// Abstract

Transforming growth factor-β (TGF-β) is a multifunctional cytokine that regulates a plethora of functions including cell growth, development, and differentiation. TGF-β plays a major role in the inhibition of normal cell growth and proliferation. Quite dichotomously, at a late stage of cancer progression cells become refractory to the cytostatic actions of TGF-β, and in turn start to proliferate, invade, and metastasize beyond their tissue of origin. Acquisition of metastatic phenotypes by cancer cells is critically dependent upon their ability to undergo epithelial-mesenchymal transition (EMT). Pathophysiologic ally, EMT is a critical step in mediating the acquisition of metastatic phenotypes by localized carcinomas. TGF-β is a potent inducer of these two lethal aspects of cancer mainly EMT and metastasis. However, it is not very clear how TGF-β suppresses growth in normal epithelial cells and promotes metastatic processes like EMT in their malignant counterparts. TGF-β is known to mediate its function by binding to its cognate serine/threonine kinase receptor and activate downstream signaling mediated by the canonical Smad dependent as well as non-canonical Smad independent pathways. Through the activation of these pathways, TGF-β regulates the expression of several target genes either positively or negatively in a cell type and context dependent manner. TGF-β induced both canonical and non-canonical signaling has been implicated in mediating its pro-tumorigenic functions. However, there is no clear understanding of the mechanism (s) responsible for the differential responses of various cell types to TGF-β. Therefore, more studies are warranted to understand how TGF-β regulates differential gene expression to induce EMT and metastasis in cancer. This understanding will enable the development of novel targeted therapies, thereby improving the clinical course of cancer patients.

Over the past few years, several independent transcriptome analyses revealed cell type dependent differential regulation of genes in response to TGF-β. A previous transcriptome analysis data from our laboratory identified several novel genes regulated by TGF-β in normal and transformed cell types including S100A2. S100A2 belongs to the S100 family of EF hand calcium binding proteins, which are heavily implicated in the progression of cancer. Differential expression of S100A2 has been reported in several cancers like breast, lung, gastric, ovarian, prostrate, endometrial, and pancreatic cancer. Over expression of S100A2 in lung and pancreatic cancer has been correlated with poor prognosis and recently, S100A2 has been reported to induce metastasis in the xenograft murine model of non-small cell lung cancer (NSCLC). However, the exact role of S100A2 in tumorigenesis remains elusive. Therefore, in view of the S100A2 and TGF-β involvement in the complex process of metastasis and tumor development, the present study is designed to understand the mechanism of S100A2 gene regulation by TGF-β and its function (s) with respect to tumor progression. The following sections highlight the mechanistic aspects of S100A2 regulation and function.

- *Characterization of S100A2 regulation by TGF-β induced signaling pathways*

In order to investigate the mechanism of S100A2 gene regulation by TGF-β, normal and transformed cell types of different tissue origins were treated with TGF-β and the levels of S100A2 transcript and protein were examined. The S100A2 mRNA expression increased as early as 1h and persisted until 24h, post TGF-β treatment. Maximal stimulation was observed at 6h after TGF-β treatment in immortalized HaCaT cell line. Similar regulation of S100A2 mRNA was observed in Hep3B and MDAMB-231 cells in response to TGF-β. In addition, increase in S100A2 mRNA and protein expression by TGF-β was completely blocked in the presence of actinomycin D, suggesting S100A2 as a transcriptional target of TGF-β in epithelial cells.

To delineate the transcriptional regulation of S100A2 expression by TGF-β, we characterized 2.1kb 5' flanking region of S100A2 gene. Treatment of cells with TGF-β induced S100A2 promoter activity in all the above-mentioned cell lines. *In silico* analysis revealed potential transcription factor binding sites, including one Smad binding Element (SBE), three each of AP-1 and p53 binding sites amongst others in the 2.1kb region of S100A2 promoter. Characterization of the promoter revealed activator protein-1(AP-1) element at -1161 to -1151 as most critical for TGF-β1 response. Chromatin immunoprecipitation and electrophoretic mobility shift assay (EMSA) confirmed the functional binding of AP-1 complex, predominantly JunB, to S100A2 promoter in response to TGF-β1. JunB over expression markedly stimulated S100A2 promoter which was blocked by dominant-negative JunB and MEK1 inhibitor, PD98059 suggesting involvement of TGF-β induced MEK/ERK signaling in this regulation. Intriguingly, despite the presence of a putative SBE, S100A2 regulation by TGF-β1 was found to be independent of SBE element. This was confirmed by absence of Smad protein complex binding on the SBE element. Further, promoter deletion analysis and blocking Smad3 signaling had no effect on the induction of S100A2 mRNA by TGF-β1. Most importantly, our study demonstrates a unique synergistic regulation of S100A2 promoter by p53 and TGF-β induced MEK/ERK signaling, without direct involvement of Smads.

Collectively, our findings demonstrate S100A2 gene as a direct transcriptional target of TGF-β in epithelial cells. TGF-β mediated regulation of S100A2 is dependent on the AP-1 binding to its consensus site in the promoter under the influence of MEK/ERK signaling. Further, our study demonstrates a novel interplay between TGF-β induced MEK/ERK and p53 signaling in the synergism of S100A2 promoter.

Functional significance of S100A2 over expression in cancer

S100A2 is an over expressed gene in several cancer including basal like breast tumors, endometrial cancer, gastric cancer, ovarian cancer, pancreatic cancer, and non-small cell lung

cancer (NSCLC). As mentioned earlier, over expression of S100A2 is correlated with increased metastasis and poor prognosis of the patients in the early stage NSCLC. Few members of S100 proteins like S100A4 actions in the process of EMT and metastasis has just begun to unravel. However, the role of S100A2 in the progression of cancer is not clear. Hence, the present study has been designed to investigate the significance of S100A2 over expression in the progression of cancer. Towards understanding the functional role of S100A2 in cancer, neomycin resistant A549 clones (lung adenocarcinoma cell line) stably over expressing S100A2 were generated in our laboratory. In the present study, we utilized these S100A2 over expressing A549 clones in different functional assays like cell proliferation, cell invasion, anchorage independent growth potential and xenograft mouse model to gain insights into S100A2 role in cancer.

In a monolayer culture, S100A2 over expression resulted in an altered morphology of cells from polarized cuboidal to spindle shaped mesenchymal cells, suggestive of EMT. In addition, in anchorage independent growth assay S100A2 over expressing clones resulted in increased number of colonies with a distinct “spread out” and loose appearance. This morphological feature of colonies in soft agar has been reported to be suggestive of increased metastatic potential of the cells. Further, S100A2 clones invaded the Matrigel more efficiently than the control cells in the Matrigel invasion assay. Effect of S100A2 in mediating EMT was biochemically confirmed by looking at the mRNA and protein expression of EMT markers. We observed an increase in the expression of mesenchymal markers like vimentin, N-cadherin, alpha-smooth muscle actin and a concomitant decrease in the expression of epithelial markers like E-cadherin and occluding. In addition, the transcription factor Snail, which is a negative regulator of E-cadherin expression, was induced in S100A2 clones compared to the control. S100A2 preferentially increased the activation of phospho-AKT in the clones compared to the vector clones. To corroborate these observations *in vivo*, these cells were injected subcutaneously in the flanks of immunocompromised mice and studied the phenotype of resultant tumors. Subcutaneous injection of S100A2 clones formed significantly large tumors which histologically appeared to have undergone EMT as seen by the hematoxylin and eosin staining. S100A2 induced EMT *in vivo* was observed by the altered mRNA expression of EMT markers as seen by RT-PCR and protein expression of few mesenchymal markers by immunohistochemistry. Increased AKT phosphorylation was also observed in the resected tumors over expressing S100A2 suggesting a role for AKT in S100A2 mediated invasive phenotype. To substantiate that these effects were specific to S100A2, we depleted the expression of S100A2 in Hep3B cell line using shRNA. In good correlation, knockdown of S100A2 reversed the EMT phenotype by repressing the expression of mesenchymal markers as well as significantly reduced phospho-AKT levels. Collectively, our data demonstrate the pro-tumorigenic role of S100A2 in cancer.

- *Mechanism of S100A2 mediated pro-tumorigenic actions*

Our data revealed that over expression of S100A2 regulates expression of genes involved in matrix modulation like MMP-9, MMP-1, MMP-7, TGM2, uPA, and EMT modulators like vimentin, Snail and E-cadherin, respectively. Interestingly, these gene targets are also shown to be regulated by TGF-β/Smad3 signaling. In addition, the role of TGF-β and Smad3 in mediating EMT has been well documented. Our observation on the involvement of S100A2 in the enhancement of EMT prompted us to evaluate a possible interaction of S100A2 with Smad3. By GST-pull down and co-immunoprecipitation experiments, we demonstrate a physical interaction between S100A2 and Smad3 proteins. In the presence of high calcium and TGF-β, S100A2-Smad3 interaction was further enhanced. Moreover, S100A2 positively induced Smad3 dependent transcriptional activation. Supporting this, knockdown of S100A2 expression abolished TGF-β/Smad3 mediated gene induction, which mainly included direct Smad3 targets like p21, PAI-1 and vimentin. Most importantly, loss of S100A2 significantly reduced TGF-β induced cancer cell migration and invasion.

In summary, our study has for the first time delineated the mechanism of S100A2 gene regulation in the context of TGF-β and p53. Further, we established a pro-tumorigenic role of S100A2 through induction of EMT. Moreover, this study highlights a novel interaction of S100A2 with Smad3, which may have important implications in the progression of cancer. In our study, PI3/AKT signaling emerged as the important signaling pathway altered upon S100A2 over expression. This substantiates the well-documented role of AKT signaling in mediating EMT and increased survival of cancer cells. Finally, our study has identified S100A2 as a potential therapeutic target at least in lung cancer for combating EMT induced by activated TGF-β/Smad3 signaling.