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-231 cells 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-β1 gene 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
RESULTS

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
RESULTS

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 (Fig 12-Lane 2). Prominent shift
was observed for TGF-β1 probe with nuclear extract from treated cells (Fig 12-Lane 3).
Similarly, Snail probe with nuclear extract from untreated cells showed no sign of shift
(Fig 12-Lane 5). 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-Lane 2, 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-Lanes 2, 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 autofluorescence 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 cytoskeletal 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
RESULTS

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-monoxygenase 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)

<table>
<thead>
<tr>
<th>Fig No.</th>
<th>Protein suggested by PMF spectra</th>
<th>Intensity Coverage %</th>
<th>Sequence Coverage %</th>
<th>pH</th>
<th>MW kD</th>
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<tr>
<td>Fig. 32</td>
<td>Enolase (C1)</td>
<td>77.1</td>
<td>62.2</td>
<td>7.7</td>
<td>47.4</td>
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<tr>
<td>Fig. 33</td>
<td>α Enolase (T1)</td>
<td>79.0</td>
<td>43.0</td>
<td>7.7</td>
<td>47.4</td>
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<tr>
<td>Fig. 34</td>
<td>α Tubulin (C2)</td>
<td>21.5</td>
<td>32.5</td>
<td>4.8</td>
<td>50.5</td>
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<td>Fig. 35</td>
<td>β Tubulin (C5)</td>
<td>76.0</td>
<td>43.6</td>
<td>4.6</td>
<td>50.3</td>
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<td>Fig. 36</td>
<td>Calreticulin precursor protein (C3)</td>
<td>35.3</td>
<td>26.6</td>
<td>4.1</td>
<td>47.1</td>
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<td>Fig. 37</td>
<td>β Actin (C4)</td>
<td>27.1</td>
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<td>Fig. 38</td>
<td>β Actin (T4)</td>
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<td>30.0</td>
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<td>Fig. 39</td>
<td>TUBB protein (C7)</td>
<td>68.6</td>
<td>38.5</td>
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<td>Fig. 40</td>
<td>TUBB protein (T7)</td>
<td>68.6</td>
<td>38.5</td>
<td>4.6</td>
<td>38.7</td>
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<td>Fig. 41</td>
<td>Triose phosphate isomerase1 isoform (D1)</td>
<td>7.7</td>
<td>38.1</td>
<td>5.6</td>
<td>31.1</td>
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<td>Fig. 42</td>
<td>Triose phosphate isomerase1 isoform (D2)</td>
<td>3.8</td>
<td>37.4</td>
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<td>Fig. 43</td>
<td>Heat shock protein-27 (D3)</td>
<td>15.9</td>
<td>29.3</td>
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<td>Fig. 44</td>
<td>14-3-3ζ protein (D4)</td>
<td>13.8</td>
<td>33.6</td>
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<td>Fig. 45</td>
<td>Type 1 Keratin 16 (D5)</td>
<td>22.5</td>
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<td>Fig. 46</td>
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<td>Elongin A2 (D6)</td>
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<td>Fig. 47</td>
<td>Ubiquitin (D7)</td>
<td>43.8</td>
<td>50.0</td>
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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 24hrs (Fig 49-Lane4), 48hrs (Fig 49-Lane5) and at 72hrs (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 Snail 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-231 cells, 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.
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**

GGATCCTGATTGGAGCTAAATTGACACGGGACGGGGAGTATTCCGCTTAATGACTGCTTACTGCTCCTCTGTCCCCCACCCTTACTGCCCGCTCTCCAGCTAGAACCAGGGGAGGACGATTTTGTTCA

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' ccc ttc cat ccc tca ggt gtc ctag tgc cc 3'
3' ggg aag gta ggg agt cca cag gac aac gg 5'
```

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<th>MDA-MB231</th>
<th>MCF-7</th>
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<tr>
<td>TGF-β1 probe (2ng)</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Nuclear extract (5μg)</td>
<td>+</td>
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<td>KCl 300mM</td>
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<tr>
<td>KCl 150mM</td>
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Fig.6: EMSA profile of TGF-β1 with various concentration of KCl. Band shift was observed at various concentration of KCl. Lanes 1, 2, Nuclear extract from MDA-MB-231 cells (5μg) was used, band shift was observed at 300mM concentration of KCl. Lanes 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.
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 losing their cell-cell junctions after 24hrs and 36hrs of TGF-β1 treatment (5ng/ml) respectively (10x magnification).
**Prob e Sequence:** 5' ggctgaggtttcaccctgcgccacagcc3'  
3'ccgactccaaagttggacgcgggtgtcgg5'

<table>
<thead>
<tr>
<th></th>
<th>Lane 1</th>
<th>Lane 2</th>
<th>Lane 3</th>
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<tr>
<td>Nuclear extract (11μg)</td>
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<td>+</td>
<td>+</td>
</tr>
<tr>
<td>TGF-β1 treatment (5ng/ml, 72 hrs)</td>
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**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 72 hrs. Nuclear extract (11μg protein) from both cells were incubated separately with E-cadherin probe for 30 minutes on ice. **Lane 1,** Free E-cadherin probe. **Lane 2,** 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.
**Probe Sequence:** 5' cacctg at cacctg at cacctg at cacctg 3'  
3' gttgac tta gttgac tta gttgac tta gttgac 5'

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<tr>
<td>TGF-β1 treatment (5ng/ml, 72 hrs)</td>
<td>-</td>
<td>-</td>
<td>+</td>
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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 9 gat ta t t 9 gat ta t t 9 gat 5'

Sequence repeat Probe (2ng)  +  +  +
Nuclear extract (11μg)        -  +  +
TGF-β1 treatment             -  -  +
(5ng/ml, 72 hrs)

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. Lane 1, Free TGF-β1 probe. Lane 2, TGF-β1 probe with nuclear extract from untreated cells showed no prominent shift. Lane 3, Prominent shift was observed for TGF-β1 probe with nuclear extract from treated cells. Lane 4, Free SNAIL probe. Lane 5, SNAIL probe with nuclear extract from untreated cells showed no prominently visible shift. Lane 6, 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 fluorescence 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 autofluorescence 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 autofluorescence. C, Overlay plot of autofluorescence and FITC staining.
Fig. 18: Expression analysis of vimentin in TGF-β1 treated and untreated MCF-7 cells. 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 nonsignificant 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 cytokeratin 18 (CK18) in TGF-β1 treated and untreated MCF-7 cells.

Cells were treated with TGF-β1 (5 ng/ml) for 72 hrs. Prim.Ab. against cytokeratin 18 was employed for intracellular probing of cytokeratin 18 in both untreated and treated cells. FITC-labeled sec. antibody was used against cytokeratin 18 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. (Focused on 7 cm strip)
Total cytosolic protein was extracted from cells and 60μg of estimated protein was loaded on 7 cm, 3-10 linear IEF-strip for electrofocussing. SDS-PAGE (12%) was run for second dimension along with marker proteins.
C-Utreated
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 mass 163.681 corresponds to HSP-27, LFDQAFGLPR along with sequence coverage map showing intensity coverage-53.5%, sequence coverage-46.3%, pi-6.0, kDa-22.8.
Fig. 26: MS/MS spectrum of m/z 1163.681 (LFDQAFGLPR) corresponds to HSP-27.
Peptide peak of mass 1805.120 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. 27: PMF spectrum suggests human Enolase.
Fig. 28: MS/MS spectrum of m/z 1805.120 (AAVPSGASTGIYEALELR) corresponds to enolase.
Fig. 29: 2D electrophoresis profile of cytosolic proteins of MCF-7 cells. (Focussed on 7 cm strip)

Total cytosolic protein was extracted from cells and 200 µg 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. (Focused 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-Monoxygenase 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.
### Protein: TUBB protein (Homo sapiens)

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**Fig. 40:** PMF spectrum suggests human TUBB protein.
Protein: Triosephosphate isomerase 1, isoform CRAb (Homo sapiens)

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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.