Activin-A is a member of transforming growth factor-β (TGF-β) superfamily of cytokines which includes TGF-βs, Activins, Nodal, bone morphogenetic proteins (BMPs), growth and differentiation factors (GDFs) and anti-Mullerian hormone (AMH). TGF-β, Activin and Nodal are known to activate SMAD2/3, while BMPs and GDFs are known to activate SMAD1/5/8 signaling pathways. Activin-A binds to type II transmembrane serine threonine kinase receptor (ActRIIA or ActRIIB), which in turn activates type I receptor (ActRIB) leading to phosphorylation of SMAD2/SMAD3. Upon phosphorylation, SMAD2/3 forms a complex with SMAD4, which then translocates to nucleus. In the nucleus, SMAD2/3/4 complex, along with other co-factors regulates expression of a large number of genes.

Unlike TGF-β, role of Activin in cancer is not well understood. Activin has been shown to be overexpressed in several cancers including metastatic prostate cancer, colorectal cancer, lung cancer, hepatocellular carcinoma and pancreatic cancer. Activin signaling has been shown to promote aggressiveness of esophageal squamous cell carcinoma and enhancing skin tumorigenesis and progression. Nodal, which binds to the same set of receptors, has also been shown to be overexpressed in several cancers. However, role of Activins in breast cancer progression is not well studied. Activin is expressed by normal breast epithelium and is known to play a role in mammary gland development. Earlier, a study had reported downregulation of Activin signaling in breast tumors. On the contrary, increased serum level of Activin has been reported in women with metastatic breast cancers. It is pertinent to mention here that TGF-β, which has been implicated in the progression and metastatic spread of breast cancers, also functions through the same set of downstream effectors- SMAD2 and SMAD3. Hence we wanted to evaluate the status of Activin signaling pathway in breast tumors and investigate its functional role in cancer progression.

Gene expression profiling of 80 breast tumors and 20 normal samples was earlier performed in our laboratory revealed overexpression of INHBA in tumors compared to normal tissue samples. An independent set of 30 tumor and 15 normal samples were used to verify these results. Real-time PCR analysis revealed around 11.31 fold upregulation ($p<0.001$) of INHBA in breast tumors in comparison to normals. While no change in expression of INHA was observed, INHBB was found to be significantly downregulated in tumor samples. These results indicated upregulation of Activin-A in breast tumors. Further, a significant upregulation of ACVR2A and SMAD2 which act as signal

transducers of Activin signaling pathway, was observed in breast tumors. Interestingly, while an increase in the expression of TGF-β1 was observed, TGFBR2 was found to be significantly downregulated in breast tumors. In addition, PCR analysis revealed significant downregulation of FST, β-glycan, IGSF1 and IGSF10, which act as negative regulators of Activin signaling pathway. Functional antagonism between TGF-β/Activin and BMP signaling pathway has been shown in both development and disease. Further analysis revealed that various BMPs including BMP2, BMP4 and BMP6 are downregulated in breast tumors compared to normal tissue samples. Various components and regulators of BMP signaling pathway were also found to be deregulated, indicating suppression of BMP signaling in breast tumors. To evaluate whether Activin signaling is active in breast tumor cells, immunohistochemistry with another set of 13 normal and 29 tumor samples was performed. Immunohistochemistry analysis revealed that most of the tumors have higher levels of Activin-A compared to normals tissues. Interestingly, no significant changes in expression of Activin-A was observed between normals and low grade tumors, suggesting that Activin-A may play an important role towards the late stages of the disease. In good correlation, breast tumors showed increased phospho SMAD2 and phospho SMAD3 levels compared to normal tissues. Also, in the same set of tumors, BMP2 staining showed a reduced expression pattern compared to normal tissues. Expression of inhibin in some normal and breast tumor samples revealed that most of the tumor samples have lower levels of inhibin compared to normal tissues.

In order to understand the role of Activin-A in cancer progression, a panel of cell lines was selected. Treatment of cells with Activin-A resulted in activation of canonical SMAD as well as non-canonical Erk1/2 and PI3K signaling pathways. However, Activin-A treatment did not lead to activation of TAK1/p38 MAPK pathway. To begin with, it was important to evaluate effect of Activin-A on proliferation of various cell lines. Primarily, SMAD2/3 signaling pathway inhibits proliferation of normal epithelial cells, and hence, it is considered to have a tumor suppressive role. However, this signaling pathway remains intact in most (~ 98%) of the breast cancers. BrdU incorporation assay showed that Activin-A does not promote proliferation of cells under monolayer culture conditions. However, soft agar assay results showed that Activin signaling promotes anchorage independent growth of cancer cells. TGF-β is widely known as an inducer of epithelial mesenchymal transition (EMT). Also, EMT is considered to be a prerequisite for epithelial cells to undergo migration and invasion. During EMT, cells loose epithelial

characteristics and acquire mesenchymal features along with cytoskeletal rearrangement. Treatment of cells with Activin-A resulted in downregulation of E-cadherin and upregulation of various mesenchymal markers. In addition, confocal microscopy imaging revealed a mesenchymal morphology of cells treated with Activin-A. Also, collagen gel contraction assay results indicated that Activin-A enhances the contractile property of HaCaT cells significantly. Cells undergone EMT are believed to acquire migratory and Invasive behaviour. In agreement with this, both scratch assay and trans-well migration assay showed that Activin-A enhances the migration of various cell lines. Further, Trans-well matrigel invasion assays were performed to assess how Activin affects invasion of various cancer cells. Matrigel invasion assay results showed that Activin-A enhances invasion of various cancer cell lines significantly. Also, RT-PCR, zymography and Luciferase assay results showed that Activin-A induces MMP2 expression. As described earlier, Activin-A activates both canonical as well as non canonical signaling pathways. In this direction, it was interesting to investigate the contribution of SMAD signaling pathway in pro-tumorigenic actions of Activin-A. Inhibiting SMAD3 activity either by its stable knockdown or by using a SMAD3 specific small molecule inhibitor revealed that Activin-A regulation of EMT markers is SMAD3 dependent. Further, it was observed that SMAD3 contributes significantly in mediating Activin-A induced migration and invasion. Hence, it is likely that SMADs may play an important role in breast tumor progression.

Next, stable overexpression of Activin-A in MCF-7 or its knockdown in MDA-MB-231 and H460 cells was performed to assess the effect of Activin-A on the behaviour of these cells. BrdU assay indicated no change in proliferation of cells upon overexpression or knockdown of Activin-A. However, soft agar assay results showed that Activin-A expression affects anchorage independent growth of these cells. MCF-7 cells are generally considered to be less aggressive in their tumor forming ability. Activin-A overexpressing MCF7 cells and control cells were respectively injected into right and left flank of immunocompromised mice and followed till the tumors reached to a prominent size. Our results show that Activin-A overexpressing MCF-7 cells have better tumor forming ability in comparison to control cells. In contrast to MCF-7 cells, MDA-MB-231 cells are known to be aggressive in their tumorigenic potential. In order to understand the effect of Activin-A knockdown on the tumor forming ability in MDA-MB-231 cells, 0.5 million cells (optimal cell number generally used is 1-2 million) were injected subcutaneously in immunocompromised mice. The results showed that while control cells

gave rise to a tumor in 7 out of 10 animals, Activin-A knockdown cells could form a tumor in only 3 out of 10 animals. Also, the tumors formed by control cells were significantly larger by weight as compared to tumors formed by knockdown cells. Further, immunohistochemistry showed that tumors formed by MCF-7 cells overexpressing Activin-A have higher Ki-67 percentage as compared to control tumors. One of the factors known to be important for tumor growth is VEGF, which leads to recruitment of blood vessels and hence providing nourishment to the tumor cells. Hence Activin-A regulation of VEGF expression was evaluated next. Activin-A treatment or its stable overexpression in MCF-7 cells resulted in increased VEGF expression in these cells. This was also confirmed by VEGF promoter activity assay. To assess if Activin-A can play a role in metastatic spread of cancer cells, tail vein injection of Activin-A overexpressing MCF-7 cells was performed in immunocompromised mice. Even though no significant difference was found in the number of nodules formed by control or Activin-A overexpressing cells, it was observed that Activin-A overexpressing cells formed much bigger nodules as compared to the control cells. This suggests that Activin-A may play an important part in the establishment of metastases from the disseminated cancer cells. Tumor forming ability of cancer cells and aggressiveness of various cancers has been associated with the presence of cells having stem-like phenotype. In this direction, $CD44^{high}$ and $CD24^{low}$ expression status was analysed upon overexpression and knockdown of Activin-A in MCF-7 and MDA-MB-231 cells respectively. FACS analysis of Activin-A overexpressing MCF-7 cells and Activin-A knockdown MDA-MB-231 cells shows that Activin-A expression leads to enrichment of breast cancer stem-like cells.

In conclusion, this study highlights the importance of Activin-A signaling pathway in the progression of breast tumors. It is also important to note the role of SMAD signalling in the progression of breast cancers since these effectors are common between TGF-β, Activin and nodal factors, which have been shown to be involved in cancer progression in a context dependent manner.