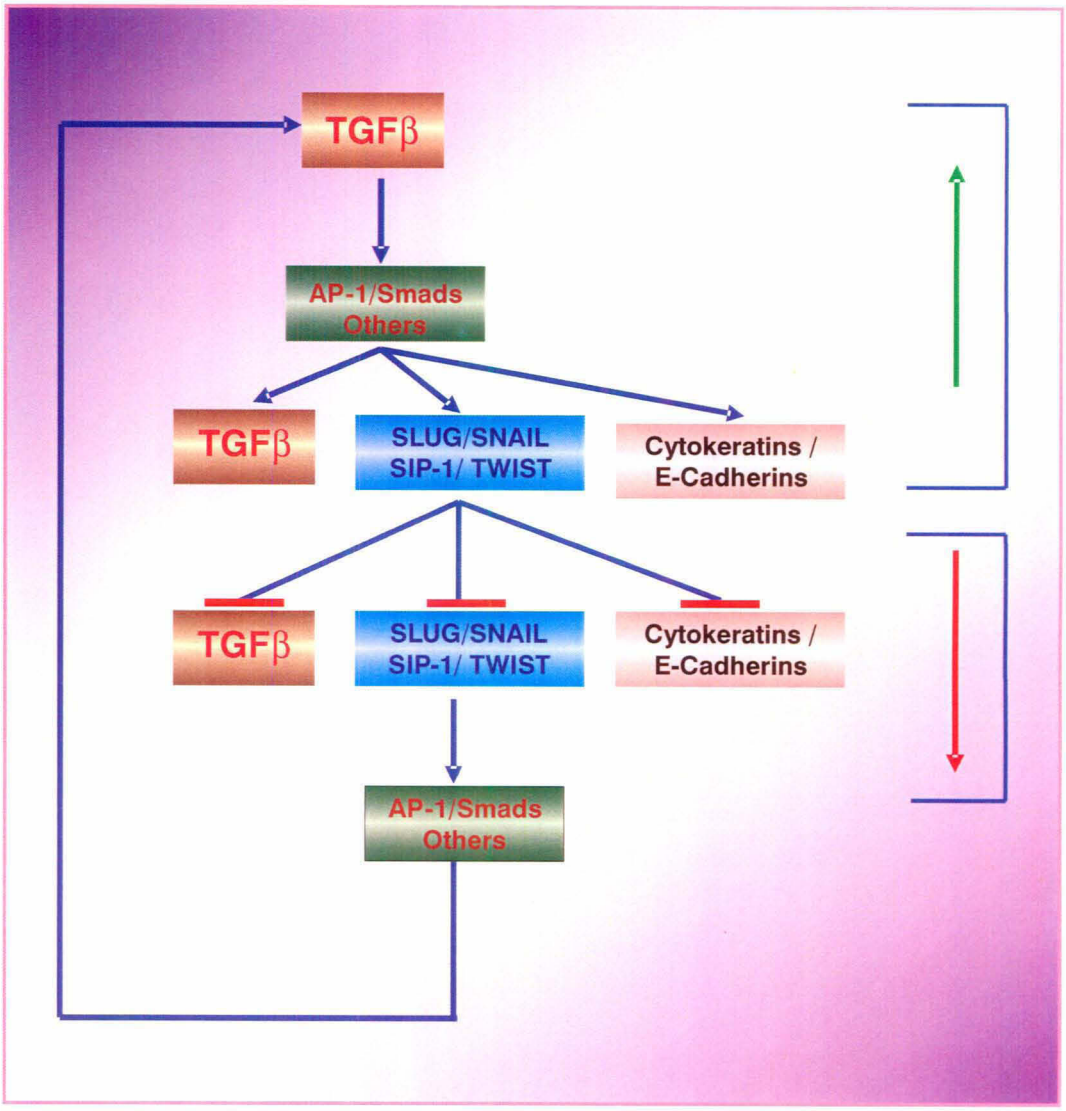


Plate XII: TGF-β mediated one of the circuits may involve in progression of cancer.

Cycle of feed-back regulation of TGF-β through Slug/ Snail, AP-1 may trigger cytoskeletal rearrangement through downregulation of cytokeratins and E-cadherin.

6 SUMMARY & CONCLUSIONS

Conversion of epithelial cells into mesenchymal cells results from a series of events during which epithelial cells undergo complex changes in cell architecture and behavior. Loss of E-cadherin expression during EMT is an essential event needed in both embryogenesis and tumor progression. As a critical molecule of epithelial phenotype, E-cadherin plays a crucial role in the suppression of tumor invasion, since E-cadherin is involved in various cell-cell junctions required for normal epitheloid structure and polarity of epithelial cells. Loss of expression or altered E-cadherin expression is associated with increased risk of local invasion and metastasis along with recurrence and poor prognosis in a variety of malignancies, for example, breast, uterine cervix, gastric and ovarian cancer. Snail a member of the Snail superfamily of zinc-finger transcription factors is considered as a key regulator of EMT and an important effector of the process of invasiveness and tumorigenecity. It is known for its potential role in E-cadherin repression essentially through interaction with E-box elements found in the proximal promoter region of E-cadherin that eventually result into EMT with the acquisition of invasive and tumorigenic properties. Aggressive carcinoma cell lines and metastatic tumors of human and mouse origin express Snail protein and consequently repress E-cadherin protein. Both the molecules are involved in EMT during embryonic development as well as in tumor advancement. Various observations support the fact that Snail plays crucial role in onset and progression of breast cancer. Expression of Snail has been detected at the invasive regions concomitant with downregulation of E-cadherin associated with lymph node status and tumor recurrence. Different affinities towards binding with E-boxes have been seen in mouse E-cadherin promoter with different repressors such as Snail, Slug and E-47. Snail has the highest affinity for E-box binding followed by E-47 and Slug. The expression patterns of these factors in vitro in carcinoma cell lines and in vivo during embryonic development support the idea that the modulation by different factors of E-cadherin expression can be attained by relative concentrations of these factors as well as by specific cellular or tumor contexts.

Snail protein is shown to be regulated by GSK-3, PAK1 kinase and PI3 kinase. However, regulation of Snail takes place through these pathways in epithelial cells and the specificity of expression and regulation of Snail in mesenchymal cells yet to be established.

Epithelial cells essentially undergo EMT to acquire epithelial plasticity during embryogenesis and progression of tumor formation. TGF-β is known to promote EMT in various physiological backgrounds. In human tumors, TGF-β enhances expression of transcription repressors such as Snail, Slug, SIP1 and Twist. These repressors selectively target constituents of the intermediate filaments such as cytokeratin and desmoplakin and cell adhesion molecules such as E-cadherin through interactions with E-boxes commonly present in promoter regions of respective genes. E-box with a core-consensus of CACCTG motif is also present in promoter regions of Snail, Slug and TGF-β1 along with other binding motifs likely to be involved in regulation of their own expression through auto-regulatory control.

Various studies on human TGF-β1 gene have revealed potential sites for binding of different transcription factors such as AP-1, transcription factor SP-1, nuclear factor-1 (NF-1) and highly G+C rich regions like eleven CCGCCC repeats. Sequence spanning upstream region of transcription initiation site (-1400 to -300 bp) account for binding with most of the positive and negative regulatory factors. Further analyses have shown five distinct regulatory regions together with two different promoter regions. Among them, two regions correspond to negative regulatory activity and one region corresponds to enhancer-like activity. These two negative regulatory regions (-1362 to -1132 and -731 to -453) mediate strong repression of transcription with the possibility of counter action by enhancer-like element located between -1132 and -731. Another promoter has also been characterized in region of transcription initiation site (+1 to +271) as a second promoter involved in the expression and regulation of the TGF-β1 independently. In addition to this, promoter analysis studies in JNK deficient cells also showed involvement of AP-1 in TGF-β1 gene regulation in mouse and human cells. Deletion analysis of the TGF-β1 promoter in *JNK* deficient cells demonstrated that the distal negative regulatory region (-1799 to -1027) was required for transcription regulation activity through AP-1. These AP-1 sites are located close to tandem SBE elements (CAGAC) in the TGF-β1 promoter and these SBE sites are considered to be positive regulatory elements. Further studies on TGF-β1 regulation suggest another mechanism mediated through HDAC3. Recruitment of HDAC3 to promoter complexes is involved in negative regulation of TGF-β1. Overall observations in JNK deficient cells, point out negative regulation of TGF-β1 through JNK mediated pathway. It permits increased AP-1 binding to the distal regulatory region attributed for negative regulation in the TGF-β1 promoter. Simultaneously it facilitates inhibition of Smad pathway responsible for

positive regulation of TGF-β1 expression. JNK signaling pathway thus represents an additional regulatory mechanism for TGF-β1 expression. Regulation of TGF-β1 at transcriptional level in contexts with EMT with respect to repressor proteins Snail, Slug and SIP1have not been explored.

With this background information available in literature, our study put a proposal that Snail, Slug, SIP1 and Twist may be the key regulatory molecules for the expression of TGF-β1 and their own auto-regulation through TGF-β1 mediated feedback regulatory cycle in context with EMT (**Plate XII**). We thought this may be one of the TGF-β1 mediated circuits responsible for the manifestation and progression of EMT.

We observed EMT like morphological changes in MCF-7 cells under normal proliferative conditions. We repeatedly observed these changes in MCF-7 cells over certain passages in culture, suggestive of epithelial to mesenchymal transition. This manifestation was further confirmed by TGF-β1 treatment at different time points. The transition is itself a morphological change and we probed for mesenchymal specific markers of transition. We employed antibody against marker protein vimentin in control cells and TGF-β1 treated cells in immunocytochemistry, immunofluorescence and western blot experiments. Expression of vimentin was found in both cytosol and nucleus irrespective of TGF-β1 treatment. Presence of vimentin in nucleus suggested nuclear transport of vimentin irrespective of TGF-β1 treatment reflecting role of vimentin in genomic activities. Proteomic analysis of cells following TGF-β1 treatment reflects on cytoskeletal modifications in contexts with EMT. Cytoskeletal proteins such as cytokeratins, actin fibers and tubulins along with differential expression of heat shock protein-27 (HSP-27), isoforms of triose phosphate isomerase1, ubiquitin, 14-3-3 ζ protein in treated cells strongly indicated aggressive form of carcinoma that MCF-7 cells undergo after TGF-β1 treatment. Overall, mass analysis of proteins expressed in TGF-β1 treated cells indicated special roles for cytoskeletal rearrangement and structural stability associated proteins in EMT.

The time course of EMT markers following TGF-β1 treatment suggested the rearrangement of the various membrane and cytoskeletal proteins associated with several cell-cell junctions. We observed gradual loss of E-cadherin expression with increase in time, which is quite consistent with the downregulation of E-cadherin as a central event in EMT. Expression of cytokeratin18, on the contrary, showed marginal changes with time in comparison with control cells. Flowcytometric analysis of cytokeratin18 further supported the minor change in expression level in both control and TGF-β1 treated

MCF-7 cells. In case of vimentin expression, we found no significant change in TGF-β1 treated cells. Endogenous growth factors might be significantly amplified with exogenous treatment of cytokine such as TGF-β1.

In an established system of EMT in MCF-7 cells, to achieve further insight into EMT at the level of regulation of TGF-β1 and transcription repressors such as Snail, Slug, SIP1 and Twist in feedback manner, we performed DNA-protein interaction of TGF-β1 and Snail probes containing binding motif for proposed candidate molecules (Slug, Snail, SIP1 and Twist) under study. We obtained electrophoretic mobility shift for TGF-β1 and Snail probes indicating that these transcription repressors are expressed in MCF-7 cells undergoing EMT in normal culture over certain passages and they do interact with TGF-β1 and Snail probes. Similar results were obtained in TGF-β1 treated MCF-7 cells undergoing EMT. Results for the query probes (TGF-β1 and Snail probe) were further confirmed by using reference probe of E-cadherin and other probes containing four times repeat of normal and mutant element sequence. The results showed varied binding stringency and affinity of these factors towards the probes.

With the results obtained in DNA-protein interaction studies, we suggest that Snail may involve in regulation of TGF-β1 as well as its own expression through auto-regulatory feedback loop. Time kinetics of TGF-β1 expression showed gradual decrease at 24 hrs and 48 hrs and regain of expression at 72hrs. This supports the assumed regulatory interplay of TGF-β1 and Snail in feedback manner in context with EMT.

With these findings we could draw the following conclusions:

- Morphological changes in MCF-7 cells under normal proliferation conditions over certain passages with loss of typical epithelial pattern may represent spontaneous epithelial to mesenchymal transition in cells.

- Expression of vimentin in MCF-7 cells was primarily observed in dispersing cells from main population appearing as migratory front suggesting EMT.

- Increase in cytosolic vimentin in response to TGF-β1 may precede TGF-β1 promoted EMT in MCF-7 cells.

- Prominent electrophoretic mobility shift in MCF-7cells for TGF-β1 probe suggests the possible modulation at transcription level.

- Prominent electrophoretic mobility shift in MCF-7 cells for Snail probe suggests the probable modulation of Snail at transcriptional level.

- Our results support auto-regulation of Snail through inhibitory loop by binding to an E-box sequence present in its promoter.

- EMSA results with Snail and TGF-β1 probes suggest that MCF-7 cells, a representative form of aggressive carcinoma, express EMT inducing growth factors and transcription repressors essential for EMT.

- MCF-7 cells with intact junctions show low or no factor binding with TGF-β1 and Snail probe compared to those undergoing EMT.

- Our results showed prominent shift for E-cadherin probe in untreated cells undergoing EMT as well as in TGF-β1 treated cells. Binding of Snail to E-cadherin probe is a well studied and established fact in context with EMT.

- Among all candidate repressors (Snail, Slug and SIP1) our EMSA and RT-PCR results may suggest Snail as a repressor factor involved in the regulation of TGF-β1 and auto-regulation of its own expression through proposed feedback regulatory model.

- A mutant sequence probe showed strong binding compared to normal sequence probe. The binding stringency and affinity towards probe might involve a space between two motifs and orientation of sequence with core binding nucleotides.

- Proteomic analysis of expressed proteins points to cytoskeletal reorganization in aggressive form of MCF-7 cells undergoing EMT in response to TGF-β1 treatment.

- Differential expression of HSP-27 in cells undergoing EMT in response to TGF-β1, suggests its role in offering structural stability by maintaining unaltered conformation of cytoskeletal proteins.

- Expression of α and β tubulin, TUBB protein signify an attempt to restore the stability lost during EMT in response to TGF-β1.

- Expression of enzymes of glycolytic pathway, triosephosphate isomerase1 isoform (TPI) and enolase is very well justified with the increased requirement of energy for the MCF-7 cells undergoing EMT.

- Overall, expression of various molecules such as cytokeratin along with actin and tubulins collectively denote their importance in structural stability and its association with loss of stability in context with EMT.

- Collectively, differential expression of HSP-27, isoforms of triose phosphate isomerase1, ubiquitin and 14-3-3ζ protein prominantly indicates aggressive form of MCF-7 cells in response to TGF-β1 treatment.

- Mass analyses of proteins indicated special roles for cytoskeletal rearrangement and structural stability associated proteins in MCF-7 cells undergoing EMT in response to TGF-β1 treatment.

- Our results of expression of cytoskeletal proteins indicate direct or indirect interactions of vimentin with microfilaments and microtubules and more particularly with actin that are essentially required for cytoskeletal dynamics associated with structural reorganization and gain of motility offered to a cell undergoing EMT.

- Our results of flowcytometry and RT-PCR analysis suggest only marginal changes of cytokeratin 18 in both control and TGF-β1 treated MCF-7 cells.

- In case of vimentin expression no significant change was observed in response to TGF-β1 treatment.

- We observed gradual decrease of E-cadherin expression in RT-PCR analysis with time. Lowering of E-cadherin is a hallmark of onset and progression of EMT.

- Observations in TGF-β1 expression with time points strikingly match with presumed regulatory interplay of TGF-β1 and Snail in feedback manner in context with EMT and support the hypothesis of regulation of TGF-β1 and TGF-β1 mediated transcription repressors through a regulatory feedback mechanism.

Our findings further suggest the following:

- Induction of EMT in normal proliferative conditions over certain passages in a small subset of MCF-7 cells without any external treatment suggest that endogenous expression of cytokine and various molecules involved in onset and progression of EMT are sufficient to induce morphological changes like EMT.

- Presence of vimentin in the nucleus irrespective of TGF-β1 treatment, suggest its functional role in genomic activities.

- Overall, presence of vimentin in cytosol indicates EMT, whereas presence in nucleus irrespective of EMT suggests multiple roles for vimentin.

- Our findings of DNA-protein interaction with TGF-β1 probe suggests another potential site for negative regulation of TGF-β1 through E-box present in -519 to -419 region through interaction of regulatory factors (Snail, Slug and SIP1) projected in this study in context with EMT.

- We further speculate a feedback regulatory loop in TGF-β1 expression that might be operated through enhancer-like sequences at nucleotides -1132 to -731 which can be considered as positive regulatory element in TGF-β1 promoter.

- Our results support the self-regulation of Snail with proposed feed-back regulatory pathway. The regulatory module may provide an understanding of molecular interplay involved in onset and progression of EMT during developmental processes and tumour invasion.

- We suggest the involvement of Snail in downregulation of TGF-β1 at different time points (24hrs, 48hrs) and regain of expression (72hrs) through TGF-β1 mediated auto-regulatory mechanism in absence of Snail protein.

- Molecular interaction involved in EMT under influence of TGF-β may trigger in cells undergoing EMT like phenotype achieved over certain passages under normal proliferative conditions. Cells that do not show any sign of spread indicative of onset of EMT may not show these molecular events required for onset of EMT in MCF-7 cells.

- We speculate that cells showing no sign of morphological changes like EMT might not respond to a cytokine such as TGF-β1 to induce expression of mesenchymal markers like vimentin and consequently EMT.

- Further investigation with regard to the role of the ubiquitins in modulation of TGF-β signaling may provide an insight in the mechanism of many cancers, which eventually help in providing precise therapies in treating the disease.

References

REFERENCES

Afrakhte M. Induction of inhibitory Smad6 and Smad7 mRNA by TGF-β family members. Biochem Biophys Res Commun **1998**; 249: 505–511.

Ahmed AA, Mills AD, Ibrahim AEK, Temple J, Blenkiron C, Vias M, Massie C E, Iyer N.G, McGeoch A, Crawford R, Nicke B, Downward J, Swanton C, Bell SD, Earl HM, Laskey RA, Caldas C, Brenton JD. The extracellular matrix protein TGFBI induces microtubule stabilization and sensitizes ovarian cancers to paclitaxel. Cancer Cell **2007**; 12: 514–527.

Ahmed S, Liu CC, Nawshad A. Mechanisms of palatal epithelial seam disintegration by transforming growth factor (TGF) b3. Dev Biol **2007**; 309: 193–207.

Akhurst RJ, Balmain A. Genetic events and the role of TGF-β in epithelial tumor progression. J Pathol **1999**; 187: 82-90.

Akhurst RJ. TGF-β antagonists: why suppress a tumor suppressor? J Clin Invest **2002**; 109:1533–1536.

Akhurst RJ. TGF-β signaling in epithelial–mesenchymal transition and invasion/metastasis. The TGF-β Family (eds Derynck, R. & Miyazono, K.) (Cold Spring Harbor Press, Cold Spring Harbor, NY, **2007**).

Akiyoshi S, et al. c-Ski acts as a transcriptional co-repressor in transforming growth factor-β signaling through interaction with Smads. J Biol Chem **1999**; 274: 35269–35277.

Alliston T, Choy L, Ducy P, Karsenty G, Derynck R. TGF-β-induced repression of CBFA1 by Smad3 decreases cbfa1 and osteocalcin expression and inhibits osteoblast differentiation. EMBO J **2001**; 20: 2254–2272.

Andreasen PA, Kjoller L, Christensen L, Duffy MJ. The urokinase-type plasminogen activator system in cancer metastasis: a review. Int J Cancer**1997**; 72: 1–22.

Ashcroft GS, Yang X, Glick AB, Weinstein M, Letterio JL, Mizel DE, Anzano M, Greenwell-Wild T, Wahl SM, Deng C, et al. Mice lacking Smad3 show accelerated wound healing and an impaired local inflammatory response. Nat Cell Biol **1999**; 1: 260–266.

Auersperg N, Maines-Bandiera SL, Dyck HG, Kruk PA. Characterization of cultured human ovarian surface epithelial cells: phenotypic plasticity and premalignant changes. Lab Invest **1994**; 71(4):510-518.

Bachelder RE, Yoon SO, Franci C, de Herreros AG, Mercurio AM. Glycogen synthase kinase-3 is an endogenous inhibitor of Snail transcription: implications for the epithelial-mesenchymal transition. J Cell Biol **2005**; 168: 29–33.

Bakin AV, Rinehart C, Tomlinson AK, Arteaga CL. p38 mitogen-activated protein kinase is required for TGF beta-mediated fibroblastic transdifferentiation and cell migration. J Cell Sci **2002**; 115:3193-206.

Bakin AV, Tomlinson AK, Bhowmick NA, Moses HL, Arteaga CL. Phosphatidylinositol 3-Kinase function is required for transforming growth factor beta - mediated epithelial to mesenchymal transition and cell migration. J Biol Chem **2000**; 275(47): 36803 - 36810.

Barbera MJ, Puig I, Dominguez D, Julien-Grille S, Guaita-Esteruelas S, Peiro S, Baulida J, Franci C, Dedhar S, Larue L, Garcia de Herreros A. Regulation of Snail transcription during epithelial to mesenchymal transition of tumor cells. Oncogene **2004**; 23: 7345–7354.

Bardeesy N, Cheng KH, Berger JH, et al. Smad4 is dispensable for normal pancreas development yet critical in progression and tumor biology of pancreas cancer. Genes Dev **2006**; 20: 3130–3146.

Barrios-Rodiles M, et al. High-throughput mapping of a dynamic signaling network in mammalian cells. Science **2005**; 307: 1621–1625.

Bates RC, Mercurio AM. Tumor necrosis factor-alpha stimulates the epithelial-to-mesenchymal transition of human colonic organoids. Mol Biol Cell **2003**; 14(5):1790-1800.

Batlle E, Sancho E, Franci C, Dominguez D, Monfar M, Baulida J, Garcia de Herreros A. The transcription factor snail is a repressor of E-cadherin gene expression in epithelial tumour cells. Nat Cell Biol **2000**; 2:84–89.

Behrens J, Lowrick O, Klein-Hitpass L, Birchmeier W. The E-cadherin promoter: functional analysis of a G.C-rich region and an epithelial cell-specific palindromic regulatory element. Proc Natl Acad Sci USA **1991**; 88: 11495-11499.

Berx G, Van Roy F. The E-cadherin/catenin complex: an important gatekeeper in breast cancer tumorigenesis and malignant progression. Breast Cancer Res **2001**; 3: 289–293.

Bhowmick NA, Chytil A, Plieth D, et al. TGF-β signaling in fibroblasts modulates the oncogenic potential of adjacent epithelia. Science **2004**; 303: 848–851.

Bhowmick NA, Ghiassi M, Bakin A, Aakre M, Lundquist CA, Engel ME, Arteaga CL, Moses HL. Transforming growth factor-beta1 mediates epithelial to mesenchymal

transdifferentiation through a RhoA-dependent mechanism. Mol Biol Cell **2001**; 12: 27–36.

Bhowmick NA, Zent R, Ghiassi M, McDonnell M, Moses HL. Integrin beta 1 signaling is necessary for transforming growth factor-beta activation of p38MAPK and epithelial plasticity. J Biol Chem **2001**; 276(50):46707-46713.

Bindels S, Mestdagt M, Vandewalle C, Jacobs N, Volders L, Noel A, van Roy F, Berx G, Foidart JM, Gilles C. Regulation of vimentin by SIP1 in human epithelial breast tumor cells. Oncogene **2006**; 25:4975–4985.

Bitzer M. et al. A mechanism of suppression of TGF-β/SMAD signaling by NF- κB/Rel A. Genes Dev **2000**; 14: 187–197.

Blanco MJ, Moreno-Bueno G, Sarrio D, Locascio A, Cano A, Palacios J, Nieto MA. Correlation of Snail expression with histological grade and lymph node status in breast carcinomas. Oncogene **2002**; 21: 3241–3246.

Blechschmidt K, Kremmer E, Hollweck R, Mylonas I, Hofler H, Kremer M, Becker KF. The E-cadherin repressor Snail plays a role in tumor progression of endometrioid adenocarcinomas. Diagn Mol Pathol **2007** (in press)

Bolos V, Peinado H, Perez-Moreno MA, Fraga MF, Esteller M, Cano A. The transcription factor Slug represses E-cadherin expression and induces epithelial to mesenchymal transitions: a comparison with Snail and E47 repressors. J Cell Sci **2002**; 116:499–511.

Border WA, Noble NA. Transforming growth factor β in tissue fibrosis. N Engl J Med **1994**; 331(19):1286-1292.

Bottinger EP, Bitzer M.TGF-beta signaling in renal disease. J Am Soc Nephrol. **2002**; 13(10):2600-1610.

Boyer AS, Ayerinskas I I, Vincent EB, McKinney LA, Weeks DL, Runyan RB. TGFb2 and TGFb3 have separate and sequential activities during epithelial-mesenchymal cell transformation in the embryonic heart. Dev Biol **1999**; 208: 530–545.

Boyer B, Thiery JP. Epithelium-mesenchyme interconversion as example of epithelial plasticity. APMIS **1993**; 101: 257–268.

Cano A, Perez-Moreno MA, Rodrigo I, Locascio A, Blanco MJ, del Barrio MG, Portillo F, Nieto MA. The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. Nat Cell Biol **2000**; 2:76–83.

Carver EA, Jiang R, Lan Y, Oram KF, Gridley T. The mouse snail gene encodes a key regulator of the epithelial-mesenchymal transition. Mol Cell Biol **2001**; 21: 8184–8188.

Cavallaro U, Christofori G. Cell adhesion and signalling by cadherins and Ig-CAMs in cancer. Nat Rev Cancer **2004**; 4:118–132.

Chang E, Goldberg H. Requirements for transforming growth factor-β regulation of the pro-α 2(I) collagen and plasminogen activator inhibitor-1 promoters. J Biol Chem **1995**; 270: 4473–4477.

Chen C, Ware SM, Sato A, Houston-Hawkins DE, Habas R, Matzuk MM, Shen MM, Brown CW. The Vg1-related protein Gdf3 acts in a Nodal signaling pathway in the pre-gastrulation mouse embryo. Development **2006**; 133: 319–329.

Chen D, Zhao M, Mundy GR. Bone morphogenetic proteins. Growth Factors **2004**; 22(4):233-241.

Chen J, Fabry B, Schiffrin EL, Wang N. Twisting integrin receptors increases endothelin-1 gene expression. Am J Physiol Cell Physiol **2001**; 280: C1475–C1484.

Chen Y, Rittling SR. Novel murine mammary epithelial cell lines that form osteolytic bone metastases: effect of strain background on tumor homing. Clin Exp Metastasis **2003**; 20:111–120.

Cheng L, Pan CX, Zhang JT, et al. Loss of 14-3-3 sigma in prostate cancer and its precursors. Clin Cancer Res **2004**; 10: 3064–3068.

Cheng N, Bhowmick NA, Chytil A, et al. Loss of TGF-β type II receptor in fibroblasts promotes mammary carcinoma growth and invasion through upregulation of TGF-α,-MSP- and HGF-mediated signaling networks. Oncogene **2005**; 24: 5053–5068.

Christ B, Ordahl CP. Early stages of chick somite development. Anat Embryol (Berl) **1995**; 191: 381–396.

Christiansen JJ, Rajasekaran AK. Reassessing epithelial to mesenchymal transition as a prerequisite for carcinoma invasion and metastasis. Cancer Res **2006**; 66: 8319–8326.

Chung KY, Agarwal A, Uitto J, Mauviel A. An AP-1 binding sequence is essential for regulation of the human α2 (I) collagen (COL1A2) promoter activity by transforming growth factor-β. J Biol Chem **1996**; 271: 3272–3278.

Ciruna B, Rossant J. FGF signaling regulates mesoderm cell fate specification and morphogenetic movement at the primitive streak. Dev Cell **2001**; 1: 37–49.

Come C, Magnino F, Bibeau F, et al. Snail and slug play distinct roles during breast carcinoma progression. Clin Cancer Res **2006**; 12:5395–5402.

Comijn J, Berx G, Vermassen P, Verschueren K, van Grunsven L, Bruyneel E, Mareel M, Huylebroeck D, van Roy F. The two-handed E box binding zinc finger protein SIP1 downregulates E-cadherin and induces invasion. Mol Cell **2001**; 7:1267–1278.

Conacci-Sorrell M, Zhurinsky J, Ben-Zeev A. The cadherin–catenin adhesion system in signaling and cancer. J Clin Invest **2002**; 109: 987–991.

Cook TA, Nagasaki T, Gundersen GG. Rho guanosine triphosphatase mediates the selective stabilization of microtubules induced by lysophosphatidic acid. J Cell Biol **1998**; 141:175–185.

Cordenonsi M, et al. Integration of TGF-β and Ras/ MAPK signaling through p53 phosphorylation. Science **2007**; 315: 840–843.

Cornford PA, Dodson AR, Parsons KF, Desmond AD, Woolfenden A, Fordham M, Neoptolemos JP, Ke Y, Foster CS. Heat shock protein expression independently predicts clinical outcome in prostate cancer. Cancer Res **2000**; 60: 7099–7105.

Correia AC, Costa M, Moraes F, Bom J, Novoa A, Mallo M. Bmp2 is required for migration but not for induction of neural crest cells in the mouse. Dev Dyn **2007**; 236: 2493–2501.

Crawford SE, et al. Thrombospondin-1 is a major activator of TGF-β1 in vivo. Cell **1998**; 93: 1159–1170.

Cui W, Fowlis DJ, Bryson S, Duffie E, Ireland H, Balmain A, Akhurst RJ. TGFbeta1 inhibits the formation of benign skin tumors, but enhances progression to invasive spindle carcinomas in transgenic mice. Cell **1996**; 86(4):531-542.

Czernobilsky B, Moll R, Levy R, Franke WW. Co-expression of cytokeratin and vimentin filaments in mesothelial, granulosa and rete ovarii cells of the human ovary. Eur J Cell Biol **1985**; 37: 175–190.

Dale JK, Malapert P, Chal J, Vilhais-Neto G, Maroto M, Johnson T, Jayasinghe S, Trainor P, Herrmann B, Pourquier O. Oscillations of the Snail genes in the presomitic mesoderm coordinate segmental patterning and morphogenesis in vertebrate somitogenesis. Dev Cell **2006**; 10: 355–366.

Dandachi N, Hauser-Kronberger C, More E, Wiesener B, Hacker GW, Dietze O, Wirl G. Co-expression of tenascin-C and vimentin in human breast cancer cells indicates phenotypic transdifferentiation during tumour progression: correlation with histopathological parameters, hormone receptors, and oncoproteins. J Pathol **2001**; 193: 181–189.

Davies JA. Mesenchyme to epithelium transition during development of the mammalian kidney tubule. Acta Anat (Basel) **1996**; 156: 187–201.

De Craene B, Gilbert B, Stove C, Bruyneel E, van Roy F, Berx G. The transcription factor snail induces tumor cell invasion through modulation of the epithelial cell differentiation program. Cancer Res **2005a**; 65:6237– 6244.

De Craene B, van Roy F, Berx G. Unraveling signalling cascades for the Snail family of transcription factors. Cell Signal **2005b**; 17: 535–547.

de Jong JS, et al. Expression of growth factors, growthinhibiting factors, and their receptors in invasive breast cancer. II: Correlations with proliferation and angiogenesis. J Pathol **1998**; 184; 53–57.

De Wever O, Westbroek W, Verloes A, et al. Critical role of N-cadherin in myofibroblast invasion and migration in vitro stimulated by colon-cancercell- derived TGF-β or wounding. J Cell Sci **2004**; 117: 4691–4703.

Deckers M, van Dinther M, Buijs J, et al. The tumor suppressor Smad4 is required for transforming growth factor β-induced epithelial to mesenchymal transition and bone metastasis of breast cancer cells. Cancer Res **2006**; 66: 2202–2209.

Demlehner MP, Schafer S, Grund C, Franke WW. Continual assembly of half-desmosomal structures in the absence of cell contacts and their frustrated endocytosis: a coordinated Sisyphus cycle. J Cell Biol **1995**; 131:745–760.

Dennler S, et al. Direct binding of Smad3 and Smad4 to critical TGF β-inducible elements in the promoter of human plasminogen activator inhibitor-type 1 gene. EMBO J **1998**; 17: 3091–3100.

Dennler S, Prunier C, Ferrand N, Gauthier JM, Atfi A. c-Jun inhibits transforming growth factor beta-mediated transcription by repressing Smad3 transcriptional activity. J Biol Chem **2000**; 275: 28858–28865.

Derynck R, Akhurst RJ, Balmain A. TGF-β signaling in tumor suppression and cancer progression. Nat Genet **2001**; 29: 117-129.

Derynck R, Akhurst RJ. Differentiation plasticity regulated by TGF-β family proteins in development and disease. Nat Cell Biol **2007**; 9:

Derynck R, et al. Synthesis of messenger RNAs for transforming growth factors α and β and the epidermal growth factor receptor by human tumors. Cancer Res **1987**; 47: 707–712.

Derynck R, Zhang Y, Feng X-H. Smads: transcriptional activators of TGF-β responses. Cell **1998**; 95: 737–740.

Derynck R, Zhang YE. Smad-dependent and Smad-independent pathways in TGF-β family signalling. Nature **2003**; 425: 577–584.

Desgrosellier JS, Mundell NA, McDonnell MA, Moses HL, Barnett JV. Activin receptor-like kinase 2 and Smad6 regulate epithelial–mesenchymal transformation during cardiac valve formation. Dev Biol **2005**; 280: 201–210.

Di K, Wong Y C, Wang X. Id-1 promotes TGF-β1-induced cell motility through HSP27 activation and disassembly of adherens junction in prostate epithelial cells. Exp Cell Res **2007**; 313:3983 – 3999.

Dickson RB, et al. Activation of growth factor secretion in tumorigenic states of breast cancer induced by 17β-estradiol or v-Ha-ras oncogene. Proc Natl Acad Sci USA**1987**; 84: 837–841.

Dumont N, Arteaga CL. Targeting the TGF beta signaling network in human neoplasia. Cancer Cell **2003**; 3: 531–536.

Eastham AM, Spencer H, Soncin F, Ritson S, Merry CLR, Stern PL, Ward CM. Epithelial-mesenchymal transition events during human embryonic stem cell differentiation. Cancer Res **2007**; 67(23):11254-11262.

Eckes B, Colucci-Guyon E, Smola H, Nodder S, Babinet C, Krieg T, et al. Impaired wound healing in embryonic and adult mice lacking vimentin. J Cell Sci **2000**; 113: 2455–2462.

Eckes B, Dogic D, Colucci-Guyon E, Wang N, Maniotis A, Ingber D, Merckling A, Langa F, Aumailley M, Delouvee A, Koteliansky V, Babinet C, Krieg T. Impaired mechanical stability, migration and contractile capacity in vimentin-deficient fibroblasts. J Cell Sci **1998**; 111: 1897–1907.

Eger A, Aigner K, Sonderegger S, Dampier B, Oehler S, Schreiber M, Berx G, Cano A, Beug H, Foisner R. Delta EF1 is a transcriptional repressor of E-cadherin and regulates epithelial plasticity in breast cancer cells. Oncogene **2005**; 24; 2375–2385.

Eger A, Stockinger A, Park J, Langkopf E, Mikula M, Gotzmann J, Mikulits W, Beug H, Foisner R. beta-Catenin and TGF beta signalling cooperate to maintain a mesenchymal phenotype after FosER-induced epithelial to mesenchymal transition. Oncogene **2004**; 23: 2672–2680.

Eger A, Stockinger A, Schaffhauser B, Beug H, Foisner R. Epithelial mesenchymal transition by c-Fos estrogen receptor activation involves nuclear translocation of beta-catenin and upregulation of beta-catenin/lymphoid enhancer binding factor-1 transcriptional activity. J Cell Biol **2000**; 148: 173–188.

Eisaki A, Kuroda H, Fukui A, Asashima M. XSIP1, a member of two-handed zinc finger proteins, induced anterior neural markers in Xenopus laevis animal cap. Biochem Biophys Res Commun **2000**; 271: 151–157.

Ellenrieder V, Hendler SF, Boeck W, Seufferlein T, Menke A, Ruhland C, Adler G, Gress TM. Transforming growth factor beta1 treatment leads to an epithelial-mesenchymal transdifferentiation of pancreatic cancer cells requiring extracellular signal-regulated kinase 2 activation. Cancer Res **2001**; 61: 4222–4228.

Elloul S, Elstrand MB, Nesland JM, Trope CG, Kvalheim G, Goldberg I, Reich R, Davidson B. Snail, Slug, and Smad interacting protein 1 as novel parameters of disease aggressiveness in metastatic ovarian and breast carcinoma. Cancer **2005**; 103:1631–1643.

Elloul S, Silins I, Trope CG, Benshushan A, Davidson B, Reich R. Expression of E-cadherin transcriptional regulators in ovarian carcinoma. Virchows Arch **2006**; 449:520-528.

Engel ME, McDonnell MA, Law BK, Moses HL. Interdependent SMAD and JNK signaling in transforming growth factor-β-mediated transcription. J Biol Chem **1999**; 274: 37413–37420.

Fan T, Li R, Todd NW, Qiu Q, Fang H-B, Wang H, Shen J, Zhao RY, Caraway NP, Katz RL, Stass SA, Jiang F. Up-regulation of 14-3-3ζ in lung cancer and its implication as prognostic and therapeutic target. Cancer Res **2007**; 67(16): 7901–7906.

Faraldo ML, Rodrigo I, Behrens J, Birchmeier W, Cano A. Analysis of the E-cadherin and P-cadherin promoters in murine keratinocyte cell lines from different stages of mouse skin carcinogenesis. Mol Carcinog **1997**; 20: 33-47.

Feng XH, Derynck R. Specificity and versatility in TGF-β signaling through Smads Annu Rev Cell Dev Biol **2005**; 21:659–693.

Feng XH, Lin X, Derynck R. Smad2, Smad3 and Smad4 cooperate with Sp1 to induce p15INK4B transcription in response to TGF-β. EMBO J **2000**; 19: 5178–5193.

Feng XH, Zhang Y, Wu RY, Derynck R. The tumor suppressor Smad4/DPC4 and transcriptional adaptor CBP/p300 are coactivators for Smad3 in TGF-β- induced transcriptional activation. Genes Dev **1998**; 12: 2153–2163.

Ferguson AT, Evron E, Umbricht CB, et al. High frequency of hypermethylation at the 14–3-3 sigma locus leads to gene silencing in breast cancer. Proc Natl Acad Sci USA **2000**; 97:6049–6054.

Fischer AN, Fuchs E, Mikula M, Huber H, Beug H, Mikulits W. PDGF essentially links TGF-β signaling to nuclear β-catenin accumulation in hepatocellular carcinoma progression. Oncogene **2007**; 26: 3395–3405.

Freeman M. Feedback control of intercellular signalling in development. Nature **2000**; 408: 313–319.

Funayama N, Sato Y, Matsumoto K, Ogura T, Takahashi Y. Coelom formation: binary decision of the lateral plate mesoderm is controlled by the ectoderm. Development **1999**; 126: 4129–4138.

Fu H, Subramanian RR, Masters SC. 14-3-3 proteins: structure, function, and regulation. Annu Rev Pharmacol Toxicol **2000**; 40:617–647.

Gadue P, Huber TL, Paddison PJ, Keller GM. Wnt and TGF-β signaling are required for the induction of an *in vitro* model of primitive streak formation using embryonic stem cells. Proc Natl Acad Sci USA **2006**; 103: 16806–16811.

Garrod DR. Desmosomes and hemidesmosomes. Curr Opin Cell Biol **1993**; 5: 30–40.

Gasco M, Bell AK, Heath V, et al. Epigenetic inactivation of 14-3-3 sigma in oral carcinoma: association with p16(INK4a) silencing and human papillomavirus negativity. Cancer Res **2002**; 62:2072–2076.

Gasco M, Sullivan A, Repellin C, et al. Coincident inactivation of 14-3-3sigma and p16INK4a is an early event in vulval squamous neoplasia. Oncogene **2002**; 21: 1876–1881.

Ge R, Rajeev V, Subramanian G, Reiss KA, Liu D, Higgins L, Joly A, Dugar S, Chakravarty J, Henson M, McEnroe G, Schreiner G and Reiss M. Selective inhibitors of type I receptor kinase block cellular transforming growth factor-beta signaling. Biochem Pharmacol **2004**; 68: 41–50.

Geiser AG, Kim SJ, Roberts AB, Sporn MB. Characterization of the mouse transforming growth factor-β1 promoter and activation by the Ha-ras oncogene. Mol Cell Biol **1991**; 11: 84–92.

Gerthoffer WT, Gunst SJ. Invited review: focal adhesion and small heat shock proteins in the regulation of actin remodeling and contractility in smooth muscle. J Appl Physiol **2001**; 91:963–972.

Gilles C, Polette M, Mestdagt M, Nawrocki-Raby B, Ruggeri P, Birembaut P, et al. Transactivation of vimentin by beta-catenin in human breast cancer cells. Cancer Res **2003**; 63: 2658–2664.

Gilles C, Polette M, Zahm JM, Tournier JM, Volders L, Foidart JM, et al. Vimentin contributes to human mammary epithelial cell migration. J Cell Sci **1999**; 112: 4615–4625.

Giroldi LA, Bringuier PP, de Weijert M, Jansen C, van Bokhoven A, Schalken JA. Role of E boxes in the repression of Ecadherin expression. Biochem Biophys Res Commun **1997**; 241: 453-458.

Glasgow E, Mishra L. Transforming growth factor-β signaling and ubiquitinators in cancer. Endocrine Related Cancer **2008**; 15: 59–72.

Goldman RD, Chou YH, Prahlad V, Yoon M. Intermediate filaments: dynamic processes regulating their assembly, motility, and interactions with other cytoskeletal systems. FASEB J **1999**; 13(Suppl 2): S261–S265.

Gonzales M, Weksler B, Tsuruta D, Goldman RD, Yoon KJ, Hopkinson SB, et al. Structure and function of a vimentin-associated matrix adhesion in endothelial cells. Mol Biol Cell **2001**; 12: 85–100.

Gonzalez MA, Pinder SE, Wencyk PM, Bell JA, Elston CW, Nicholson RI, Robertson JF, Blamey RW, Ellis IO. An immunohistochemical examination of the expression of E-cadherin, alpha- and beta/ gamma-catenins, and alpha2- and beta1-integrins in invasive breast cancer. J Pathol **1999**; 187: 523–529.

Gotzmann J, Huber H, Thallinger C, Wolschek M, Jansen B, Schulte-Hermann R, Beug H, Mikulits W. Hepatocytes convert to a fibroblastoid phenotype through the cooperation of TGF-beta1 and Ha-Ras: steps towards invasiveness. J Cell Sci **2002**; 115: 1189–1202.

Gotzmann J, Mikula M, Eger A, Schulte-Hermann R, Foisner R, Beug H, Mikulits W. Molecular aspects of epithelial cell plasticity: implications for local tumor invasion and metastasis. Mutat Res **2004**; 566:9–20.

Greenburg G, Hay ED. Epithelia suspended in collagen gels can lose polarity and express characteristics of migrating mesenchymal cells. J. Cell Biol **1982**; 95: 333–339.

Grille SJ, Bellacosa A, Upson J, Klein-Szanto AJ, Van Roy F, Lee-Kwon W, Donowitz M, Tsichlis PN, Larue L. The protein kinase Akt induces epithelial mesenchymal transition and promotes enhanced motility and invasiveness of squamous cell carcinoma lines. Cancer Res **2003**; 63: 2172–2178.

Grooteclaes ML, FrischSM. Evidence for a function of CtBP in epithelial gene regulation and anoikis. Oncogene **2000**; 19: 3823-3828.

Grotegut S, von Schweinitz D, Christofori G, Lehembre F. Hepatocyte growth factor induces cell scattering through MAPK/Egr-1-mediated upregulation of Snail. EMBO J **2006**; 25: 3534–3545.

Grunert S, Jechlinger M, Beug H. Diverse cellular and molecular mechanisms contribute to epithelial plasticity and metastasis. Nat Rev Mol Cell Biol **2003**; 4: 657–665.

Guaita S, Puig I, Franci C, Garrido M, Dominguez D, Batlle E, Sancho E, Dedhar S, Garcia de Herreros A, Baulida J. Snail induction of epithelial to mesenchymal transition in tumor cells is accompanied by MUC1 repression and ZEB1 expression. J Biol Chem **2002**; 277: 30209–39216.

Guarino M. Epithelial-to-mesenchymal change of differentiation. From embryogenetic mechanism to pathological patterns. Histol Histopathol **1995**; 10: 171–184.

Guayet J, Lambert H, Gingras-Breton G, Lavoie JN, Huot J, Landry J. Regulation of actin filament dynamics by p38 map kinase-mediated phosphorylation of heat shock protein 27. J Cell Sci **1997**; 110:357–368.

Gundersen GG, Kalnoski MH, Bulinski JC. Distinct populations of microtubules: Tyrosinated and nontyrosinated alpha tubulin are distributed differently in vivo. Cell **1984**; 38:779–789.

Gundersen GG, Kim I, Chapin CJ. Induction of stable microtubules in 3T3 fibroblasts by TGF-beta and serum. J Cell Sci **1994**; 107:645–659.

Hagedorn HG, et al. Synthesis and degradation of basement membranes and extracellular matrix and their regulation by TGF-β in invasive carcinomas**.** Int J Oncol **2001**; 18: 669–681.

Hajra KM, Chen DY, Fearon ER. The SLUG zinc-finger protein represses E-cadherin in breast cancer. Cancer Res **2002b**; 62:1613–1618.

Hajra KM, Fearon ER. Cadherin and catenin alterations in human cancer. Genes Chromosomes Cancer **2002a**; 34:255–268.

Hajra KM, Ji X, Fearon ER. Extinction of E-cadherin expression in breast cancer via a dominant repression pathway acting on proximal promoter elements. Oncogene **1999**; 18: 7274-7279.

Hanafusa H, et al. Involvement of the p38 mitogen-activated protein kinase pathway in transforming growth factor-β-induced gene expression. J Biol Chem **1999**; 274: 27161–27167.

Hanahan D, Weinberg RA. The hallmarks of cancer. Cell **2000**; 100:57–70.

Hanai J, et al. Interaction and functional cooperation of PEBP2/CBF with Smads. Synergistic induction of the immunoglobulin germline Cα promoter. J Biol Chem **1999**; 274: 31577–31582.

Hanz S, Fainzilber M. Retrograde signalingin injured nerve - the axon reaction revisited. J Neurochem **2006**; 99: 13–19.

Hartig R, Shoeman RL, Janetzko A, Tolstonog G, Traub P. DNA-mediated transport of the intermediate filament protein vimentin into the nucleus of cultured cells. J Cell Sci **1998**; 111: 3573-3584.

Hartsough MT, Mulder KM. Transforming growth factor beta activation of p44mapk in proliferating cultures of epithelial cells. J Biol Chem **1995**; 270: 7117–7124.

Hartwell KA, Muir B, Reinhardt F, Carpenter AE, Sgroi DC, Weinberg RA. The Spemann organizer gene, Goosecoid, promotes tumor metastasis. Proc Natl Acad Sci USA **2006**; 103: 18969–18974.

Hay ED. An overview of epithelio-mesenchymal transformation. Acta Anat (Basel) **1995**; 154: 8–20.

Hay ED. The mesenchymal cell, its role in the embryo, and the remarkable signaling mechanisms that create it. Dev Dyn **2005**; 233: 706–720.

Hayashi H, Abdollah S, Qiu Y, Cai J, Xu Y-Y, et al. The MAD-related protein Smad7 associates with the TGFβ receptor and functions as an antagonist of the TGF β signaling. Cell **1997;** 89: 1165–1173.

Hayes S A, et al. Smad3 represses androgen receptor-mediated transcription. Cancer Res**2001**; 61: 2112–2118.

Helfand BT, Chang L, Goldman RD. Intermediate filaments are dynamic and motile elements of cellular architecture. J Cell Sci **2004**; 117:133–141.

Helfand BT, Chou YH, Shumaker DK, Goldman RD. Intermediate filament proteins participate in signal transduction. Trends Cell Biol **2005**; 15: 568–570.

Hemavathy K, Ashraf SI, Ip YT. Snail/slug family of repressors: slowly going into the fast lane of development and cancer. Gene **2000**; 257: 1–12.

Hendrix MJ, Seftor EA, Seftor RE, Trevor KT. Experimental co-expression of vimentin and keratin intermediate filaments in human breast cancer cells results in phenotypic interconversion and increased invasive behavior. Am J Pathol **1997**; 150: 483– 495.

Hennig G, Behrens J, Truss M, Frisch S, Reichmann E, Birchmeier W. Progression of carcinoma cells is associated with alterations in chromatin structure and factor binding at the E-cadherin promoter in vivo. Oncogene **1995**; 11: 475-484.

Hennig G, Lowrick O, Birchmeier W, Behrens J. Mechanisms identified in the transcriptional control of epithelial gene expression. J Biol Chem **1996**; 271: 595-602.

Hocevar BA, Brown TL, Howe PH. TGF-β induces fibronectin synthesis through a c-Jun N-terminal kinase-dependent, Smad4-independent pathway. EMBO J **1999**; 18: 1345–1356.

Hinck AP, Archer SJ, Qian SW, Roberts AB, Sporn MB, Weatherbee JA, Tsang ML-S, Lucas R, Zhang B-L, Wenker J, Torchia DA. Transforming growth factor β1: Three-dimensional structure in solution and comparison with the X-ray structure of transforming growth factor β2. Biochemistry **1996**; 35: 8517-8534.

Hojo M, et al. Cyclosporine induces cancer progression by a cell-autonomous mechanism. Nature **1999**; 397: 530–534.

Homan SM, Mercurio AM, LaFlamme SE. Endothelial cells assemble two distinct alpha6beta4-containing vimentin-associated structures: roles for ligand binding and the beta4 cytoplasmic tail. J Cell Sci **1998**; 111: 2717–2728.

Hosobuchi M, Stampfer MR. Effects of transforming growth factor β on growth of human mammary epithelial cells in culture. In Vitro Cell Dev Biol **1989**; 25: 705–713.

Hu L, Lau SH, Tzang CH, Wen JM, Wang W, Xie D, Huang M, Wang Y, Wu MC, Huang JF, Zeng WF, Sham JS, Yang M, Guan XY. Association of Vimentin overexpression and hepatocellular carcinoma metastasis. Oncogene **2004**; 23: 298–302.

Hua X, Liu X, Ansari DO, Lodish HF. Synergistic cooperation of TFE3 and Smad proteins in TGF-β-induced transcription of the plasminogen activator inhibitor-1 gene. Genes Dev **1998**; 12: 3084–3095.

Hua X, Miller ZA, Wu G, Shi Y, Lodish H F. Specificity in transforming growth factor β-induced transcription of the plasminogen activator inhibitor-1 gene: interactions of promoter DNA, transcription factor μE3, and Smad proteins. Proc Natl Acad Sci USA **1999**; 96: 13130–13135.

Huber MA, Azoitei N, Baumann B, et al. NF-kappa B is essential for epithelialmesenchymal transition and metastasis in a model of breast cancer progression. J Clin Invest **2004**; 114: 569-581.

Huber MA, Kraut N, Beug H. Molecular requirements for epithelial mesenchymal transition during tumor progression. Curr Opin Cell Biol **2005**; 17: 548–558.

Ikenouchi J, Matsuda M, Furue M, Tsukita S. Regulation of tight junctions during the epithelium-mesenchyme transition: direct repression of the gene expression of claudins/occludin by Snail. J Cell Sci **2003**; 116: 1959–1967.

Imamura T, Takase M, Nishihara A, Oeda E, Hanai J, et al. Smad6 inhibits signaling by the TGF-*β* superfamily. Nature **1997**; 389:622–626.

Ito N, et al. Positive correlation of plasma transforming growth factor-β1 levels with tumor vascularity in hepatocellular carcinoma. Cancer Lett **1995**; 89: 45–48.

Iwano M, Plieth D, Danoff TM, et al. Evidence that fibroblasts derive from epithelium during tissue fibrosis. J Clin Invest **2002**; 110: 341-350.

Iwata N, Yamamoto H, Sasaki S, et al. Frequent hypermethylation of CpG islands and loss of expression of the 14-3-3 sigma gene in human hepatocellular carcinoma. Oncogene **2000**; 19:5298–5302.

Jaggi M, Nazemi T, Abrahams NA, Baker JJ, Galich A, Smith LM, Balaji KC. N-cadherin switching occurs in high Gleason grade prostate cancer. Prostate **2006**; 66: 193–199.

Jakowlew SB. Transforming growth factor-beta in cancer and metastasis. Cancer Metastasis Rev **2006**; 25:435–457.

Janda E, Lehmann K, Killisch I, et al. Ras and TGF beta cooperatively regulate epithelial cell plasticity and metastasis: dissection of Ras signaling pathways. J Cell Biol **2002**; 156: 299- 313.

Janknecht R, Wells NJ, Hunter T. TGF-β stimulated cooperation of Smad proteins with the coactivators CBP/p300. Genes Dev **1998**; 12: 2114–2119.

Jechlinger M, Grunert S, Tamir IH, et al. Expression profiling of epithelial plasticity in tumor progression. Oncogene **2003**; 22: 7155–7169.

Jiao W, Miyazaki K, Kitajima Y. Inverse correlation between E-cadherin and Snail expression in hepatocellular carcinoma cell lines in vitro and in vivo. Br J Cancer **2002**; 86:98–101.

Ju W, Ogawa A, Heyer J, et al. Deletion of Smad2 in mouse liver reveals novel functions in hepatocyte growth and differentiation. Mol Cell Biol **2006**; 26: 654–667.

Kalluri R, Neilson EG. Epithelial mesenchymal transition and its implications for fibrosis. J Clin Invest **2003**; 112: 1776-1784.

Kang HY, et al. From transforming growth factor-β signaling to androgen action: identification of Smad3 as an androgen receptor coregulator in prostate cancer cells. Proc Natl Acad Sci USA **2001**; 98: 3018–3023.

Kang Y, Chen CR, Massague J. A self-enabling TGF*β* response coupled to stress signaling: Smad engages stress response factor ATF3 for Id1 repression in epithelial cells. Mol Cell **2003**; 11:915–926.

Kang Y, Massague J. Epithelialmesenchymal transitions: twist in development and metastasis. Cell **2004**; 118: 277-279.

Kang Y, Siegel PM, Shu W, et al. A multigenic program mediating breast cancer metastasis to bone. Cancer Cell **2003**; 3: 537–549.

Kano MR, et al. Improvement of cancer-targeting therapy, using nanocarriers for intractable solid tumors by inhibition of TGF-β signaling. Proc Natl Acad Sci USA **2007**; 104: 3460–3465.

Kemler R, Hierholzer A, Kanzler B, et al. Stabilization of β-catenin in the mouse zygote leads to premature epithelial-mesenchymal transition in the epiblast. Development **2004**; 131: 5817–5824.

Keshamouni VG, Michailidis G, Grasso CS, et al. Differential protein expression profiling by iTRAQ-2DLC-MS of lung cancer cells undergoing epithelial mesenchymal transition reveals a migratory/invasive phenotype. J Proteome Res **2006**; 5:1143–1154.

Kim KK, et al. Alveolar epithelial cell mesenchymal transition develops *in vivo* during pulmonary fibrosis and is regulated by the extracellular matrix. Proc Natl Acad Sci USA **2006**; 103:13180–13185.

Kim RH, Wang D, Tsang M, Martin J, Huff C, de Caestecker M P, Parks WT, Meng X, Lechleider RJ, Wang T, Roberts AB. A novel Smad nuclear interacting protein, SNIP1, suppresses p300-dependent TGF-β signal transduction. Genes Dev **2000**; 14: 1605-1616.

Kim S-J, Glicks A, Sporn MB, Roberts AB. Characterization of the promoter region of the human transforming growth factor-β1 gene. J Biol chem **1989**; 264: 402-408.

Kim S-J, Angel P, Lafyatis R, Hattori K, Kim KY, Sporn MB, Karin M, Roberts AB. Autoinduction of transforming growth factor beta 1 is mediated by the AP-1 complex. Mol Cell Biol **1990**; 10(4): 1492-1497.

Kimelman D. Mesoderm induction: from caps to chips. Nat Rev Genet **2006**; 7: 360–372.

Kouklis PD, Hutton E, Fuchs E. Making a connection: direct binding between keratin intermediate filaments and desmosomal proteins. J Cell Biol **1994**; 127:1049–1060.

Kowanetz M, Valcourt U, Bergstrom R, Heldin C-H, Moustakas A. Id2 and Id3 define the potency of cell proliferation and differentiation responses to tranforming growth factor β and bone morphogenetic protein. Mol Cell Biol **2004**; 24: 4241–4254.

Kreis S, Schonfeld HJ, Melchior C, Steiner B, Kieffer N. The intermediate filament protein vimentin binds specifically to a recombinant integrin alpha2/beta1 cytoplasmic

tail complex and co-localizes with native alpha2/beta1 in endothelial cell focal adhesions. Exp Cell Res **2005**; 305: 110–121.

Kurokawa M, et al. The oncoprotein Evi-1 represses TGF-β signalling by inhibiting Smad3. Nature **1998**; 394: 92–96.

LaGamba D, Nawshad A, Hay ED. Microarray analysis of gene expression during epithelial-mesenchymal transformation. Dev Dyn **2005**; 234: 132–142.

Lage H. Proteomics in cancer cell research: an analysis of therapy resistance. Pathol Res Pract **2004**; 200:105–117.

Lan M, Kojima T, Osanai M, Chiba H, Sawada N. Oncogenic Raf-1 regulates epithelial to mesenchymal transition via distinct signal transduction pathways in an immortalized mouse hepatic cell line. Carcinogenesis **2004**; 25: 2385–2395.

Lang SH, Hyde C, Reid IN, Hitchcock IS, Hart CA, Bryden AA, Villette JM, Stower MJ, Maitland NJ. Enhanced expression of vimentin in motile prostate cell lines and in poorly differentiated and metastatic prostate carcinoma. Prostate **2002**; 52:253–263.

Larsson J, Karlsson S. The role of Smad signaling in hematopoiesis. Oncogene **2005**; 24:5676-5692.

Lehmann K, Janda E, Pierreux CE, Rytomaa M, Schulze A, McMahon M, Hill CS, Beug H and Downward J. Raf induces TGFβ production while blocking its apoptotic but not invasive responses: a mechanism leading to increased malignancy in epithelial cells. Genes Dev **2000**; 14: 2610–2622.

Leptin M, Grunewald B. Cell shape changes during gastrulation in *Drosophila.* Development **1990**; 110: 73–84.

Lewis MP, Lygoe KA, Nystrom ML, et al. Tumour-derived TGF-β1 modulates myofibroblast differentiation and promotes HGF/SF-dependent invasion of squamous carcinoma cells. Br J Cancer **2004**; 90: 822–832.

Li W, Qiao W, Chen L, et al. Squamous cell carcinoma and mammary abscess formation through squamous metaplasia in Smad4/Dpc4 conditional knockout mice. Development **2003**; 130: 6143–6153.

Liberati NT, Datto MB, Frederick JP, Shen X, Wong C, et al. Smads bind directly to the Jun family of AP-1 transcription factors. Proc Natl Acad Sci USA **1999**; 96:4844–4849.

Liu Y, Chen Q, Zhang JT. Tumor suppressor gene 14-3-3 sigma is down-regulated whereas the proto-oncogene translation elongation factor 1delta is up-regulated in non-small cell lung cancers as identified by proteomic profiling. J Proteome Res **2004**; 3:728–735.

Liu Y, Liu H, Han B, Zhang J-T. Identification of 14-3-3α as a contributor to drug resistance in human breast cancer cells using functional proteomic analysis. Cancer Res **2006**; 66: 3248-3255.

Locascio A, Nieto M A. Cell movements during vertebrate development: integrated tissue behavior versus individual cell migration. Curr Opin Genet Dev **2001**; 11: 464–469.

Luo K, et al. The Ski oncoprotein interacts with the Smad proteins to repress TGFβ signaling. Genes Dev **1999**; 13: 2196–2206.

Lyons RM, Gentry LE, Purchio AF, Moses H.L. Mechanism of activation of latent recombinant transforming growth factor β1 by plasmin. J Cell Biol **1990**; 110: 1361–1367.

Madri JA, et al. Phenotypic modulation of endothelial cells by transforming growth factor-β depends upon the composition and organization of the extracellular matrix. J Cell Biol **1988**; 106: 1375–1384.

Maeda M, Johnson KR, Wheelock MJ. Cadherin switching: essential for behavioral but not morphological changes during an epithelium-to-mesenchyme transition. J Cell Sci **2005**; 118: 873–887.

Maestro R, Dei Tos AP, Hamamori Y, et al. Twist is a potential oncogene that inhibits apoptosis. Genes Dev **1999**; 13: 2207-2217.

Mani SA, Yang J, Brooks M, Schwaninger G, Zhou A, Miura N, Kutok JL, Hartwell K, Richardson AL, Weinberg RA. Mesenchyme Forkhead 1 (FOXC2) plays a key role in metastasis and is associated with aggressive basal-like breast cancers. Proc Natl Acad Sci USA **2007**; 104: 10069–10074.

Maniotis AJ, Chen CS, Ingber DE. Demonstration of mechanical connections between integrins, cytoskeletal filaments, and nucleoplasm that stabilize nuclear structure. Proc Natl Acad Sci USA **1997**; 94: 849–854.

Manotham K, Tanaka T, Matsumoto M, Ohse T, Inagi R, Miyata T, Kurokawa K, Fujita T, Ingelfinger JR, Nangaku M. Transdifferentiation of cultured tubular cells induced by hypoxia. Kidney Int **2004**; 65: 871–880.

Massague J, Blain SW, Lo RS. TGF-β signaling in growth control, cancer, and heritable disorders. Cell **2000c**; 103: 295–309.

Massague J, Seoane J, Wotton D. Smad transcription factors. Genes Dev **2005**; 19: 2783–2810.

Massague J, Wotton D. Transcriptional control by the TGF-β/Smad signaling system. EMBO J **2000b**; 19: 1745–1754.

Massague J. How cells read TGF-β signals. Nat Rev Mol Cell Biol **2000a**; 1: 169–178.

McDowell N, Gurdon JB. Activin as a morphogen in Xenopus mesoderm induction. Semin. Cell Dev Biol **1999**; 10: 311–317.

Medici D, Hay ED, Goodenough DA. Cooperation between Snail and LEF-1 transcription factors is essential for TGF-β1 induced epithelial–mesenchymal transition. Mol Biol cell **2006**; 17:1871-1879.

Mercado-Pimentel ME, Hubbard AD, Runyan RB. Endoglin and ALK5 regulate epithelial–mesenchymal transformation during cardiac valve formation. Dev Biol **2007a**; 304: 420–432.

Mercado-Pimentel ME, Runyan RB. Multiple transforming growth factor-b isoforms and receptors function during epithelial-mesenchymal cell transformation in the embryonic heart. Cells Tissues Organs **2007b**; 185: 146–156.

Meulemans D, Bronner-Fraser M. Gene–regulatory interactions in neural crest evolution and development. Dev Cell **2004**; 7: 291–309.

Micke P, Ostman A. Exploring the tumour environment: cancer-associated fibroblasts as targets in cancer therapy. Expert Opin Ther Targets **2005**; 9: 1217–1233.

Miettinen PJ, Ebner R, Lopez AR and Derynck R. TGF-β induced transdifferentiation of mammary epithelial cells to mesenchymal cells: involvement of type I receptors. J Cell Biol **1994**; 127: 2021–2036.

Mishra L, Derynck R, Mishra B. Transforming growth factor–β signaling in stem cells and cancer. Science **2005**; 310: 68-70.

Miyazono K, Ichijo H, Heldin C-H. Transforming growth factor-β: latent forms, binding proteins and receptors. Growth Factors **1993**; 8: 11–22.

Miyazono K, ten Dijke P, Heldin C-H. TGF-β signaling by Smad proteins. Adv Immunol **2000**; 75: 115-157.

Miyoshi A, Kitajima Y, Sumi K, Sato K, Hagiwara A, Koga Y, Miyazaki K. Snail and SIP1 increase cancer invasion by upregulating MMP family in hepatocellular carcinoma cells. Br J Cancer **2004**; 90:1265–1273.

Mohamed OA, Clarke HJ, Dufort D. B-catenin signaling marks the prospective site of primitive streak formation in the mouse embryo. Dev Dyn **2004**; 231: 416–424.

Moreno-Bueno G, Cubillo E, Sarrio D, Peinado H, Rodrıguez-Pinilla SM, Villa S, Bolos V, Jorda M, Fabra A, Portillo F, Palacios J, Cano A. Genetic profiling of

epithelial cells expressing E-Cadherin repressors reveals a distinct role for Snail, Slug, and E47 factors in epithelial-mesenchymal transition.Cancer Res **2006**; 66:

Moustakas A, Heldin C-H. Non-Smad TGF-β signals. J Cell Sci **2005**; 118: 3573–3584.

Moustakas A, Heldin C-H. Signaling networks guiding epithelial–mesenchymal transitions during embryogenesis and cancer progression. Cancer Sci **2007**; 98:1512-1520.

Munger J, et al. Latent transforming growth factor-β: structural features and mechanisms of activation. Kidney Int **1997**; 51: 1376–1382.

Munger JS, et al. The integrin αvβ6 binds and activates latent TGF β1: a mechanism for regulating pulmonary inflammation and fibrosis. Cell **1999**; 96: 319–328.

Muraoka RS, Dumont N, Ritter CA, Dugger TC, Brantley DM, Chen J, Easterly E, Roebuck LR, Ryan S, Gotwals PJ, Koteliansky V, Arteaga CL. Blockade of TGF-beta inhibits mammary tumor cell viability, migration, and metastases. J Clin Invest **2002**; 109(12):1551-1559.

Muslin AJ, Xing H. 14-3-3 proteins: regulation of subcellular localization by molecular interference. Cell Signal **2000**; 12:703–709.

Nakao A, Afrakhte M, Moren A, Nakayama T, Christian J, et al. Identification of Smad7, a TGF*β*-inducible antagonist of TGF-*β* signalling. Nature **1997**; 389:631–635.

Nakashima K, et al. Synergistic signaling in fetal brain by STAT3–Smad1 complex bridged by p300. Science **1999**; 284: 479–482.

Nakayama KI, Nakayama K. Ubiquitin ligases: cell-cycle control and cancer. Nat Rev Cancer **2006**; 6: 369–381.

Niemantsverdriet M, Wagner K, Visser M, Backendorf C. Cellular functions of 14-3-3 zeta in apoptosis and cell adhesion emphasize its oncogenic character. Oncogene **2008**; 27(9): 1315-1319.

Nieto MA. The early steps of neural crest development. Mech Dev **2001**; 105: 27-35.

Nieto MA. The snail superfamily of zincfinger transcription factors. Nat Rev Mol Cell Biol **2002**; 3:155-166.

Niimi H, Pardali K, Vanlandewijck M, Heldin CH, Moustakas A. Notch signaling is necessary for epithelial growth arrest by TGF-β. J Cell Biol **2007**; 176: 695–707.

Nollet F, Berx G, van Roy F. The role of the E-cadherin/ catenin adhesion complex in the development and progression of cancer. Mol Cell Biol Res Commun **1999**; 2:77–85.

Oft M, Akhurst RJ, Balmain A. Metastasis is driven by sequential elevation of H-Ras and Smad2 levels. Nat Cell Biol **2002**; 4: 487–494.

Oft M, Heider KH, Beug H. TGF beta signaling is necessary for carcinoma cell invasiveness and metastasis. Curr Biol **1998**; 8: 1243–1252.

Oft M, Peli J, Rudaz C, Schwarz H, Beug H, Reichmann E. TGF-beta1 and Ha-Ras collaborate in modulating the phenotypic plasticity and invasiveness of epithelial tumor cells. Genes Dev **1996**; 10:2462–2477.

Oklu R, Hesketh R. The latent transforming growth factor β binding protein (LTBP) family. Biochem J **2000**; 352; 601–610.

Oldfield MD, Bach LA, Forbes JM, Nikolic-Paterson D, McRobert A, Thallas V, Atkins RC, Osicka T, Jerums G, Cooper ME. Advanced glycation end products cause epithelial-myofibroblast transdifferentiation via the receptor for advanced glycation end products (RAGE). J Clin Invest **2001**; 108: 1853–1863.

Olmeda D, Moreno-Bueno G, Flores JM, Fabra A, Portillo F, Cano A. SNAI1 is required for yumor growth and lymph node metastasis of human breast carcinoma MDA-MB-231 cells. Cancer Res **2007**; 67**:**

Owen RD, Ostrowski MC. Transcriptional activation of a conserved sequence element by Ras requires a nuclear factor distinct from c-Fos or c-Jun. Proc Natl Acad Sci USA **1990**; 87: 3866–3870.

Ozdamar B, Bose R, Barrios-Rodiles M, Wang HR, Zhang Y and Wrana JL. Regulation of the polarity protein Par6 by TGF beta receptors controls epithelial cell plasticity. Science **2005**; 307: 1603-1609.

Palazzo AF, Eng CH, Schlaepfer DD, Marcantonio EE, Gundersen GG. Localized stabilization of microtubules by integrin- and FAK-facilitated Rho signaling. Science **2004**; 303:836–839.

Pardali K, Kurisaki A, Moren A, ten Dijke P, Kardassis D, et al. Role of Smad proteins and transcription factor Sp1 in p21Waf1/Cip1 regulation by transforming growth factor-β. J Biol Chem **2000**; 275:29244–29256.

Park C, Afrikanova I, Chung YS, Zhang WJ, Arentson E, Fong Gh G, Rosendahl A, Choi K. A hierarchical order of factors in the generation of FLK1- and SCL-expressing hematopoietic and endothelial progenitors from embryonic stem cells. Development **2004**; 131: 2749-2762.

Paruchuri S, et al. Human pulmonary valve progenitor cells exhibit endothelial/mesenchymal plasticity in response to vascular endothelial growth factor-A and transforming growth factor-β2. Circ Res **2006**; 99: 861–869.

Peinado H, Olmeda D, Cano A. Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype? Nat Rev Cancer **2007**; 7:415–428.

Peinado H, Portillo F, Cano A. Transcriptional regulation of cadherins during development and carcinogenesis. Int J Dev Biol **2004**; 48: 365–375.

Peinado H, Quintanilla M, Cano A. Transforming growth factor beta-1 induces snail transcription factor in epithelial cell lines: mechanisms for epithelial mesenchymal transitions. J Biol Chem **2003**; 278: 21113–2123.

Peiro S, Escriva M, Puig I, Barbera MJ, Dave N, Herranz N, Larriba MJ, Takkunen M, Franci C, Munoz A, Virtanen I, Baulida J, Garcia de Herreros A. Snail1 transcriptional repressor binds to its own promoter and controls its expression. Nucleic Acids Res **2006**; 34: 2077–2084.

Pera EM, Ikeda A, Eivers E, De Robertis EM. Integration of IGF, FGF, and anti-BMP signals via Smad1 phosphorylation in neural induction. Genes Dev **2003**; 17:3023–3028.

Perez-Moreno M, Jamora C, Fuchs E. Sticky business: orchestrating cellular signals at adherens junctions. Cell **2003**; 112: 535 –548.

Perez-Moreno MA, Locascio A, Rodrigo I, Dhondt G, Portillo F, Nieto MA, Cano A. A new role for E12/E47 in the repression of E-cadherin expression and epithelial-mesenchymal transitions. J Biol Chem **2001**; 276: 27424-27431.

Perl AK, Wilgenbus P, Dahl U, Semb H, Christofori G. A causal role for E-cadherin in the transition from adenoma to carcinoma. Nature **1998**; 392: 190–193.

Perlson E, Hanz S, Ben-Yaakov K, Segal-Ruder Y, Seger R, Fainzilber M. Vimentin-dependent spatial translocation of an activated MAP kinase in injured nerve. Neuron **2005**; 45: 715–726.

Perlson E, Michaelevski I, Kowalsman N, Ben-Yaakov K, Shaked M, Seger R, Eisenstein M, Fainzilber M. Vimentin binding to phosphorylated Erk sterically hinders enzymatic dephosphorylation of the kinase. J Mol Biol **2006**; 364: 938–944.

Peron P, et al. Potentiation of Smad transactivation by Jun proteins during a combined treatment with epidermal growth factor and transforming growth factor-β in rat hepatocytes. Role of phosphatidylinositol 3-kinase-induced AP-1 activation. J Biol Chem **2001**; 276: 10524–10531.

Pertovaara L, et al. Vascular endothelial growth factor is induced in response to transforming growth factor-β in fibroblastic and epithelial cells. J Biol Chem **1994**; 269: 6271–6274.

Petersen OW, Nielsen HL, Gudjonsson T, et al. Epithelial to mesenchymal transition in human breast cancer can provide a nonmalignant stroma. Am J Pathol **2003**; 162: 391–402.

Piek E, Heldin C-H, ten Dijke P. Specificity, diversity, and regulation in TGF-β superfamily signaling. FASEB J **1999**; 13: 2105–2124.

Portella G, et al. Transforming growth factor β is essential for spindle cell conversion of mouse skin carcinoma *in vivo*: implications for tumor invasion. Cell Growth Differ **1998**; 9: 393–404.

Poser I, Dominguez D, de Herreros AG, Varnai A, Buettner R, Bosserhoff AK. Loss of E-cadherin expression in melanoma cells involves up-regulation of the ranscriptional repressor Snail. J Biol Chem **2001**; 276:24661–24666.

Postigo AA, Depp JL, Taylor JJ, Kroll KL. Regulation of Smad signaling through a differential recruitment of coactivators and corepressors by ZEB proteins. EMBO J **2003**; 22: 2453–2462.

Prasad GL, Valverius EM, McDuffie E, Cooper HL. Complementary DNA cloning of a novel epithelial cell marker protein, HME1 that may be down-regulated in neoplastic mammary cells. Cell Growth Differ **1992**; 3: 507–513.

Prindull G. Hypothesis: cell plasticity, linking embryonal stem cells to adult stem cell reservoirs and metastatic cancer cells? Exp Hematol **2005**; 33: 738–746.

Proetzel G, et al. Transforming growth factor-β3 is required for secondary palate fusion. Nat Genet **1995**; 11: 409–414.

Qing J, Zhang Y, Derynck R. Structural and functional characterization of the transforming growth factor-β-induced Smad3/c-Jun transcriptional cooperativity. J Biol Chem **2000**; 275: 38802–38812.

Radisky D C. Epithelial to mesenchymal transition. J Cell Sci **2005**; 118: 4325-4326.

Raible DW. Development of the neural crest: achieving specificity in regulatory pathways. Curr Opin Cell Biol **2006**; 18: 698–703.

Ramaekers FC, Haag D, Kant A, Moesker O, Jap PH, Vooijs GP. Coexpression of keratin- and vimentin-type intermediate filaments in human metastatic carcinoma cells. Proc Natl Acad Sci USA **1983**; 80: 2618–2622.

Rastaldi MP, Ferrario F, Giardino L, Dell' Antonio G, Grillo C, Grillo P, Strutz F, Muller GA, Colasanti G, D'Amico G. Epithelial-mesenchymal transition of tubular epithelial cells in human renal biopsies. Kidney Int **2002**; 62: 137–146.

Reichmann E, Schwarz H, Deiner EM, Leitner I, Eilers M, Berger J, Busslinger M, Beug H. Activation of an inducible c-FosER fusion protein causes loss of epithelial polarity and triggers epithelial-fibroblastoid cell conversion. Cell **1992**; 71: 1103–1116.

Reiss M. TGF-beta and cancer. Microbes Infect **1999**; 1:1327–1347.

Rhyu DY, Yang Y, Ha H, Lee GT, Song JS, Uh ST, Lee HB. Role of reactive oxygen species in TGF-beta1-induced mitogen-activated protein kinase activation and epithelial-mesenchymal transition in renal tubular epithelial cells. J Am Soc Nephrol **2005**; 16: 667–675.

Roberts AB, et al. Smad3 is key to TGF-β-mediated epithelial-to-mesenchymal transition, fibrosis, tumor suppression and metastasis. Cytokine Growth Factor Rev **2006**; 17: 19–27.

Roberts AB, Wakefield LM. The two faces of transforming growth factor beta in carcinogenesis. Proc Natl Acad Sci USA **2003**; 100: 8621–8623.

Rocchi P, So A, Kojima S, Signaevsky M, Beraldi E, Fazli L, Hurtado-Coll A, Yamanaka K, Gleave M. Heat shock protein-27 increases after androgen ablation and plays a cytoprotective role in hormone-refractory prostate cancer. Cancer Res **2004**; 64: 6595–6602.

Rodrigo I, Cato AC, Cano A. Regulation of E-cadherin gene expression during tumor progression: the role of a new Ets-binding site and the E-pal element. Exp Cell Res **1999**; 248: 358-371.

Rosenquist M. 14-3-3 proteins in apoptosis. Braz J Med Biol Res **2003**; 36: 403–408.

Rosivatz E, Becker I, Specht K, Fricke E, Luber B, Busch R, Hofler H, Becker KF. Differential expression of the epithelial–mesenchymal transition regulators snail, SIP1 and twist in gastric cancer. Am J Pathol **2002**; 161: 1881– 1891.

Rosivatz E, Becker KF, Kremmer E, Schott C, Blechschmidt K, Hofler H, Sarbia M. Expression and nuclear localization of Snail, an E-cadherin repressor, in adenocarcinomas of the upper gastrointestinal tract. Virchows Arch **2006**; 448: 277–287.

Rousseau S, Dolado I, Beardmore V, Shpiro N, Marquez R, Nebreda AR, Arthur JS, Case LM, Tessier-Lavigne M, Gaestel M, Cuenda A, Cohen P. CXCL12 and C5a trigger cell migration via a PAK1/2-p38alpha MAPK-MAPKAP-K2-HSP27 pathway. Cell Signal **2006**; 6:6.

Ruscetti FW, Akel S, Bartelmez SH. Autocrine transforming growth factor-beta regulation of hematopoiesis: many outcomes that depend on the context. Oncogene **2005**; 24:5751-5763.

Saika S, Ikeda K, Yamanaka O, et al. Adenoviral gene transfer of BMP-7, Id2 or Id3 suppresses injury-induced epithelial-to-mesenchymal transition of lens epithelium in mice. Am J Physiol Cell Physiol **2006**; 290: C282–309.

Sakai D, et al. Regulation of Slug transcription in embryonic ectoderm by β-catenin-Lef/Tcf and BMP Smad signaling. Dev Growth Differ **2005**; 47: 471–482.

Sakai D, Suzuki T, Osumi N, Wakamatsu Y. Cooperative action of Sox9, Snail2 and PKA signaling in early neural crest development. Development **2006**; 133: 1323–1333.

Salnikov AV, et al. Inhibition of TGF-β modulates macrophages and vessel maturation in parallel to a lowering of interstitial fluid pressure in experimental carcinoma. Lab Invest **2005**; 85: 512–521.

Sano Y, Harada J, Tashiro S, Gotoh-Mandeville R, Maekawa T, et al. ATF-2 is a common nuclear target of Smad and TAK1 pathways in transforming growth factor-*β* signaling. J Biol Chem **1999**; 274: 8949–8957.

Sato M, Muragaki Y, Saika S, Roberts AB, Ooshima A. Targeted disruption of TGF-β1/Smad3 signaling protects against renal tubulointerstitial fibrosis induced by unilateral ureteral obstruction. J Clin Invest **2003**; 112: 1486–1494.

Sato Y, Rifkin DB. Inhibition of endothelial cell movement by pericytes and smooth muscle cells: activation of a latent transforming growth factor-β1-like molecule by plasmin during co-culture. J Cell Biol **1989**; 109: 309–315.

Savagner P, Yamada KM, Thiery JP. The zinc-finger protein Slug causes desmosome dissociation, an initial and necessary step for growth factor–induced epithelial–mesenchymal transition. J Cell Biol **1997**; 137: 1403-1419.

Savagner P. Leaving the neighborhood; molecular mechanism involved during epithelial to mesenchymal transition. Bioessay **2001**; 23: 912-923.

Scandura JM, Boccuni P, Massague J, Nimer SD. Transforming growth factor beta-induced cell cycle arrest of human hematopoietic cells requires p57KIP2 up-regulation. Proc Natl Acad Sci USA **2004**; 101:15231-15236.

Schuldiner M, Yanuka O, Itskovitz-Elder J, Melton DA, Benvenisty N. Effects of eight growth factors on the differentiation of cells derived from human embryonic stem cells. Proc Natl Acad Sci USA **2000**; 97: 11307-11312.

Schultz-Cherry S, Murphy-Ullrich JE. Thrombospondin causes activation of latent transforming growth factor-β secreted by endothelial cells by a novel mechanism. J Cell Biol **1993**; 122: 923–932.

Shen X, et al. TGF-β-induced phosphorylation of Smad3 regulates its interaction with coactivator p300/CREB-binding protein. Mol Biol Cell **1998**; 9: 3309–3319.

Shi Y, et al. Crystal structure of a Smad MH1 domain bound to DNA: insights on DNA binding in TGF-β signaling. Cell **1998**; 94: 585–594.

Shi Y, Massague J. Mechanisms of TGF-*β* signaling from cell membrane to the nucleus. Cell **2003**; 113: 685–700.

Shin KD, Lee MY, Shin DS, Lee S, Son KH, Koh S, Paik YK, Kwon BM, Han DC. Blocking tumor cell migration and invasion with biphenyl isoxazole derivative KRIBB3, a synthetic molecule that inhibits Hsp27 phosphorylation. J Biol Chem **2005**; 280: 41439–41448.

Shioda T, et al. Transcriptional activating activity of Smad4: roles of SMAD hetero-oligomerization and enhancement by an associating transactivator. Proc Natl Acad Sci USA **1998**; 95: 9785–9790.

Shook D, Keller R. Mechanisms, mechanics and function of epithelial-mesenchymal transitions in early development. Mech Dev **2003**; 120: 1351-1383.

Singh S, Sadacharan S, Su S, Belldegrun A, Persad S, Singh G. Overexpression of vimentin: role in the invasivephenotype in an androgen-independent model of prostate cancer. Cancer Res **2003**; 63: 2306–2311.

Sinha P, Hutter G, Kottgen E, Dietel M, Schadendorf D, Lage H. Increased expression of epidermal fatty acid binding protein, cofilin, and 14-3-3 sigma (stratifin) detected by two-dimensional gel electrophoresis, mass spectrometry and microsequencing of drug-resistant human adenocarcinoma of the pancreas. Electrophoresis **1999**; 20: 2952–2960.

Sirard C, et al. The tumor suppressor gene Smad4/ Dpc4 is required for gastrulation and later for anterior development of the mouse embryo. Genes Dev **1998**; 12: 107–119.

Sleeman JP. The lymph node as a bridgehead in the metastatic dissemination of tumors. Recent Results Cancer Res **2000**; 157: 55–81.

Smith DE, del Amo FF, Gridley T. Isolation of Sna, a mouse gene homologous to the *Drosophila* genes *snail* and *escargot*: its expression pattern suggests multiple roles in postimplantation development. Development **1992**; 116: 1033–1039.

Song CZ, Tian X, Gelehrter TD. Glucocorticoid receptor inhibits transforming growth factor-β signaling by directly targeting the transcriptional activation function of Smad3. Proc Natl Acad Sci USA **1999**; 96: 11776–11781.

Sorokin L, Sonnenberg A, Aumailley M, Timpl R, Ekblom P. Recognition of the laminin E8 cell-binding site by an integrin possessing the α6 integrin subunit is essential for epithelial polarization in developing kidney. J Cell Biol **1990**; 111: 1265–1273.

Spaderna S, Schmalhofer O, Wahlbuhl M, Dimmler A, Bauer K, Sultan A, Hlubek F, Jung A, Strand D, Eger A, Kirchner T, Behrens J, Brabletz T. The Transcriptional Repressor ZEB1 Promotes Metastasis and Loss of Cell Polarity in Cancer. Cancer Res **2008**; 68(2):537-544.

Spagnoli FM, Cicchino C, Tripodi M, Weiss MC. Inhibition of MMH (Met murine hepatocyte) cell differentiation by TGF(beta) is abrogated by pre-treatment with the heritable differentiation effector FGF1. J Cell Sci **2000**; 113: 3639–3647.

Spencer HL, Eastham AM, Merry CLR, Southgate TD, Perez-Campo F, Soncin F, Ritson S, Kemler R, Stern PL, Ward CM. E-Cadherin inhibits cell surface localization of the pro-migratory 5T4 oncofetal antigen in mouse embryonic stem cells. Mol Biol Cell **2007**; 18: 2838-2851.

Stamenkovic I. Matrix metalloproteinases in tumor invasion and metastasis. Semin Cancer Biol **2000**; 10: 415–433.

Stappenbeck TS, Bornslaeger EA, Corcoran CM, Luu HH, Virata ML, Green KJ. Functional analysis of desmoplakin domains:specification of the interaction with keratin versus vimentin intermediate filament networks. J Cell Biol **1993**; 123:691–705.

Stappenbeck TS, Lamb JA, Corcoran CM, Green KJ. Phosphorylation of the desmoplakin COOH terminus negatively regulates its interaction with keratin intermediate filament networks. J Biol Chem **1994**; 269:29351–29354.

Steinert PM, Roop DR. Molecular and cellular biology of intermediate filaments. Annu Rev Biochem **1988**; 57: 593–625.

Stetler-Stevenson WG, Yu AE. Proteases in invasion: matrix metalloproteinases. Semin Cancer Biol **2001**; 11: 143–153.

Stroschein SL, Wang W, Zhou S, Zhou Q, Luo K. Negative feedback regulation of TGF-β signaling by the SnoN oncoprotein. Science **1999**; 286: 771–774.

Subramanian G, Schwarz RE, Higgins L, McEnroe G, Chakravarty S, Dugar S, Reiss M. Targeting endogenous transforming growth factor beta receptor signaling in SMAD4-deficient human pancreatic carcinoma cells inhibits their invasive phenotypel. Cancer Res **2004**; 64: 5200–5211.

Sunderkotter C, et al. Macrophage-derived angiogenesis factors. PharmacolTher **1991**; 51: 195–216.

Svitkina TM, Verkhovsky AB, Borisy GG. Plectin sidearms mediate interaction of intermediate filaments with microtubules and other components of the cytoskeleton. J Cell Biol **1996**; 135: 991–1007.

Taipale J, Saharinen J, Keski-Oja J. Extracellular matrix-associated transforming growth factor-β: role in cancer cell growth and invasion. Adv Cancer Res **1998**; 75: 87–134.

Takano S, Kanai F, Jazag A, et al. Smad4 is essential for down-regulation of E-cadherin induced by TGF-β in pancreatic cancer cell line PANC-1. J Biochem (Tokyo) **2007**; 141: 345–351.

Takeichi M. Cadherins in cancer: implications for invasion and metastasis. Curr Opin Cell Biol **1993**; 5: 806-811.

Takeichi M. Cadherin cell adhesion receptors as a morphogenetic regulator. Science **1991**; 251: 1451– 1455.

Taki M, Kamata N, Yokoyama K, Fujimoto R, Tsutsumi S, Nagayama M. Down-regulation of Wnt-4 and up-regulation of Wnt-5a expression by epithelial-mesenchymal transition in human squamous carcinoma cells. Cancer Sci **2003**; 94: 593–597.

Tan C, Costello P, Sanghera J, Domınguez D, Baulida J, Garcia de Herreros A. Dedhar S. Inhibition of integrin linked kinase (ILK) suppresses beta-catenin-Lef/Tcf-dependent transcription and expression of the E-cadherin repressor, snail, in APC-/- human colon carcinoma cells. Oncogene **2001**; 20: 133–140.

Tatjana S-S, Bronner-Fraser M. Development and evolution of the migratory neural crest: a gene regulatory perspective. Curr Opin Genet Dev **2006**; 16:360–366.

ten Dijke P, Miyazono K, Heldin C-H. Signaling inputs converge on nuclear effectors in TGF-β signaling. Trends Biochem Sci **2000**; 25: 64–70.

Thiery JP, Chopin D. Epithelial cell plasticity in development and tumor progression. Cancer Metastasis Rev **1999**; 18: 31–42.

Thiery JP, Sleeman JP. Complex networks orchestrate epithelial-mesenchymal transitions. Nat Rev Mol Cell Biol **2006**; 7:131–142.

Thiery JP. Epithelial–mesenchymal transitions in development and pathologies. Curr Opin Cell Biol **2003**; 15: 740–746.

Thiery JP. Epithelial–mesenchymal transitions in tumour progression. Nat Rev Cancer **2002**; 2: 442–454.

Thuault S, Valcourt U, Petersen M, Manfioletti G, Heldin C-H, Moustakas A. Transforming growth factor-β employs HMGA2 to elicit epithelialmesenchymal transition. J Cell Biol **2006**; 174: 175–183.

Tomita K, van Bokhoven A, van Leenders GJ, Ruijter ET, Jansen CF, Bussemakers MJ, Schalken JA. Cadherin switching in human prostate cancer progression. Cancer Res. **2000**; 60:3650–3654.

Trainor PA, Melton KR, Manzanares M. Origins and plasticity of neural crest cells and their roles in jaw and craniofacial evolution. Int J Dev Biol **2003**; 47: 541–553.

Traub P, Shoeman RL. Intermediate filament proteins: Cytoskeletal elements with gene-regulatory function? Int Rev Cytol **1994**; 54:1-103.

Troyanovsky SM, Eshkind LG, Troyanovsky RB, Leube RE, Franke WW. Contributions of cytoplasmic domains of desmosomal cadherins to desmosome assembly and intermediate filament anchorage. Cell **1993**; 72:561–574.

Troyanovsky SM, Troyanovsky RB, Eshkind LG, Leube RE, Franke WW. Identification of amino acid sequence motifs in desmocollin, a desmosomal glycoprotein, that are required for plakoglobin binding and plaque formation. Proc Natl Acad Sci USA **1994**; 91:10790–10794.

Tsuruta D, Jones JC. The vimentin cytoskeleton regulates focal contact size and adhesion of endothelial cells subjected to shear stress. J Cell Sci **2003**; 116: 4977–4984.

Ullmann U, In't Veld P, Gilles C, et al. Epithelial-mesenchymal transition process in human embryonic stem cells cultured in feeder-free conditions. Mol Hum Reprod **2007**; 13: 21–32.

Ulloa L, Doody J, Massague J. Inhibition of transforming growth factor-*β*/SMAD signaling by the interferon-*γ* /STAT pathway. Nature **1999**; 397:710–713.

Valcourt U, Kowanetz M, Niimi H, Heldin C-H, Moustakas A. TGF-β and the Smad signaling pathway support transcriptomic reprogramming during epithelial-mesenchymal cell transition. Mol Biol Cell **2005**; 16: 1987–2002.

Van de Putte T, Maruhashi M, Francis A, Nelles L, Kondoh H, Huylebroeck D, et al. Mice lacking ZFHX1B, the gene that codes for Smad-interacting protein-1, reveal a role for multiple neural crest cell defects in the etiology of Hirschsprung disease-mental retardation syndrome. Am J Hum Genet **2003**; 72: 465–470.

van Grunsven LA, Papin C, Avalosse B, Opdecamp K, Huylebroeck D, Smith JC, et al. XSIP1, a Xenopus zinc finger/homeodomain encoding gene highly expressed during early neural development. Mech Dev **2000**; 94: 189–193.

van Hemert MJ, Steensma HY, van Heusden GP. 14-3-3 proteins: key regulators of cell division, signaling and apoptosis. Bioessays **2001**; 23: 936–946.

Van Obberghen-Schilling E, Roche NS, Flanders KC, Sporn MB, Roberts AB. Transforming growth factor β1 positively regulates its own expression in normal and transformed cells. J Biol Chem **1988**; 263: 7741–7746.

Vandewalle C, Comijn J, De Craene B, et al. SIP1/ZEB2 induces EMT by repressing genes of different epithelial cell-cell junctions. Nucleic Acids Res **2005**; 33: 6566–6578.

Vega S, Morales AV, Ocana OH, et al. Snail blocks the cell cycle and confers resistance to cell death. Genes Dev **2004**; 18: 1131-1143.

Ventura J-J, Kennedy NJ, Flavell RA, Davis R J. JNK regulates autocrine expression of TGF-β1. Mol Cell **2004**; 15: 269–278.

Ventura J-J, Kennedy NJ, Lamb JA, Flavell RA, Davis RJ. c-Jun NH(2)-terminal kinase is essential for the regulation of AP-1 by tumor necrosis factor. Mol Cell Biol **2003**; 23: 2871–2882.

Verrecchia F, Pessah M, Atfi A, Mauviel A. Tumor necrosis factor-alpha inhibits transforming growth factor-beta/Smad signaling in human dermal fibroblasts via AP-1 activation. J Biol Chem **2000**; 275: 30226–30231.

Verrecchia F, Tacheau C, Wagner EF, Mauviel A. A central role for the Jun-N-terminal kinase pathway in mediating the antagonistic activity of pro-inflammatory cytokines against transforming growth factor-beta-driven Smad3/4-specific gene expression. J Biol Chem **2002**; 278: 1585–1593.

Verrecchia F, Vindevoghel L, Lechleider RJ, Uitto J, Roberts AB, Mauviel A. Smad3/AP-1 interactions control transcriptional responses to TGF-beta in a promoter-specific manner. Oncogene **2001**; 20: 3332–3340.

Vincent-Salomon A, Thiery JP. Host microenvironment in breast cancer development: epithelial-mesenchymal transition in breast cancer development. Breast Cancer Res **2003**; 5: 101–106.

Vleminckx K, Vakaet L, Mareel M, Fiers W, Van Roy F. Genetic manipulation of E-cadherin expression by epithelial tumor cells reveals an invasion suppressor role. Cell **1991**; 66:107–119.

Vongwiwatana A, Tasanarong A, Rayner DC, Melk A, Halloran PF. Epithelial to mesenchymal transition during late deterioration of human kidney transplants: the role of tubular cells in fibrogenesis. Am. J. Transplant **2005**; 5: 1367–1374.

Wang HR, et al. Regulation of cell polarity and protrusion formation by targeting RhoA for degradation. Science **2003**; 302: 1775–1779.

Wang J, et al. Atrioventricular cushion transformation is mediated by ALK2 in the developing mouse heart. Dev Biol **2005**; 286: 299–310.

Wang J, et al. Defective ALK5 signaling in the neural crest leads to increased postmigratory neural crest cell apoptosis and severe outflow tract defects. BMC Dev Biol **2006**; 6: 51.

Weijzen S, Rizzo P, Braid M, Vaishnav R, Jonkheer SM, Zlobin A, Osborne BA, Gottipati S, Aster JC, Hahn WC, Rudolf M, Siziopikou K, Kast WM, Miele L. Activation of Notch-1 signaling maintains the neoplastic phenotype in human Ras-transformed cells. Nat Med **2002**; 8: 979–986.

Weiss C, Schneider S, Wagner EF, Zhang X, Seto E, Bohmann D. JNK phosphorylation relieves HDAC3-dependent suppression of the transcriptional activity of c-Jun. EMBO J **2003**; 22: 3686–3695.

Willis BC, et al. Induction of epithelial–mesenchymal transition in alveolar epithelial cells by transforming growth factor-β1: potential role in idiopathic pulmonary fibrosis. Am J Pathol **2005**; 166: 1321–1332.

Wong C, et al. Smad3-Smad4 and AP-1 complexes synergize in transcriptional activation of the c-Jun promoter by transforming growth factor β. Mol Cell Biol **1999**; 19: 1821–1830.

Wotton D, Lo RS, Lee S, Massague J. A Smad transcriptional corepressor. Cell **1999**; 97: 29–39.

Wu AL, Wang J, Zheleznyak A, Brown EJ. Ubiquitin-related proteins regulate interaction of vimentin intermediate filaments with the plasma membrane. Mol Cell **1999**; 4: 619–625.

Wu Z, Zang X, Salmon M, Lin X, Zehner ZE. TGFβ1 regulation of vimentin gene expression during differentiation of the C2C12 skeletal myogenic cell line requires Smads, AP-1 and Sp1 family members. Biochim Biophys Act **2007**; 1773:427-439.

Xie L, Law BK, Aakre ME, Edgerton M, Shyr Y, Bhowmick NA and Moses HL. Transforming growth factor β-regulated gene expression in a mouse mammary gland epithelial cell line. Breast Cancer Res **2003**; 5: R187–198.

Xie L, Law BK, Chytil AM, Brown KA, Aakre ME, Moses HL. Activation of the Erk pathway is required for TGF-beta1-induced EMT in vitro. Neoplasia **2004**; 6: 603–610.

Xu L, Chen S, Bergan RC. MAPKAPK2 and HSP27 are downstream effectors of p38 MAP kinase-mediated matrix metalloproteinase type 2 activation and cell invasion in human prostate cancer. Oncogene **2006**; 25:2987-2998.

Yahata T, et al. The MSG1 non-DNA-binding transactivator binds to the p300/CBP coactivators, enhancing their functional link to the Smad transcription factors. J Biol Chem **2000**; 275: 8825–8834.

Yanagisawa J, et al. Convergence of transforming growth factor-β and vitamin D signaling pathways on SMAD transcriptional coactivators. Science **1999**; 283: 1317–1321.

Yanez-Mo M, Lara-Pezzi E, Selgas R, Ramirez-Huesca M, Dominguez-Jimenez C, Jimenez-Heffernan JA, Aguilera A, Sanchez-Tornero JA, Bajo MA, Alvarez V, Castro MA, del Peso G, Cirujeda A, Gamallo C, Sanchez-Madrid F, Lopez-Cabrera M. Peritoneal dialysis and epithelial-to-mesenchymal transition of mesothelial cells. N Engl J Med **2003**; 348: 403–413.

Yang J, Mani SA, Donaher JL, Ramaswamy S, Itzykson RA, Come C, Savagner P, Gitelman I, Richardson A, Weinberg RA. Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. Cell **2004**; 117: 927–939.

Yang J, Weinberg RA. Epithelial-Mesenchymal Transition: At the crossroads of development and tumor metastasis. Developmental Cell 14, June **2008**; 818-829. DOI 10.1016/j.devcel.2008.05.009.

Yang Z, Rayala S, Nguyen D, Vadlamudi RK, Chen S, Kumar R. Pak1 phosphorylation of snail, a master regulator of epithelial-to-mesenchyme transition, modulates snail's subcellular localization and functions. Cancer Res **2005**; 65: 3179–3184.

Yatabe Y, Osada H, Tatematsu Y, Mitsudomi T, Takahashi T. Decreased expression of 14-3-3 sigma in neuroendocrine tumors is independent of origin and malignant potential. Oncogene **2002**; 21:8310–8319.

Yates B, Zetterberg C, Rajeev V, Reiss M, Rittling SR. Promoter-independent regulation of vimentin expression in mammary epithelial cells by val12 ras and TGFβ. Exp Cell Res **2007**; 313:3718-3728.

Yeaman C, Grindstaff KK, Hansen MD, Nelson WJ. Cell polarity: Versatile scaffolds keep things in place. Curr Biol **1999**; 9: R515- R517.

Yin JJ, et al. TGF-β signaling blockade inhibits PTHrP secretion by breast cancer cells and bone metastases development. J Clin Invest **1999**; 103: 197–206.

Yingling JM, Blanchard KL, Sawyer JS. Development of TGF-beta signalling inhibitors for cancer therapy. Nat Rev Drug Discov **2004**; 3: 1011–1022.

Yingling JM, et al. Tumor suppressor Smad4 is a transforming growth factor β-inducible DNA binding protein. Mol Cell Biol **1997**; 17: 7019–7028.

Yokoyama K, Kamata N, Fujimoto R, Tsutsumi S, Tomonari M, Taki M, Hosokawa H, Nagayama M. Increased invasion and matrix metalloproteinase-2 expression by snail-induced mesenchymal transition in squamous cell carcinomas. Int J Oncol **2003**; 22:891–898.

Yokoyama K, Kamata N, Hayashi E, Hoteiya T, Ueda N, Fujimoto R, Nagayama M. Reverse correlation of Ecadherin and snail expression in oral squamous cell carcinoma cells in vitro. Oral Oncol **2001**; 37:65–71.

Yu L, Hebert MC, Zhang YE. TGF-beta receptor-activated p38 MAP kinase mediates Smad-independent TGF-beta responses. EMBO J **2002**; 21: 3749–3759.

Yu Q, Stamenkovic I. Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-β and promotes tumor invasion and angiogenesis. Genes Dev **2000**; 14: 163–176.

Yue J, Mulder KM. Requirement of Ras/MAPK pathway activation by transforming growth factor β for transforming growth factor β1 production in a Smad-dependent pathway. J Biol Chem **2000**; 275: 30765–30773.

Zavadil J, Bitzer M, Liang D, Yang YC, Massimi A, Kneitz S, Piek E, Bottinger E P. Genetic programs of epithelial cell plasticity directed by transforming growth factor-β. Proc Natl Acad Sci USA **2001**; 98: 6686-6691.

Zavadil J, Bottinger EP. TGF-beta and epithelial-to-mesenchymal transitions. Oncogene **2005**; 24:5764–5774.

Zavadil J, Cermak L, Soto-Nieves N, Bottinger EP. Integration of TGF-β/ Smad and Jagged1/Notch signalling in epithelial-to-mesenchymal transition. EMBO J **2004**; 23: 1155–1165.

Zawel L, Dai JL, Buckhaults P, Zhou S, Kinzler KW, Vogelstein B, Kern SE. Human Smad3 and Smad4 are sequence-specific transcription activators. Mol Cell **1998**; 1: 611-617.

Zeisberg M, Kalluri R. The role of epithelial-to-mesenchymal transition in renal fibrosis. J Mol Med **2004**; 82: 175–81.

Zeisberg M, Shah AA, Kalluri R. Bone morphogenic protein-7 induces mesenchymal to epithelial transition in adult renal fibroblasts and facilitates regeneration of injured kidney. J Biol Chem **2005**; 280: 8094–8100.

Zhang D, Tai LK, Wong LL, Chiu L-L, Sethi SK, Koay ESC. Proteomic study reveals that proteins involved in metabolic and detoxification pathways are highly expressed in HER-2/neu-positive breast cancer. Mol Cell Proteom **2005**; 4:1686–1696.

Zhang D, Wong LL, Koay ESC. Phosphorylation of Ser78 of Hsp27 correlated with HER-2/*neu* status and lymph node positivity in breast cancer. Mol Cancer **2007**; 6:52.

Zhang Y, Chang C, Gehling DJ, Hemmati-Brivanlou A, Derynck R. Regulation of Smad degradation and activity by Smurf2, an E3 ubiquitin ligase. Proc Natl Acad Sci **2001**; 98: 974–979.

Zhang Y, Derynck R. Regulation of Smad signalling by protein associations and signalling crosstalk. Trends Cell Biol **1999**; 9: 274–279.

Zhang Y, Derynck R. Transcriptional regulation of the transforming growth factor-β-inducible mouse germ line Igα constant region gene by functional cooperation of Smad, CREB, and AML family members. J Biol Chem **2000**; 275: 16979–16985.

Zhang Y, Feng XH, Derynck R. Smad3 and Smad4 cooperate with c-Jun/c-Fos to mediate TGF-β-induced transcription. Nature **1998**; 394: 909–913.

Zhou BP, Deng J, Xia W, Xu J, Li YM, Gunduz M, Hung MC. Dual regulation of Snail by GSK-3 beta-mediated phosphorylation in control of epithelial–mesenchymal transition. Nat Cell Biol **2004**; 6:931–940.

Zhu H, Kavsak P, Abdollah S, Wrana JL, Thomsen GH. A SMAD ubiquitin ligase targets the BMP pathway and affects embryonic pattern formation. Nature **1999**; 400: 687–693.

Zoltan-Jones A, Huang L, Ghatak S, Toole BP. Elevated hyaluronan production induces mesenchymal and transformed properties in epithelial cells. J Biol Chem **2003**; 278: 45801–45810.