Assessing the Role of Activins / Inhibins in Human Oral Cancers

By

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Recommendations of the Viva Voce Committee

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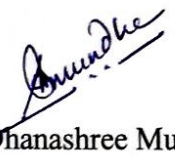

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DECLARATION

I, hereby declare that the investigation presented in the thesis has been carried out by me. The work is original and has not been submitted earlier as a whole or in part for a degree/diploma at this or any other Institute / University.

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p63 promotes oral cancer cell migration by regulating Activin A expression. Rupa Mishra, **Dhanashree Mundhe**, Sanjeev Kumar, Srikanta Basu, Sorab Dalal, Tanuja Teni. Experimental Cell Research. (RM and DM are equal contributors).

Conferences

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DEDICATED TO

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Chapter 7

Summary and Conclusions

This study was designed to comprehensively analyse the expression of all components of Activin/Inhibin signaling pathway with an aim to determine the Activins/Inhibins that are differentially expressed in oral cancers and their contribution to oral oncogenesis. We demonstrate here that Activin A ligand from this molecular subfamily is significantly overexpressed in oral cancer cell lines and tissue samples; and that the hyperactivated Activin A signaling exhibits oncogenic functions in oral cancer by promoting epithelial-mesenchymal transition, cell migration and invasion, phenotypes that influence cancer metastases. In oral cancer patients, this reflects on the clinicopathological characteristics of their tumors as well as on patient survival. Patients with higher expression of Activin A exhibit tumors with higher T stage, positive lymph node metastases and a shorter recurrence-free and overall survival. Activin A overexpression in these tumors serves as an independent predictor of recurrence and survival of patients.

Our study is also the first to demonstrate a correlation between Activin A and p63 expression in oral tumors and a direct transcriptional regulation of the Activin A gene by p63 transcription factor in oral cancer cells. Notably, the correlation between Activin A and p63 was p53 dependent; tumors with high or stabilized p53 expression exhibited a negative correlation between Activin A and p63, while the tumors with low p53 expression did not exhibit such a correlation. This correlation between Activin A and p63 was associated with survival in patients; tumors concomitantly overexpressing Activin A and p63 were associated with shorter recurrence-free and overall survival. When taken together, the combined overexpression of these two proteins could independently predict recurrence in oral cancer patients, more efficiently than Activin A overexpression alone. Thus, we postulate that Activin A and p63 combined expression can be used as a marker for relapse and poor prognosis in oral cancers.

We also analysed the effect of inhibiting the signaling pathway downstream of Activin A on the phenotypes of oral cancer cells. Knockdown of the canonical pathway component, SMAD2/3 as well as inhibition of the activity of Activin A receptor, ALK4 using EW-7197 inhibitor, both resulted in attenuation of cell migration and invasion. A reverse effect on these phenotypes was observed when oral cancer cells lacking Activin A expression were treated with recombinant Activin A. We could also demonstrate that Activin A signaling stimulates the phosphorylation of SMAD2/3, p38-MAPK and JNK, thus activating both canonical and non-canonical downstream signaling pathways in oral cancer. Overall, we conclude that Activin A signaling, through its canonical SMAD pathway and non-canonical MAPK pathways, promotes oncogenic functions in oral cancers. Attenuating the action of Activin A in this cancer subtype using orally bioavailable inhibitors such as the one used in this study may aid in achieving loco-regional control of cancer, a long-known hindering factor in treatment success and one of the significant contributors of therapy failure in oral cancers.

The observations and inferences of this study are summarized in Figure 30 as below.

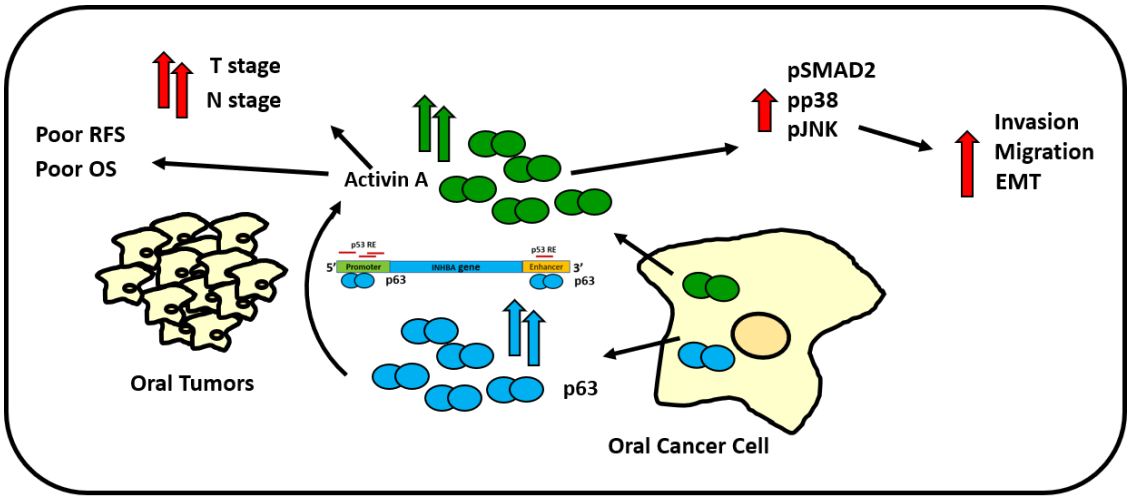

Figure 30: Role of Activin A signaling in Oral Cancers

Thesis Title: Assessing the Role of Activins / Inhibins in Human Oral Cancers

Oral cancers are one of the most prevalent cancers in the Indian population, and a leading cause of mortality due to cancers. Frequent loco-regional metastases are seen at diagnosis and is one of the important factors contributing to the poor prognosis associated with this disease. Cellular processes such as epithelial-mesenchymal transition (EMT), cell migration and invasion underly this process of regional metastases and are important in determining the spread of cancers. TGF-β signaling pathway plays a crucial role in these cell processes. The role of TGF-β subfamily proteins, Activins / Inhibins, in oral cancers was however, unexplored. This study aimed to evaluate the expression of Activin / Inhibin signaling components and determine the role of this signaling in oral cancers, specifically in the context of its oncogenic functions and mechanisms.

Using real-time PCR, western blotting and Immunohistochemistry, we performed a comprehensive expression analysis of all components of the Activin / Inhibin signaling pathway in oral cancer cell lines &/or patient tumor tissue samples, where we found aberrant expression of these proteins in oral cancer. Particularly, Activin A ligand was found to be highly overexpressed in oral cancers and its overexpression was associated with higher tumor stage, lymph node metastases and poor survival. The expression of Activin A in these oral tumors was correlated to that of p63, a p53 family transcription factor that is also overexpressed in oral cancers. Tumors concomitantly overexpressing Activin A and p63 were associated with shorter recurrence-free and overall survival; the overexpression of these two proteins together could independently predict relapse in these patients. Hence, this study determined new prognostic markers in oral cancers that are linked to a poor outcome in patients. We also studied the regulation of Activin gene expression by p63 and demonstrated that p63 is capable of transcriptionally regulating Activin A gene expression by binding to its promoter and enhancer. Further, recombinant Activin A rescued the loss in migration capacity of oral cancer cells upon p63 knockdown. Thus, p63 is overexpressed in oral cancers, underlining one of the probable ways by which overexpression of Activin A is achieved, which further mediates the oncogenic functions of p63 such as cell migration.

We assessed the functional implications of Activin A overexpression in oral cancer cells. Recombinant Activin A could promote EMT, cell migration and invasion in these cells, demonstrating an oncogenic role. Mechanistically, Activin A induced phosphorylation (activation) of SMAD 2/3, the canonical pathway of Activins. It also activated the non-canonical MAPK pathways, particularly p38 kinase, in oral cancer cells. Blockade of the Activin signaling, by knockdown of the downstream signal transducers, SMAD 2/3 or by using Vactosertib, an upstream inhibitor of Activin signaling, both attenuated these oncogenic phenotypes of Activin A. Our study identifies new molecular mechanism that contributes to migratory & invasive properties of oral cancer cells, phenotypes that may eventually lead to loco-regional metastases in oral cancer patients, and deciphers the probable underlying mechanisms. The results of this study suggest the testing of Vactosertib inhibitor to assess its effectiveness in containing loco-regional metastases in oral cancers.

Chapter 1

Introduction

Oral cancer is a global health concern owing to its poor prognosis and high mortality rate. In India, this is the most prevalent cancer and the leading cause of death due to cancer among the males, and the second most common cancer among females [1]. Despite advances in understanding the aetiologies and risk factors associated with oral cancers, the overall mortality rate and prognosis of this cancer type is poor and has remained unchanged over decades. Diagnosis at advanced stage is one of the factors contributing to this poor prognosis. Another significant contributor is the presence of regional lymph node metastases at the time of diagnosis. In most cases of oral cancers, patients present with the cancer spread to local lymph nodes in the neck region, thus diagnosing it at an advanced stage. These loco-regional metastases are largely responsible for therapy failure and relapses post treatment [2]. Hence there is an unmet need to understand the molecular mechanisms involved in metastases of oral cancers, and identify molecules or pathways that could be targeted to prevent or restrict this loco-regional spread of cancer. Targeting such pathways along with existing therapies would help achieve better loco-regional control, eventually improving the mortality rate and prognosis of this disease.

Several molecular pathways have been implicated till date in cancer metastases or processes underlying it, such as epithelial-mesenchymal transition (EMT), tumour cell invasion & migration and tumour cell-microenvironment interactions. Certain molecules and their downstream effector pathways have been reported to be both tumour promoting and tumour suppressive, in a cell-type or tumour-stage specific manner. One such pathway that is important in processes like EMT & cell migration and has been attributed a dual role in tumorigenesis is the Transforming Growth Factor-β (TGF-β) signalling pathway [3]. The TGF-β superfamily of secreted ligand molecules comprises of broadly four sub-families namely, TGF-βs, Activins/Inhibins, Growth & Differentiation Factors (GDFs) and Bone Morphogenetic Proteins (BMPs). All of these ligands are secreted

extracellularly, and exhibit their cellular functions by binding to heterotypic transmembrane receptor complexes to initiate a cascade of intracellular signalling through SMADs. Specific receptors and SMADs are activated downstream of each ligand, determining the specificity of each sub-family and the genes regulated by them. The TGF-β sub-family has been widely studied in cancers and has been demonstrated to be both tumour promoting and tumour suppressive in cell-context dependent manner [3]. The role of other three subfamilies in cancers is however not much explored.

Preliminary studies from our lab suggested a deregulation or differential expression of some of the Activin/Inhibin sub-family ligands in oral cancers. The Activin/Inhibin subfamily is itself comprised of two types of ligands, Activins and Inhibins, with opposing functions [4]. The secreted ligands of this pathway are dimeric molecules and the composition of the dimer determines the function. Monomers of this sub-family include an α monomer and four β monomers namely βA, βB, βC and βE. A dimer formed by an association between any two of the four β monomers is termed as an Activin. Activin (β-β) dimers bind to transmembrane receptors and trigger the phosphorylation of SMAD 2/3. These activated SMADs are TGF-β and Activin sub-family specific, and they further associate with a common SMAD4, to translocate to the nucleus and regulate transcription of specific genes. On the other hand, a combination of the α monomer with any one of the four βs generates an Inhibin dimer. This Inhibin (α-β) dimer binds to the receptors on cell membrane, but does not transduce any signal downstream, thereby blocking the Activin signalling [4]. Thus, the function (activation or inactivation status) of this self-regulatory pathway is dependent upon formation of Activin (β-β) or Inhibin (α-β) dimers; which in turn is dependent upon the relative expression of each monomer or their stoichiometry. The more abundant monomers expressed will form respective dimers to activate or inactivate the signalling through SMAD 2/3. Expression of some of

these monomers forming Activins/Inhibins, particularly βA, βB or α, has been demonstrated by individual studies to be deregulated in certain cancers [5-8]. Preliminary studies in our lab demonstrated a deregulation in the expression pattern of some these monomers in a few oral cancer cell lines. However, comprehensive studies evaluating the expression of all monomers together and demonstrating the functionality of this pathway are lacking and thus required to conclusively determine its deregulation in oral cancer. The aim of this study was to perform such a comprehensive expression analysis of all components of the Activin/Inhibin signalling pathway including ligand monomers, receptors and intracellular signal transducers, SMADs. We have used a panel of oral cancer cell lines and paired oral tumour tissues samples from oral cancer patients to analyse the deregulation in expression of this pathway.

Another aspect that remains unexplored is the regulation of Activin/Inhibin monomers expression. Since these molecules are shown to be deregulated in certain cancers, and our preliminary studies also suggest a differential expression in oral cancer, it was interesting to explore what regulates their expression in oral cancers. Through a parallel study ongoing in the lab, we observed that Activin βA monomer expression was deregulated upon p63 knockdown in oral cancer cell lines. P63 is a p53 family member, whose role in development and differentiation is well established [9]. Additionally, p63 has been demonstrated to promote tumorigenesis in several human tissues including oral [10,11]. P53, on the contrary, is a well-established tumour suppressor, known to be mutated in many cancers including oral [12,13]. Mutant p53 not only loses its tumour suppressive abilities, but also gains novel oncogenic functions [14,15]. One of the mechanisms of the gain-of-function of p53 mutants is by association with and utilization of other transcription factors to bind to regulatory regions on the DNA and differentially regulate gene expression. P63 has been reported to be such a ‘partner in crime’ for mutant

p53 [14.15]. Previous studies from our lab have also demonstrated the binding of mutant p53 with p63 in oral cancer cells. The observation that Activin βA levels altered post p63 knockdown in oral cancer cells harbouring a mutant p53, and the differential expression pattern of this Activin monomer in our cell line panel with differential p53 mutant and p63 expression background made us postulate that p53 and p63 could regulate Activin βA gene expression and possibly its oncogenic functions. We explore this hypothesis in the present study using combined expression analysis of Activin βA, p53 and p63 in oral cancer tissue samples and comparing their prognostic significance. The possible regulatory mechanisms by which p53 &/or p63 could govern Activin expression are also explored using oral cancer cell lines.

Activins have been implicated earlier to be promoting cancer-associated phenotypes [5,7,8]. However, their role in oral cancer and the downstream mechanisms by which Activins may promote loco-regional metastases remain largely unknown. To this end, we assessed the phenotypes affected by TGF-β and Activin pathways in oral cancer by attenuating these pathways using commercially available inhibitor and knockdown of essential signalling components. Apart from signalling through the SMAD molecules, Activins have also been demonstrated to regulate downstream non-canonical pathways such as p38- Mitogen-Activated Protein Kinase (MAPK), c-Jun N-terminal Kinase (JNK) and Akt-Phosphoinositide 3-Kinase (PI3K) pathways [16,5], specifically in the context of cancers. We thus test the contribution of canonical SMAD pathway and non-canonical MAPK pathways downstream of Activins in regulating these phenotypes in oral cancer cells.

Overall, this study aimed to comprehensively assess the expression of the entire Activin/Inhibin signalling pathway components and determine which Activins/Inhibins are differentially expressed in oral cancer. It also determines the association of

deregulated Activins with oral cancer stage and prognosis in patient samples. Further, this study explores the regulation of Activin expression by p53 family proteins, p53 and p63, and the impact of this regulatory axis on oncogenic functions in oral cancer. Lastly, the impact of canonical and non-canonical Activin signalling pathways on the phenotypes contributing to loco-regional spread of oral cancers is evaluated.

Chapter 2

Review of Literature

2.1.Oral Cancer

Oral or mouth cancers, are a subgroup of the head and neck cancers. They comprise of cancers of the oral cavity including those that develop on the buccal mucosa (inner epithelial lining of the lips and cheeks), oral tongue (movable front two-third part of the tongue), lips, alveolar ridge (space between the lips and gums or teeth), gingiva or gums, retromolar trigone (small area of gums behind the wisdom tooth), floor of the mouth and hard palate (Figure 1) [17]. Ninety percent of oral cancers are squamous cell carcinomas (OSCC) arising in the flat, squamous epithelial cells of the oral cavity. The remaining 10% comprise of less common subtypes such as malignant melanomas, jaw bones and soft tissue sarcomas and salivary gland cancers.

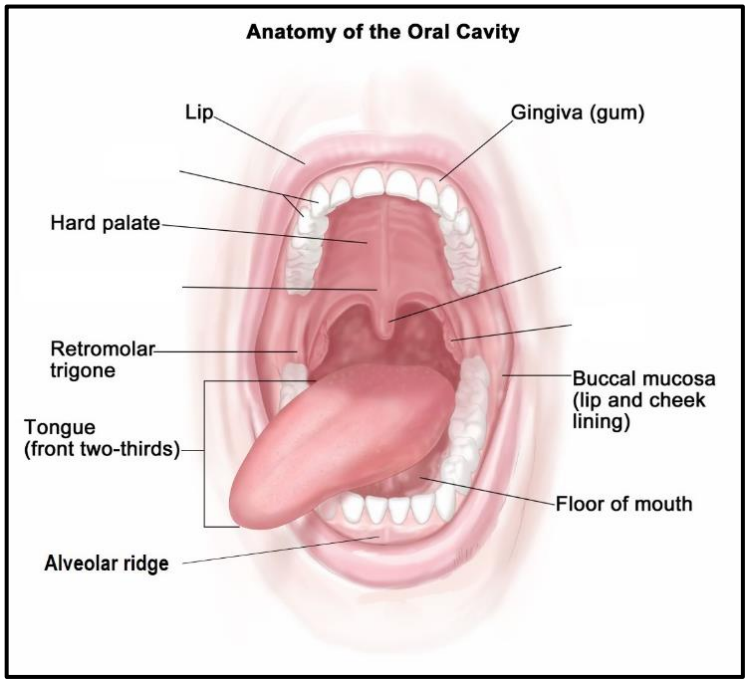

Figure 1: Oral Cancer Anatomical Sites

(Adapted from Terese Winslow, LLC, NCI) [17]

2.1.1. Incidence

Lip and oral cavity cancers rank fifteenth in terms of prevalence worldwide [1]. In terms of incidence and five-year prevalence rate, oral cancers rank second in India, considering both genders taken together [1]. As many as 1,19,992 new cases of lip and oral cancer have been estimated in India in the year 2018. They are also the second largest cause of death due to cancer in the country, with an estimated number of 72,616 deaths attributed to this cancer in 2018 [1, Figure 2]. Men are two to three times more susceptible to oral cancers than women [1]. When considered gender-wise, oral cancers are the most prevalent cancers among the male population in the country, with an estimated number of 92,011 new cases documented in 2018, while 50,812 deaths due to this cancer have been reported in that year, making it the leading cause of mortality due to cancer in the males. While in the female population, oral cancers rank fourth in terms of incidence, five-year prevalence and mortality due to cancer. As many as 27,981 new cases have been documented with 21,804 deaths due to this cancer among females in 2018 (Figure 2) [1].

2.1.2. Aetiology & Risk Factors

Tobacco-chewing or smokeless tobacco is the predominant aetiological or causative factor responsible for oral cancer in the south-east and south-central Asian countries. The habit of consuming tobacco in various forms such as chewing plain tobacco (Tambakoo), mixtures of tobacco with other ingredients such as areca nut/supari, slaked lime, catechu (Gutkha, Khaini, Paan Masala, Betel quid or Paan, Zarda, Mawa, etc.) or applying roasted tobacco powder as a dentrifice (Mishri) is highly prevalent in India; and the preferred form of tobacco

used varies among different socio-economic classes and regions [18,19]. Numerous carcinogens that have been identified in tobacco can be broadly categorized into (*i*) Tobacco-specific nitrosamines (TSNA) (from tobacco alkaloids during curing, fermentation and ageing); (*ii*) N-nitrosamine acids (from amino acids present in tobacco leaves amenable to N-nitrosation); (*iii*) Volatile N-nitrosamines; (*iv*) Polycyclic aromatic hydrocarbons; (*v*) Aldehydes (formaldehyde, acetaldehyde, acrolein, crotonaldehyde); and (*vi*) Other carcinogenic compounds (mostly heavy metals: cadmium, uranium and polonium).

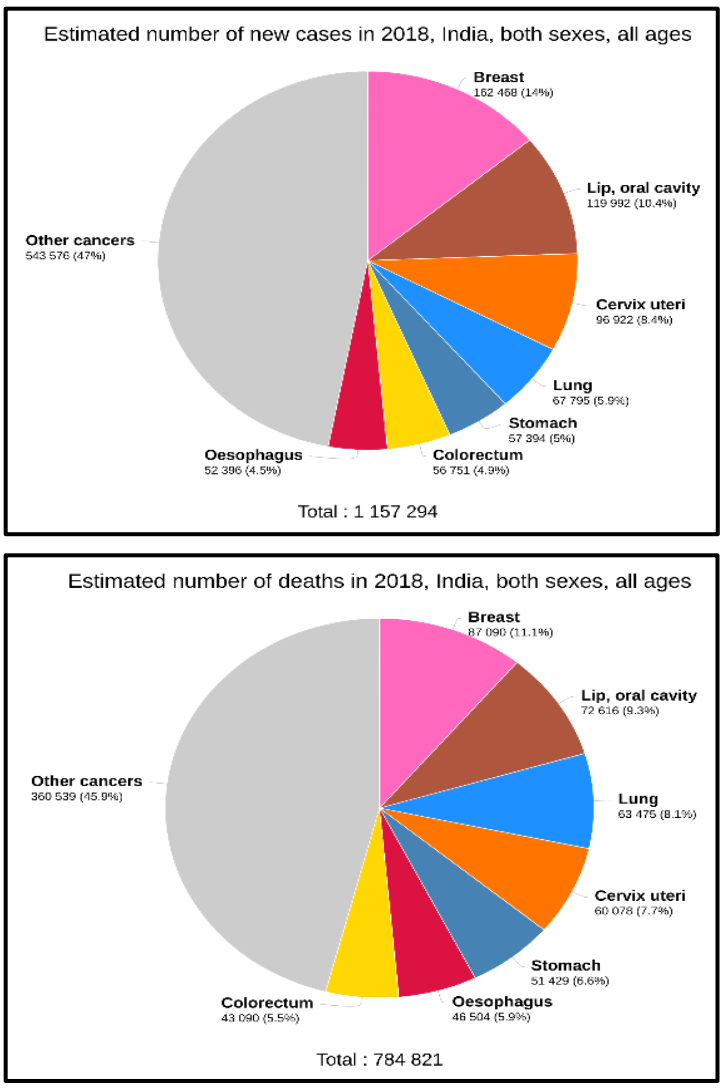

Figure 2: Incidence and Mortality of Lip and Oral Cavity Cancers in India (Adapted from Globocan Statistics 2018) [1]

Of these, tobacco-specific nitroamines (TSNA), mainly N'-nitrosonornicotine (NNN) and 4(methynitrosamino)-1-(3-pyridyl)-1- butanone (NNK) are the most potent and most abundant carcinogens in chewed tobacco [20]. These are formed by nitrosation of nicotine found in tobacco; are pro-carcinogenic in nature, are activated by metabolic enzymes in the body, and form DNA adducts to exert their carcinogenic effects [21].

Apart from smokeless tobacco consumption, smoked tobacco, alcohol consumption, areca nut/supari consumption, poor oral hygiene and ill-fitting dentures are other aetiological factors associated with the risk of developing oral cancer. Alcohol consumption along with tobacco usage potentially increases the carcinogenic effect of the latter. The most widely reported association with oral and oropharyngeal cancers is with that of Human Papilloma Virus (HPV) [22]. High-risk HPV subtypes, such as HPV16 and HPV18 among others, have been reported to express two major oncoproteins, E6 and E7, that degrade the tumour suppressor proteins p53 and Rb, respectively. This disrupts the cell cycle thereby leading to loss of control on DNA replication, repair and apoptosis, and promotion of carcinogenesis [22]. Additionally, other viruses have also been implicated in oral cancer development such as the human herpes virus (mainly Epstein– Barr virus (EBV)), human papillomavirus (HPV), and herpes simplex virus (HSV). The causal role for EBV and HSV to oral cancers has however been controversial, and these are more likely to be infections co-existing with oral cancers due to an immunosuppressed environment.

2.1.3. Pathophysiology

2.1.3.1. Field Cancerization

Oral cancer usually does not develop as an individual entity, but rather as a larger group of cells in the oral cavity being transformed simultaneously. The carcinogens from tobacco chewing that are released in and absorbed by cells of the oral tissue influence multiple cells at the same time, leading to induction of pre-cancerous and cancerous changes in them. These altered cells go on to develop tumours at varying rates thus resulting in multiple primary tumours in the oral cavity. This phenomenon of the entire oral cavity being chronically exposed to tobacco carcinogens and resulting in multiple tumours is termed as field cancerization [23]. It is one of the important factors contributing to recurrences, therapy failure and the poor prognosis associated with oral cancers. Oral tumours occur at an accessible site where surgical removal of the tumour is feasible and is routinely done, ensuring tumour free margins during surgery. However, this does not address the problem of second primary tumours arising due to field cancerization, thus leading to early relapses and a poor survival.

2.1.3.2. Oral Premalignant Disorders

In the Indian subcontinent, oral cancers are almost always preceded by unique oral potentially malignant or pre-malignant conditions, which are morphologically altered tissue regions in the oral cavity that may have a higher potential to develop carcinoma compared to the normal mucosa [24]. The development of oral carcinoma from normal oral mucosa includes these intermediary pre-cancerous lesions, mainly leukoplakias, erythroplakias and sub-mucus fibrosis. Leukoplakias (prevalence 1.1-11.7%) are white patches or

discoloration of the normal mucosa, while the far less common precancerous lesions called erythroplakias (prevalence 0.02-0.83%) are red patches on the normal mucosa. Sometimes, both conditions co-exist and is then termed as erythroleukoplakias. Both lesions have been associated with chronic tobacco usage, and although less common, erythroplakias are more likely to convert to malignancy or carcinoma (transformation rates vary between 14% - 50%) [24]. A pre-malignant condition strongly associated with chronic use of areca nut/supari is oral sub-mucus fibrosis. This is characterized by fibrosis of the mouth lining, difficulty in opening of mouth or jaw movement and difficulty in eating [24].

2.1.3.3. Molecular alterations in premalignant lesions and oral cancers

The conversion of normal oral mucosa into pre-cancerous lesions and thereon into malignant carcinoma is a multistep process involving a series of molecular alterations (Figure 3) [2]. These include genetic and epigenetic events that lead to oncogene activation, inactivation of tumour suppressors and genomic instability including loss of heterozygosity (LOH) and microsatellite instability (MSI), all of which are hallmarks of cancer [25]. Activating mutations, gene amplifications and overexpression of proto-oncogenes such as EGFR (Epidermal Growth Factor Receptor), c-Myc, Ras family proteins, Int-2, Hst, CCND1 and Bcl family proteins have been implicated in oral cancer development [2, 26]. Growth factors from the TGF family of proteins have also been reported to be deregulated in oral cancers [26]. Tumour suppressor TP53 is the most frequently inactivated gene in HNSCC and specifically oral cancers [13,26]. Tobacco use has been linked to p53 inactivation [12]. Point mutations, deletions or

rearrangements in p53 gene not only lead to the loss of its tumour suppressor activity, but may also confer gain of oncogenic functions to this molecule [13-15]. Other gene inactivations include that of SMAD4, a TGF-β / Activin regulated signalling protein [26]. Albeit, certain other genes from the TGF-β signalling pathway are rather found to be activated in oral and other cancers; thus, the role of this pathway in oral carcinogenesis is controversial.

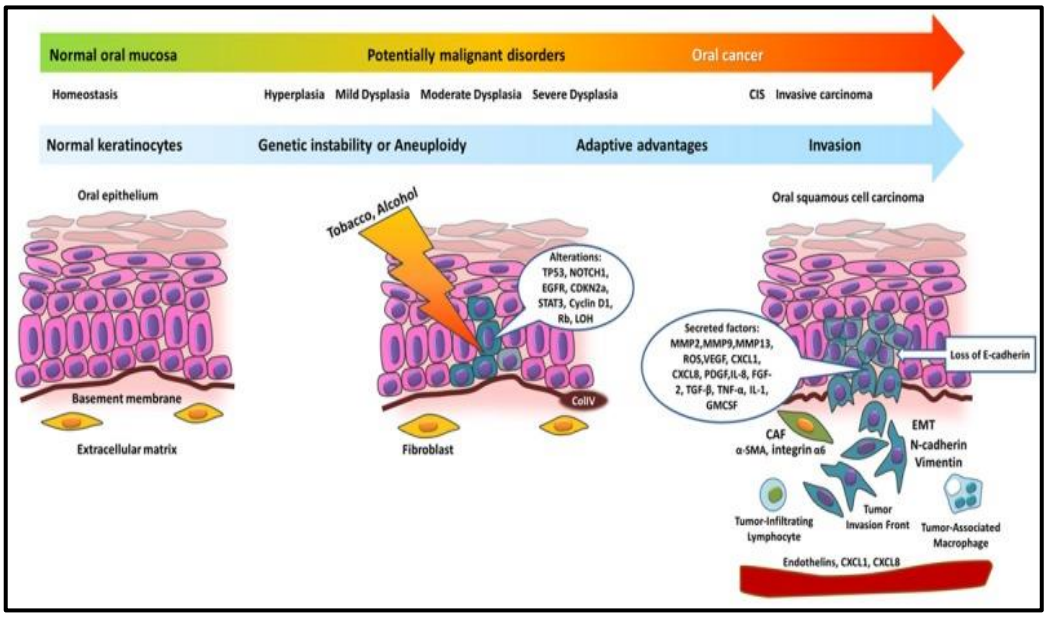

Figure 3: Molecular Alterations in Oral Cancers
(Adapted from Rivera C. 2015) [26]

2.1.4. Diagnosis & Staging

During a physical examination of the mouth, the doctor or the dentist examine the oral cavity and look for abnormal lesions such as red or white patches (erythroplakias or leukoplakias), ulcers, irritation or and burning sensation, and difficulty in opening of the mouth and difficulty in swallowing [27]. If a suspicious region is seen, a small part of it is excised with a cutting tool or needle (punch biopsy) to obtain tissue sample for biopsy. Biopsy, by far, remains the gold standard for a confirmatory diagnosis of the disease. It indicates whether

carcinoma or pre-malignant cells with potential to develop into a carcinoma are present in the tissue sample tested. Once the biopsy confirms the presence of oral cancer, additional tests including imaging techniques like CT, MRI or PET-CT scans and endoscopic techniques like panendoscopy could be performed as required to determine the extent of cancer metastases within and beyond the mouth cavity [27]. A detailed staging of the carcinoma is then done based on primary tumour characteristics and extent of loco-regional or lymph node and distant metastases, using the TNM staging system as follows [2]:

Table 1: TNM Classification system

T - Primary tumour	
TX	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Tis	Carcinoma in situ
T1	Tumour 2 cm or less in greatest dimension
T2	Tumour more than 2 cm but not more than 4 cm in greatest dimension
T3	Tumour more than 4 cm in greatest dimension
T4a (lip)	Tumour invades through cortical bone, inferior alveolar nerve, floor of mouth, or skin (chin or nose)
T4a (oral cavity)	Tumour invades through cortical bone, into deep/extrinsic muscle of tongue (genioglossus, hyoglossus, palatoglossus, and styloglossus), maxillary sinus, or skin of face
T4b (lip and oral cavity)	Tumour invades masticator space, pterygoid plates, or skull base; or encases internal carotid artery

N - Regional Lymph Nodes	
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in a single ipsilateral lymph node, 3 cm or less in greatest dimension
N2	Metastasis as specified in N2a, 2b, 2c below
N2a	Metastasis in a single ipsilateral lymph node, more than 3 cm but not more than 6 cm in greatest dimension
N2b	Metastasis in multiple ipsilateral lymph nodes, none more than 6 cm in greatest dimension
N2c	Metastasis in bilateral or contralateral lymph nodes, none more than 6 cm in greatest dimension
N3	Metastasis in a lymph node more than 6 cm in greatest dimension

M - Distant metastasis	
M0	No distant metastasis
M1	Distant metastasis

Based on the TNM criteria for oral cancer mentioned above (Table 1), the stage of an oral tumour is determined as indicated below (Table 2):

Table 2: Oral Cancer Staging based on TNM Classification

Stage grouping	T	N	M
Stage 0	Tis	N0	M0
Stage I	T1	N0	M0
Stage II	T2	N0	M0
Stage III	T1, T2	N1	M0
	T3	N0, N1	M0
Stage IVA	T1, T2, T3	N2	M0
	T4a	N0, N1, N2	M0
Stage IVB	Any T	N3	M0
	T4b	Any N	M0
Stage IVC	Any T	Any N	M1

Apart from the TNM staging as shown above, tumour grading is simultaneously determined, popularly by Broder's classification system that categorizes a tumour

based on its degree of differentiation and keratinization into well, moderately, poorly differentiated and anaplastic carcinoma or Grades I to IV respectively [28].

2.1.5. Treatment & Prognosis

Treatment for oral cancers depends upon the site of tumour, stage and grade of tumour including lymph node involvement and distant metastases. It also considers the patients' nutritional status, existing co-morbidities, and their will and ability to tolerate treatment [2]. For early stage and resectable oral tumours, the primary treatment modality is surgery and radiotherapy. Tumours at early stage, those of a smaller size and those that have not metastasized to regional lymph nodes are candidates for surgical excision. Radiotherapy could be either provided as external beam or brachytherapy (interstitial implantation of radiation source). A combination of the multiple modalities is used for treating advanced stage tumours including co-adjuvant chemotherapy alongside surgery and radiation. Commonly used chemotherapeutic drugs include cisplatin, carboplatin, 5-fluorouracil, paclitaxel and docetaxel [17].

About one third of oral cancers are diagnosed at stage I/II, where surgery or radiotherapy or a combination of the two is used to treat these patients, and a cure rate of about 80% for stage I and 65% for stage II is expected. However, most OSCC cases are diagnosed and treated at advanced stage III/IV, where surgery, radiation and chemotherapy are used in combination and the cure rate goes down to only 30%, with a 5-year survival rate of less than 50% [2]. Patients with metastatic tumours show a survival of only about 4 months if left untreated [2].

Overall, factors such as advanced stage at diagnosis with presence of loco-regional lymph node metastases, and therapy failure due to frequent recurrences on account of multiple primary tumours (that are initiated as a result of field cancerization) are majorly responsible for such a poor prognosis of this disease.

2.2. Processes underlying Cancer Metastases

Metastases or the spread of cancer is a crucial step in carcinogenesis and is an indicator or poor prognosis. 'Activation of Invasion and Metastases' is one of the hallmarks of cancer (Figure 4) [25]. It involves three important processes that occur at the tumour cell or tissue level namely Epithelial-Mesenchymal Transition (EMT), cell invasion and cell migration; whereas the interaction of these tumours with their microenvironment is also crucial for the process of metastases to occur.

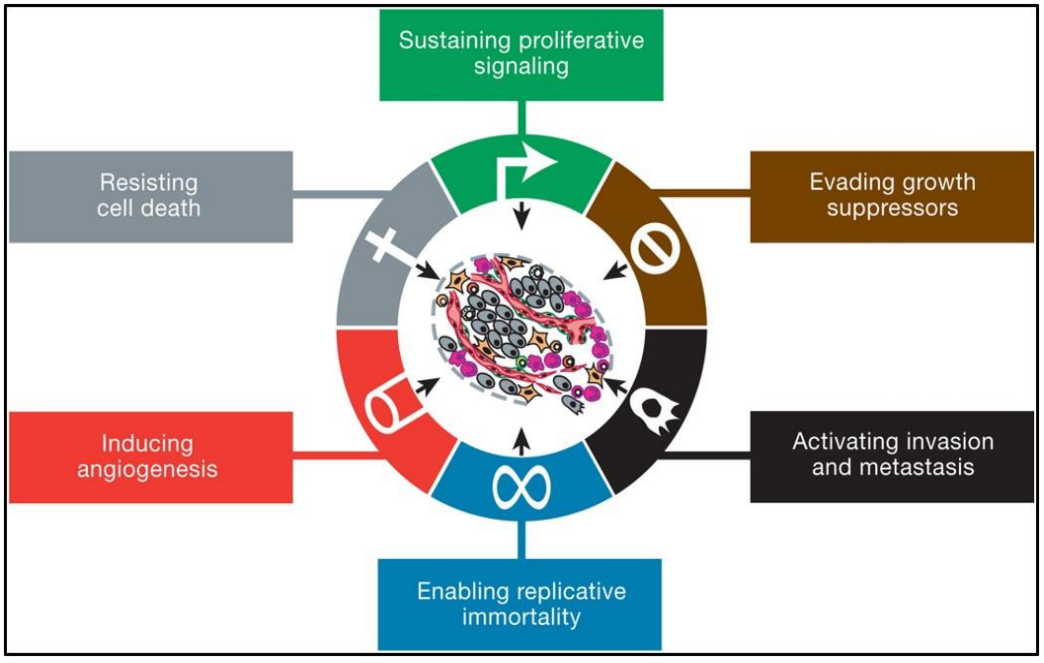

Figure 4: Hallmarks of Cancer
(Adopted from Hanahan & Weinberg, 2011) [25]

2.2.1. Epithelial-Mesenchymal Transition

Epithelial cells are characterised by an apical-basal polarity, where one end of these cells is attached to the basement membrane whereas the other apical end faces the lumen. These cells are held together to the basement membrane and to each other by tight junctions, adherent junctions and desmosomes. The loss of such cell polarity in epithelial cells to gain a mesenchymal phenotype that has lost its attachment to the basement membrane, and is thus more invasive and migratory, is termed as epithelial to mesenchymal transition (EMT) [29]. It is the de-differentiation of terminally differentiated epithelial cells into stem cell-like populations and further re-differentiation into mesenchymal lineage. EMT is one of the first events that possibly occurs during metastases, which renders the cancerous cells more invasive and migratory. Various molecules have been implicated in this process, and include activation of several transcription factors, reorganization of the cytoskeletal proteins, extracellular matrix remodelling, and changes in expression of several micro RNAs (miRNAs) and cell surface proteins [29]. Several signalling pathways including the TGF-β signalling, Integrins, Notch and Wnt/β-catenin pathways have been implicated in EMT. These pathways alter the expression of transcription factors such as Snail, Slug, Twist-1, Zeb-1 and TCF3, that repress the levels of E-cadherins, a marker protein for epithelial cell type. This is also associated with increase in N-cadherin and Vimentin expression, both mesenchymal markers. Tumour microenvironment derived secreted cytokines such as TGF-β and IL-6 have been shown to induce EMT, cell invasion and metastases in oral and head and neck cancers among others [30]. Integrins and the PI3K and MAPK pathways have also been implicated in inducing EMT in oral cancer cells [31].

2.2.2. Cancer Cell Invasion

The loss of cell-cell adhesion during the process of EMT results in cancer cells dissociating from the primary tumour. Further, the remodelling of ECM results in altered interactions between cancer cells and the matrix, enabling the cells to dissolve the surrounding stroma and penetrate or invade it, a process termed as cell invasion [32]. Cancer cells that have undergone EMT are mesenchymal-like and more migratory and invasive. They become elongated and also develop actin-rich protrusions of the cell membrane called invadopodia that help them penetrate the stroma [32]. The invaded cancer cells can undergo intravasation into the surrounding lymphatic and blood vessels, migrate through the lymph or blood, extravasate to distant organs with a niche, and form secondary tumours at the new site, thus leading to local or distant organ metastases. A process called Mesenchymal-Epithelial Transition (MET), which is the reverse of EMT, takes place at the metastatic tumour site thus establishing the new metastatic tumour that resembles the primary tumour origin and characteristics [32]. Tumour invasion is an indication of higher stage and is associated with poor prognosis. Several molecular pathways including the ones mentioned above that aid during EMT also contribute to cell invasion. In oral cancers, PI3K-AKT, FAK and TGF-β signalling has been implicated in promoting tumour cell invasion and metastases [31,33,34]. One of the important players that these pathways regulate are the Matrix Metalloproteases (MMPs) that are responsible for ECM remodelling.

2.2.3. Cancer Cell Migration

Cancer cell motility or the directed chemotactic movement of cancer cells towards specific chemical cues or signals in the stroma and with the help of invadopodia is a prerequisite for metastases. This process termed as cell migration occurs in response to cytokine and chemokine signals secreted from the cancer cells itself (autocrine action) as well as from the surrounding stromal cells (paracrine action) [32]. Several cytokines from the TGF-β / Activin protein family have been reported to be crucial in this process [32, 34]. In response to such chemotactic signals, cytoskeletal reorganization takes place in cancer cells that allows them to migrate towards the chemical signal, thus creating and following migratory tracks towards intravasation into lymphatic or blood vessels.

2.2.4. Tumour Microenvironment

The non-epithelial cells and stroma surrounding the tumour and holding it in is termed as tumour microenvironment (TME). This includes the extracellular matrix (ECM) and cellular components like the cancer-associated fibroblasts (CAFs), endothelial cells, macrophages and other immune cells. TME plays a crucial role in initiating and promoting cancer metastases [35]. Many of the chemical signals and physical cues that induce cancer cell EMT, migration and invasion originate from the tumour microenvironment. TGF-β / Activin/ BMP family of cytokines and growth factors are one of the most important and predominant molecules secreted by the stromal cells, that act in a paracrine manner on neighbouring cancer cells to induce a metastatic phenotype [35].

2.3. TGF-β Superfamily and Activins / Inhibins

Transforming Growth Factor-β superfamily of proteins comprise of cytokines and growth factors that play crucial roles in development and homeostasis [36]. These molecules are important players in diverse cellular functions such as growth, adhesion, migration, apoptosis and differentiation. Ligands of this group act as morphogens during embryo development, forming a concentration gradient and regulating cell phenotypes in a dose-dependent manner. They regulate cell proliferation, differentiation, migration, angiogenesis, wound healing and survival [37]. It is this very role of these molecules that is hijacked by cancer cells and subverted into oncogenic functions during tumorigenesis and metastases. The TGF-β superfamily comprises of over 30 members classified further into subfamilies including TGF-βs, Bone Morphogenetic Proteins (BMPs), Growth and Differentiation Factors (GDFs), Activins/ Inhibins, and Nodal [36]. The focus of this study is the Activins / Inhibins subfamily, that is described in greater details hereon.

2.3.1. Activin / Inhibin Subfamily

This subfamily of TGF-β superfamily comprises of two types of ligands: Activins and Inhibins. These ligands are secreted dimeric proteins composed of the α and β monomers or subunits. A single α subunit has been reported in humans, while four β subunits namely βA, βB, βC and βE have been reported till date in humans. Each of these subunits is encoded by a different gene, and the four β subunits share about 60-75% homology at the amino acid level [36]. A heterodimer of the single α subunit with any one of the four β subunits is termed as an Inhibin, and is named after the distinct β subunit component. For example, a heterodimer of α

and βA subunits is termed as Inhibin A, that of α and βB as Inhibin B and so on. While homo- or heterodimers composed of only β subunits are termed as Activins and named after the subunits they are composed of. For instance, the homodimers of βA-βA and βB-βB are termed as Activin A and Activin B respectively, while the heterodimer of βA-βB is termed as Activin AB, and so on [4]. These subunits are expressed in the cell as proproteins that are glycosylated by Furin-related proprotein convertases in the endoplasmic reticulum [4,38]. They are dimerized and further cleaved to be secreted as mature protein dimers. All of these dimers are linked by a single disulphide bond between the α and β subunits in Inhibins or between two β subunits in Activins. The mature secreted Activin dimers are linked by a single disulphide bond at cysteine 80 residue [4]. The mature form of Inhibin dimers thus secreted could be non-glycosylated, mono-glycosylated or di-glycosylated; while the mature Activin dimers secreted are non-glycosylated [4].

2.3.2. Canonical Activin Signalling Pathway

Activin ligands or the β-β dimers are the signalling ligands of this pathway; they bind to transmembrane receptors and initiate a cascade of signal transduction [4]. Two types of Activin receptors are present on the cell membrane, Activin Receptor Type I (ACVR1) and Activin Receptor Type II (ACVR2), of which ACVR2 are ligand binding receptors while ACVR1 transduce the signal further upon ligand binding to ACVR2. There are two ACVR2 receptors shown to bind to Activin dimers, ACVR2A and ACVR2B. ACVR1B, also known as Activin-Like Kinase 4 (ALK4), is the predominant ACVR1 type utilized by Activins. When the Activin dimer binds to ACVR2A/2B receptor, it triggers the auto-phosphorylation and hetero-tetramerization of ACVR1B / ALK4 with

ACVR2A/2B. Upon tetramerization, this ALK4 receptor induces phosphorylation of downstream signalling proteins, SMADs. The term SMAD was coined from the '*Sma'* gene from *C. elegans* and the '*mothers against decapentaplegic'* genes from *Drosophila*, based on homology of SMAD proteins to these two genes. Several SMAD proteins have been reported in humans, of which SMAD2 and SMAD3 are the Activin-responsive or Activin and TGF-β specific SMADs. SMAD4, also called as companion SMAD, is the indispensable SMAD in the Activin signalling cascade [39]. The Activin ligand binding to ACVR2A/2B and consequent tetramerization and activation of kinase activity of ACVR1B / ALK4, eventually leads to phosphorylation, activation and release of membrane anchored SMAD2 and SMAD3. These activated SMADs interact with companion SMAD4, and this complex further translocates to the nucleus. Interacting with other transcription factors, SMADs then regulate transcriptional activity of target gene promoters [4]. This canonical Activin-ACVR2-ALK4-SMAD2/3/4 signalling cascade has been illustrated in Figure 5 [40].

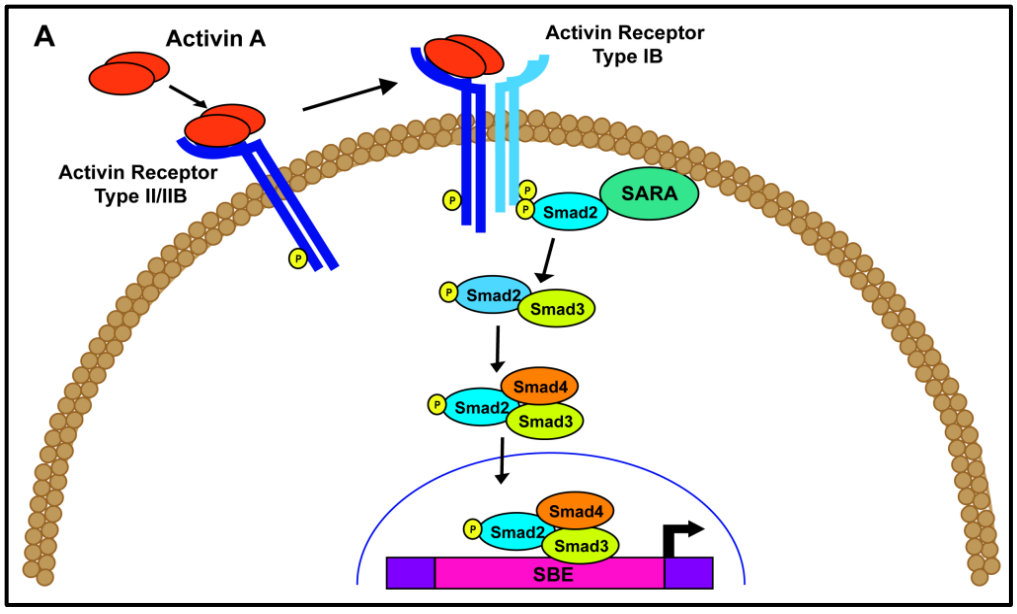

Figure 5: Activin Canonical Signalling Pathway
(Adopted from Loomans et al. 2015) [40]

Inhibin α-β dimers, on the contrary, bind to the same ACVR2A/B receptors as Activins; however, they form inert complexes that do not transduce any signal downstream, leading to the inhibition of this pathway. This serves as a negative regulatory event that balances the Activin signalling. Another level of regulation of this signalling is associated with the SMAD proteins. SMAD7, also known as inhibitory SMAD, inhibits the phosphorylation of SMAD2/3, thereby inhibiting Activin signal transduction [4].

2.3.3. Antagonists of Activin Signalling

Apart from intrinsic signalling molecules such as Inhibins and inhibitory SMAD6/7, certain secreted extracellular molecules also act as antagonists for the Activin-SMAD canonical pathway. Follistatin (FST) and Follistatin-Like 3 (FSTL3) are two such important candidates. Both FST and FSTL3 bind to Activin dimers and sequester them, thus preventing their binding to cell surface receptors ACVR2, consequently inhibiting the SMAD signalling pathway [41]. Another antagonist of this pathway is Cripto-1, a cell surface molecule that binds to Activin receptor ACVR2 and to Activin dimers to form an inert complex that does not transduce any signal further in the cell, thereby inhibiting the signalling [41]. BMP Activin Membrane-Bound Inhibitor (BAMBI) also negatively regulates Activin-SMAD signalling by acting as a pseudo-receptor and inhibiting the formation of Activin-Receptor complexes [41].

2.3.4. Non-canonical Activin Signalling Pathways

In addition to the canonical SMAD signalling, Activins also signal in a SMAD-independent manner. Activin-ACVR receptor complexes have been reported to

activate p38-MAPKs (Mitogen Activated Protein Kinases), ERK1/2 (Extracellular signal-regulated Kinase 1/2) and JNK (c-Jun N-terminal Kinase), in a cell-type specific manner [31]. Activin type I receptor ALK4 and SMAD2 complex, independent of the companion SMAD4, regulates Wnt/β-catenin pathway. Activins also crosstalk with the PI3K/AKT signalling independently of SMADs [42]. These non-canonical signalling pathways downstream of Activin receptor binding increase the complexity of the system and also contribute to Activin oncogenic functions, as discussed in detail later. Figure 6 represents a schematic of non-canonical Activin Signalling Pathways [40].

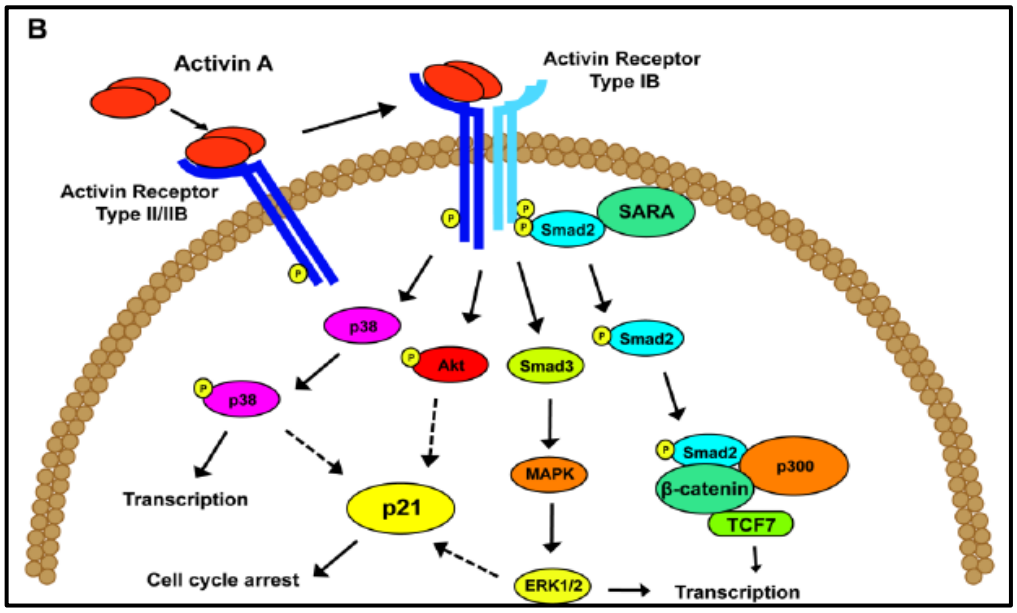

Figure 6: Activin Non-canonical Signalling Pathways (Adopted from Loomans et al. 2015) [40]

2.3.5. Physiological roles of Activins / Inhibins

Activins were initially isolated from follicular fluids and identified as molecules stimulating Follicular Stimulating Hormone (FSH) secretion from the pituitary gonadotrophs, while Inhibins inhibited this secretion [42]; and were hence named these. They were also therefore earlier described to be reproductive hormones.

With decades of research on these molecules, varied physiological and also pathological functions of these molecules were unravelled.

2.3.5.1.Development and Reproduction

It is now established that Activin signalling plays important roles in folliculogenesis, spermatogenesis and pregnancy [42]. Additionally, the developmental roles of Activins are also well established. Activins function in regulating mesoderm induction, left-right symmetry or axis determination, patterning, embryonic ovary and follicular development, testis development, and luteolysis, and the degradation of corpus luteum [42]. It is now known that Activins not only stimulate secretion of FSH from the pituitary, but are involved in its direct transcriptional activation as well as indirect trans-activation via modulating functions of gonadal steroids, glucocorticoids and Gonadotropin Releasing Hormone (GnRH). It also stimulates the transcription of luteinizing Hormone (LH) and GnRH receptor, together supporting a strong role in neuroendocrine control of reproduction [42]. Activins regulate primordial follicular pool size during female embryogenesis that determines adult stage fertility and premature ovarian failure. In adult ovarian tissues, Activins are predominantly expressed in granulosa cells, where they are shown to synergise with FSH actions and estrogen signalling to regulate granulosa cell proliferation, differentiation and steroidogenesis [42]. Activin A deficient mice are born with craniofacial and other developmental defects and die neonatally. Activin B deficient mice survive and are fertile, their offspring however, are perinatally lethal, probably due to insufficient lactation [42]. Thus, both Activins are important in ovarian and developmental functions and Activin A replacement in

Activin B deficient mice suggested that it is functionally dominant over Activin B. Activins secreted from luteinized granulosa cells stimulated MMP-2 expression by fibroblast-like cells, where MMP-2 acts as an important luteolytic agent, thus supporting the role of Activins in luteolysis [42]. Activin A is known to be expressed in the breast mammary glands, predominantly in ductal and lobular cells. They have been described to regulate growth, differentiation and morphogenesis of these primary or transformed mammary epithelium [43]. Activins regulates testes size during early testicular development by promoting Sertoli cells proliferation and thus controls fertility in adult males [42,44].

2.3.5.2.Stem Cell Differentiation

Along with other TGF-β family proteins, Activins signalling through the downstream SMAD2/3 molecules have been shown to be crucial in maintaining pluripotency and self-renewal of human embryonic stem cells (hESCs) [42]. Activin signalling co-operates with FGF signalling to regulate the expression of stem cell genes such as Nanog, Oct-4 and Sox-2 [42]. Inhibition of Activin signalling results in hESCs differentiation, and reapplication of Activins induced endoderm/mesoderm patterning. Specifically, the endodermal and mesodermal lineage cell types induced by Activins include neuroectoderm, hepatocytes, insulin-secreting β-cells and cardiomyocytes among others [42].

2.3.5.3.Glucose Metabolism

Activin A signalling has been shown to regulate pancreatic β-cell proliferation, insulin production in response to glucose availability, serum insulin levels and glucose tolerance. On the contrary, Activin B, probably functioning through the ALK7 receptor instead of ALK4, negatively regulated insulin production and

secretion in β-cells. Activins were also shown to influence the replication and differentiation of islet cell progenitors located in ductal epithelium. In the α-cells however, Activins decreased glucagon gene expression. Together, Activins may act opposite in α and β cells in pancreas, to eventually enhance glucose uptake and reduce its synthesis, actions that aid in relieving diabetes symptoms [42].

2.3.5.4.Immune Responses, Inflammation and Wound Healing

Activins and other TGF-β components are important regulators of inflammatory response. Serum Activin levels significantly increase during sepsis and are associated with release of other pro-inflammatory cytokines [42]. Monocytes, macrophages, dendritic cells, T and B lymphocytes, and mast cells all express Activins and its signalling components; their expression is further induced upon immune stimuli. Dendritic cell secreted Activins inhibit production of cytokines and chemokines to prevent their uncontrolled release and regulate recruitment of immune effectors in the local microenvironment. Activins also induce macrophage activation and production of IgG and IgE from B cells [42]. Activin expression is also elevated in inflamed and wounded tissues, where it actively participates in wound healing process. Activins have been implicated neuroprotective roles in response to brain injury, infection in the cerebrospinal fluid (CSF), and ischemia or stroke, where its role in inflammatory and immune responses is utilized [42].

2.3.5.5.*Cell Migration*

Multicellular organisms require coordinated movement or migration of cells for their generation and sustenance; for example, during gastrulation where the three germ layers attain specific relative positions within the embryo, or during physiological and pathological angiogenesis where endothelial cells from existing blood vessels move or migrate to form new vasculature, or during would healing and tissue repair to maintain homeostasis or during inflammatory and immune responses. Cell migration underlies all such processes, and occurs with the help of expression of morphogens, that in a dose dependent manner, guide migrating cells through their trajectories. This chemotaxis in eukaryotic cells, unlike bacterial chemotaxis, involves multi-step signalling that starts with the morphogen signal and initiates a cascade of signalling events in the cells, culminating into actin polymerization and cytoskeletal rearrangements, processes necessary for cell migration. Several growth factors, cytokines and chemokines have been reported to be involved in migration, including the TGF-β and Activin ligands. Activins can regulate cell migration both by canonical SMAD and the non-canonical MAPK signalling pathways [45]. Activins also induce directed migration of immature dendritic cells, another immune cell type. Along with the immune cells mentioned herein, Activins also regulate migration of non-immune cells. Activin signalling induces cytoskeletal changes that help cell motility. A change in the cytokeratin expression pattern is induced and genes such as Vimentin, α-Smooth Muscle Actin are expressed under the influence of Activin signals [45]. Besides, the differentiation of cells is also regulated by Activins in a manner that promotes EMT. Activin-induced expression of Snail, Slug, Twist-1 and Zeb-1 transcription factors facilitates the transition from epithelial to

mesenchymal cell lineage. The acquirement of a mesenchymal phenotype endows enhanced migratory capacity of these cells [45,46]. Such Activin-regulated EMT is essential during embryo development and also forms the basis of pathological EMT during tumour metastases. Activins also influence cell-cell adhesions and interaction between cells and their microenvironment. These properties are altered in pathological conditions such as cancer, where the physiological function of Activins in such processes is redirected to attain a migratory or metastatic tumour. The role of Activin signalling in tumour cell migration is emphasized in subsequent sections.

2.4. Activins / Inhibins in Cancer

2.4.1. Ovarian Cancer

Given the importance of Activin signalling in ovarian physiology, deregulation in the expression and function of components of this pathway is prominently seen in ovarian cancer. In comparison to normal ovarian epithelium, ovarian cancer over expresses Activin A; its higher expression being associated with poor prognosis in advanced and high grade serous ovarian cancers. Activin A secreted from ovarian cancer cells promotes stromal fibroblast activation through the canonical SMAD2 signalling [47]. Activin and TGF-β signalling induced EMT in both normal ovarian epithelium and ovarian cancer. Additionally, Activin signalling via SMAD2/3 induces expression of Snail and MMP-9, that promoted migration and invasion of ovarian cancer cells [48]. Another mechanism by which Activin A mediates ovarian tumorigenesis is by activating AKT and repressing GSK, thereby promoting cellular proliferation [5]. The role of Inhibins in ovarian cancer is, however, complicated. Inhibin α subunit deficient mice

develop granulosa cell tumours, suggesting that Inhibin α is a tumour suppressor gene [6]. Contradictorily, Inhibins are secreted by ovarian tumours [49]. Inhibin forming α subunit, or Inhibin A or B dimers containing this α subunit are expressed by certain types of ovarian tumours such as granulosa cell tumours and mucinous carcinoma, and their serum levels are used in diagnosis of ovarian cancer and to monitor its recurrence. Diagnostic tests routinely examine serum Inhibin levels along with the predominant ovarian cancer antigen, CA125 [50]. Mutations in both Inhibin α gene (INHA) and Activin βA gene (INHBA) have also been reported in ovarian cancers [51].

2.4.2. Endometrial and Cervical Cancer

In women with uterine and cervical cancer, serum Activin A levels were found to be significantly elevated, which decreased upon surgical removal of the endometrial or cervical tumours, suggesting that the tumours were the source of secretion [52]. Inhibin α subunit expression was demonstrated to be an independent prognostic marker in endometrial carcinoma [53]. An interesting study that compared two cervical cancer cell lines, HeLa and SiHa, demonstrated that activation of differential pathways, MAPK and SMAD respectively, occurs downstream of TGF-β treatment; and that this leads to differential effects of TGF-β treatment. The HeLa cells show enhanced proliferation due to activation of MAPK pathway that was independent of SMAD activation, while in SiHa cells, same treatment leads to SMAD activation and anti-proliferative effect (54).

2.4.3. Breast Cancer

In a study done on T47D breast cancer cell line, Activin A was shown to induce both canonical SMAD and non-canonical p38-MAPK pathways, leading to a growth inhibitory effect on these cells [55]. In another study, Activin βA levels were shown to be higher in local and metastatic breast cancer tissues compared to normal mammary tissue. Elevated serum Activin A levels were also found in women with breast cancer, and the levels depleted within the first two days after surgical excision of the tumours, suggesting these tumours to be secreting the Activin A systemically [55]. In MCF7 breast cancer cells, Activin and Estrogen pathways were shown to intersect, both dually repressing each other to suppress their transcriptional programs. The presence of estrogen suppressed Activin signalling, thus suppressing a growth arrest signal and promoting cell growth (estrogen responsive). However, if the estrogen receptors (ER) are lost, Activin signalling can resurface and induce downstream molecules such as Plasminogen Activator Inhibitor-1 (PAI-1), that induces invasion of breast cancers (56). On similar lines, Activin signalling was demonstrated to be hyperactivated in advanced stage breast tumours, indicated by higher Activin A, phospho-SMAD2 and phospho-SMAD3 levels. Further, Activin-SMAD signalling was also shown to promote anchorage-independent growth, stemness, EMT, invasion and angiogenesis of breast cancer cells, and tumour-forming ability and metastatic colonization of breast cancer in nude mice [7]. Earlier, microRNA-181 (miR-181) had been shown to act as a metastamir in breast cancer, promoting metastases downstream of Activins and TGF-β pathways [57]. Taken together, the general notion is that Activins and TGF-β signalling pathways are tumour-suppressive

during early stages of breast carcinogenesis, while they acquire an oncogenic role in advanced stage tumours, where they promote metastases.

2.4.4. Activins in HNSCC and Oral Cancer

In vivo gene expression profiling, assisted by laser captured microdissection, performed on five paired oral normal-tumour tissues detected significant Activin A upregulation in oral tumours [58]. A study done using eight HNSCC cell lines, six of which were of oral SCC origin and remaining two were from the pharynx, investigated DNA amplifications using chromosomal comparative genomic hybridization (CGH) as well as transcriptional alterations using microarray. This study found DNA copy number gains in 7p chromosomal region, along with others. The 7p14 locus carries the gene for Activin A (INHBA), as mentioned earlier. They also found a positive correlation between genomic amplification and transcriptional expression of the INHBA gene in the tumour tissues, as indicated by upregulation of Activin A transcripts. Activin A overexpression was further found to be associated with shorter disease-free survival in these patients, indicating it to be a prognostic factor in HNSCC [59]. A transcriptomic profiling of fifty-three tongue squamous cell carcinomas in comparison to twenty-two normal tissues also indicated an upregulation of Activin A (INHBA) gene [60]. Transcriptomic profiling of oral cancer parental cell line compared to its highly invasive subline counterpart indicated an upregulation of Activin B gene (INHBB) in the latter [61]. Another study that assessed transcriptome scale differences in nine oral tumours compared to a single normal gingival tissue also found significant upregulation of Activin A (INHBA) gene in the tumour samples [62]. All of these were transcriptome scale studies that indicated an upregulation

of INHBA or INHBB genes individually or the Activin / Inhibin forming βA or βB subunits alone, without the knowledge on the expression of other subunits that are required to make functional dimers other than Activin A or B homodimers. Whether the upregulated INHBA gene product, that is, βA subunit forms a homodimer with itself or heterodimers with other βs to form Activins or with the α subunit to form Inhibin A, was not addressed by these studies. To answer this question and to determine the functional dimer formed and their stoichiometry or amounts, a comprehensive study that determines the expression and relative proportions of all of these subunits simultaneously in any given model system is required. Also, some of the above-mentioned studies were suggestive of an oncogenic function for INHBA, and probably for INHBB, direct evidence for this and the mechanisms underlying these functions remained largely unknown.

More detailed studies with larger patient cohort and direct experimental evidence for the oncogenic functions of INHBA gene in oral cancer emerged lately. The first study compared the protein expression of Activin βA subunit and Follistatin, the antagonist of Activin signalling, in ninety-two paired oral normal-tumour tissue samples by Immunohistochemistry (IHC); and the serum levels of these two molecules in one hundred eleven OSCC patients and ninety-one healthy controls by Enzyme-linked Immunosorbent Assay (ELISA) [63]. An overexpression of Activin βA protein was observed by IHC in tumour tissues compared to normal counterparts, and it correlated with positive N stage, poor histological differentiation, perineural invasion, and poor survival. Follistatin expression was however, not prognostic. The serum levels of neither of these proteins were altered in OSCC patients compared to healthy controls. Further, a

transient knockdown of Activin βA subunit expression in OC3 oral cancer cells suppressed cell proliferation, migration and invasiveness, suggesting an oncogenic role for this molecule [63]. A similar study that compared seventeen OSCC tumour samples with eleven normal oral mucosa samples by quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR), and one hundred and fifteen OSCC tumour tissues by IHC, for expression of Activin βA subunit also reported its over expression in tumours that was associated with lymph node metastases, tumour differentiation and poor survival. Further, using stable knockdown of Activin βA expression, and treatment with recombinant Activin A and Follistatin proteins, Activin βA was shown to be regulating apoptosis, proliferation, EMT, migration and invasion in oral cancer cells [64]. A recent and the only study till date that assessed INHBB or Activin βB subunit expression in nine OSCC cell lines compared to human normal oral keratinocytes, and in one hundred and three OSCC paired normal-tumour tissues, by qRT-PCR, western blotting and IHC, found an upregulation of Activin βB and its association with regional lymph node metastases. Further, Activin βB knockdown promoted cell adhesion, regulated EMT and suppressed cell invasion and migration [8]. However, the three studies mentioned above assessed expression of either βA or βB subunits individually, not simultaneously or along with other subunits that are important in determining functions, for example the Inhibin forming α subunit.

2.5. P53 family proteins

The tumour suppressor gene TP53 is the most frequently altered gene in oral cancers. P53 gene alterations have been reported in about 46% of oral cancers in India [12]. These include point mutations, loss of heterozygosity (LOH), overexpression or stabilization of mutant p53 and degradation of wild-type p53 by HPV E6 antigen. About 92% concordance between missense p53 mutations and p53 protein overexpression has been reported [12], indicating that mutation in this gene could stabilize the protein. The most prevalent mutations of p53 in oral cancer or HNSCC include those in codon 248 (R248W), codon 273 (R273H) and codon 175 (R175H), the first two being DNA binding mutants and R175H being a structural mutant of the DNA binding region of p53 [14,15]. Interestingly, these p53 mutants not only lose their tumour suppressive activities, but some also gain novel oncogenic functions. The mutant p53 may lose its DNA binding ability; one of its gain-of-function mechanisms include its binding to and utilization of other transcription factors to bind to gene promoters [14,15]. One such partner protein that mutant p53 can bind to is its family member, p63 [14]. P63, which is also a transcription factor, is overexpressed in many cancers including HNSCC and oral cancer, and is demonstrated to be oncogenic in function [9-11]. P63, acting as a transcription factor, can transregulate p53 target genes by binding to p53 response elements on the gene promoters [9]. Two isoforms of p63 have been described and they function differentially; the full length TAp63 isoform that retains the N-terminal transactivation domain is reported to be tumour-suppressive in function, while the ΔNp63 isoform that lacks the N-terminal transactivation domain is known to be oncogenic in function. The ΔNp63 isoform is predominantly expressed over the TAp63 isoform in oral cancers [10].

2.5.1. Functional similarities between p63 and Activin

P63 is predominantly expressed in keratinocyte stem cells and is indispensable for epidermal differentiation. It is required to maintain keratinocytes in the immature or undifferentiated form [65]. ΔNp63 acts as a transcriptional repressor of neuronal fate in zebrafish and determines ventral specification in gastrulating embryos and in the ectoderm [66]. ΔNp63 is essential for maintaining basement membrane integrity and terminal differentiation of keratinocytes, and mice lacking this isoform of p63 fail to develop stratified epithelium and epithelial appendages and limbs [67]. P63 has been linked to adult stem cells and also possibly to the cancer stem cells [9]. It is often amplified and/or overexpressed in cancers [9].

Activins and other TGF-β superfamily molecules have also been shown to maintain hESCs (human embryonic stem cells) pluripotency, and attenuation of Activin signals lead to differentiation of hESCs [42]. Activins have been largely implicated in mesoderm/endoderm induction. Interestingly, reapplication of the Activin pathway in hESCs leads to endodermal differentiation as well as into different cell types [42]. Activins have been implicated in left-right patterning or axis determination and in deciding cell fates during development [41]. Activin A knockout mice exhibit craniofacial deformities, defects with skin development and are neonatally lethal, as mentioned earlier [41].

Thus, both ΔNp63 and Activin A have indispensable roles in development, are important in stem cell pluripotency and differentiation, and have been demonstrated to be overexpressed and having oncogenic roles in several cancers.

2.5.2. Crosstalk between Activin/ TGF-β and p53/p63 proteins

ΔNp63 has been demonstrated to be a direct transcriptional target of BMP signalling, a TGF-β superfamily protein [66]. Conversely, Activin A, from the TGF-β superfamily has been shown to be direct transcriptionally repressed targets of ΔNp63α isoform of p63, and this regulation plays a role in mesoderm induction in Xenopus [68]. An example of the association between p63 and Activin A in mesoderm induction was demonstrated in the context of cardiomyocytes. The TAp63 isoform was shown to directly activate the transcriptional expression of Activin A and Angiomodulin (AGM). Both these secreted factors could then induce cardiomyocytes generation from embryonic stem cells. However, Activin A and AGM also negatively regulate TAp63 levels as a negative feedback loop [69]. Another link between TGF-β signalling and p63 came from two reports; one that demonstrated a SMAD4-independent TGF-β-SMAD2/3-IKKα transcriptional axis that regulates multiple genes involved in keratinocyte differentiation and proliferation [70], while a second report demonstrated p63 to be a transcriptional target gene of a proposed keratinocyte-specific TGF-β signalling that required IKKα and SMAD2 binding to coregulate p63 expression [71].

P53 is an essential transcriptional partner of SMADs and these together regulate transcription of several target genes [72]. P53 interacts physically and functionally with BMP and Activin pathways to regulate transcription of homeobox genes involved in differentiation and cell fate determination in Xenopus species [73]. P53-deficient cells show an impaired response to cytostatic TGF-β signals [74]. P53 and SMAD2 physically interact to co-regulate

transcription via simultaneous promoter binding to synergistically activate certain TGF-β target genes [75]. Mutant p53 has been demonstrated to modulate TGF-β1 signalling by reducing the expression of its type II receptor, thus leaving cells unresponsive to TGF-β signals [75]. Mutant p53 interacts with SMADs, and this enables their interaction with p63. Together, this may lead to attenuation of metastatic suppressive gene transcription and thus promote tumorigenesis [76]. Recently, wild-type and mutant p53 were shown to differentially regulate Nox-4 signalling in TGF- β mediated cell migration in lung and breast cancer cells [77]. Another interesting study also delineated a mechanism by which mutant p53 could attenuate tumour-suppression and concomitantly promote oncogenic signals by TGF-β [78], supporting the role of p53 mutants in subverting the TGF-β superfamily signalling to initiate a tumour promoting phenotype rather than a tumour suppressive one.

With this background, we hypothesized that Activin signalling is deregulated in oral cancers and that this could contribute to oral carcinogenesis. This study involves a comprehensive analysis of Activin / Inhibin signalling components and its downstream signal mediators in oral cancer, their contribution to oral cancer phenotypes and prognosis, and the regulation of Activin / Inhibin signalling and functions by p53 family proteins including p53 and p63.

Chapter 3

Aims and Objectives

Aim of the Study:

To evaluate the expression of Activin signaling components & its contribution to oral cancer phenotypes & prognosis and to decipher the downstream mechanisms.

Objectives:

1. To perform expression analysis of Activin / Inhibin subunits & their receptors in OSCC
2. To study the functional role of Activins / Inhibins & their receptors in OSCC

Chapter 4

Materials and Methods

4.1. Materials

The reagents and chemicals used in this study, along with their manufacturers are provided below:

Advansta: WesternBright ECL HRP Substrate (#K-12045-D20).

Applied Biosystems: High-capacity cDNA Reverse Transcription Kit (#4368814), PowerUp SYBR Green PCR Master Mix (#A25780), 384-well qPCR plates (#4309849).

Axygen and Tarsons: Nuclease free disposable tips and Eppendorf tubes.

Corning: Cell culture inserts (#353049)

Dharmacon: p63 Smartpool siRNA, Control/non-target siRNA

Gibco: Foetal Bovine Serum (FBS), Dulbecco's Modified Eagle Medium (DMEM), Minimum Essential Media (MEM), Iscove's Modified Dulbecco's Medium (IMDM), Dulbecco's Modified Eagle (DME) and Ham's F12 Medium (DMEM-F12),

Hi-Media Laboratories: Sodium Chloride (NaCl), Puromycin dihydrochloride.

Kodak: X-ray films

Lucigen: Masterpure DNA and RNA Purification Kit (#MC85200)

Macherey-Nagel: Nucleospin Plasmid DNA Isolation kit (#740588.50)

MBI Fermentas: 6X Loading Dye, 25mM MgCl2, 10X PCR Buffer, 100X Proteo-block Protease Inhibitor Cocktail.

Merck Millipore: PVDF Membrane, ChIP Assay Kit (#17-295).

MP: Sodium Fluoride (NaF)

Nunc, BD Falcon and TPP: Tissue culture plasticwares (tubes, plates and flasks)

shRNA constructs: pLKO.1 from Addgene (Plasmid#10878) for cloning p63 shRNA

Qiagen: Blood and Cell Culture DNA Mini kit (#13323)

Qualigens: Disodium hydrogen phosphate (Na_2HPO_4), Sodium dihydrogen phosphate (NaH_2PO_4), Sodium Hydroxide (NaOH) pellets, Magnesium Chloride ($MgCl_2$), Glacial Acetic Acid, Xylene, DPX Mountant.

Roche: dNTPs, Taq DNA Polymerase.

Santacruz: SMAD 2/3 siRNA

SD Fine: Prestained protein ladder (10-250kDa range)

Selleck Chemicals: EW-7197 (Vactosertib) – S7530.

Sigma: Acrylamide: N-N' Methylene Bis-Acrylamide, Bovine Serum Albumin (BSA), Sodium Dodecyl Sulphate (SDS), β-mercaptoethanol (BME), Ethylenediaminetetraacetic acid (EDTA), Tris, Glycine, Tween-20, Paraformaldehyde (PFA), Triton X-100, Sodium Orthovandate (Na_3VO_4), Ammonium Persulphate (APS), N,N,N',N'-Tetramethylethylenediamine (TEMED), Diethyl Pyrocarbonate (DEPC), Ethidium Bromide (EtBr), Dimethyl Sulfoxide (DMSO), Coomassie Brilliant Blue (CBB), Bromophenol Blue, Ponceau Stain, Fast Green Stain, Erythrosine B, 4',6-diamidino-2-phenylindole (DAPI), Trypan Blue, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 3,3'-Diaminobenzidine (DAB), Agarose, Low melting Agarose, Proteinase K, Bradford Reagent, Hydroxyethyl PiperazineEthaneSulfonic acid (HEPES), Ampicillin sodium salt (A9518-5G), Nonidet P 40 Substitute (NP-40), Matrigel.

SISCO Research Laboratories: Methanol, Isoamyl alcohol, Molecular Biology Grade Ethanol, Phenol, Chloroform.

Takara: Taq DNA polymerase, RNA Isoplus.

Thermo Fisher Scientific: Lipofectamine-3000 (#L3000015), TRIzol Reagent

Vector labs (USA): Vectashield (Fluorescence anti-quench mounting medium), VectaStain ABC kit for IHC.

Water used for the preparation and dilutions of all solutions and reagents was obtained from MilliQ water plant (Resistivity = 18 MΩ cm, Millipore).

Primers: All the primers (3 O.D.) used in the study were obtained from either Sigma, IDT or Eurofins in lyophilized form and were reconstituted using Milli-Q. The list of primers used in this study is as follows:

Table 3: List of Primers

Sr. No.	**Primer name**	**Sequence (5'-3')**
1	INHBA For	TTCATGTGGGCAAAGTCGGG
2	INHBA Rev	TTCCTGGCTGTTCCTGACTCG
3	INHBB For	GCTTCGCCGAGACAGATGG
4	INHBB Rev	GGACGTAGGGCAGGAGTTTC
5	INHBC For	TTGATCTGGCCAAGAGAAGCA
6	INHBC Rev	CACAGGGCGGTTCAGTGTT
7	INHBE For	CTGGATGGGTTGCACCTGAC
8	INHBE Rev	TAGGCTGAAGTGGAGTCTGTG
9	INHA For	GAGCCCGAGGAAGAGGAGGATGTC
10	INHA Rev	TGTCCAGCCCGGTGTGGAAC
11	ALK4 For	CTCCTCCTTCTTCCCCCTTG

12	ALK4 Rev	CACACAGCAGAGCCTGGA
13	ACVR2A For	GGTGTCTGGGTTGAAGGAGGTT
14	ACVR2A Rev	GAAAGACGGCAAACGCCAAC
15	ACVR2B For	CAGCAGATGTGTCTTTCACG
16	ACVR2B Rev	CCTCAATTCCTGGTTACCT
17	FST For	TCGGGATGTTTTCTGTCCAGG
18	FST Rev	ACACTTTCCCTCATAGGCTAATCC
19	FLRG For	CACTTGGGGATCCCAGAACC
20	FLRG Rev	GAATCCTCCCCGGCAATAGG
21	Cripto-1 For	GCCCAAGAAGTGTTCCCTGT
22	Cripto-1 Rev	ACGAGGTGCTCATCCATCAC
23	P53 For	GCCAGACTGCCTTCCGGGTC
24	P53 Rev	TGGGACGGCAAGGGGGACAG
25	TAP63 For	GCCCTGACCCTTACATCCAGC
26	TAP63 Rev	GTGTGCTCTGGGACATGGTGG
27	DNP63 For	CTGGAAAACAATGCCCAGAC
28	DNP63 Rev	GGGTGATGGAGAGAGAGCAT
29	P63 shRNA For	CCGGAAGTTTCGGACAGTACAAAGACTC
30	P63 shRNA Rev	AATTCAAAAAAAGTTTCGGACAGTACAA
31	GAPDH For	GCATCCTGGGCTACACTGA
32	GAPDH Rev	CCACCACCCTGTTGCTGTA
33	ACTB For	ACAGAGCCTCGCCTTTGC
34	ACTB Rev	CATCACGCCCTGGTGCCT
35	INHBA Promo For	CGTGTGTGCGAGTAGTAAAAGT

36	INHBA Promo Rev	AGCCGGCTCTTGTATCATGT
37	INHBA Promo 1 For	CGTGTGTGCGAGTAGTAAAAGT
38	INHBA Promo 1 Rev	GGATTGGTTGGAATCAGCAT
39	INHBA Promo 2/3 For	CAGTCATCCGCTGATGTCAT
39	INHBA Enhr For	AATGGAGTTGGCAAAGGATG
40	INHBA Enhr Rev	CCCAGGACCACACTAACCTG
41	ZNF Promo For	GGAGGGAGCTTATCCCAGAG
42	ZNF Promo Rev	CCTCCGATTAAGCAAGCAAG

Antibodies: All antibodies used in the study were either obtained from Abcam, Cell Signalling technologies (CST) or Santacruz. List of antibodies used with dilution and source is as follows:

Table 4: List of Antibodies

Sr. No.	**Antibody & Catalogue no.**	**Source**	**Application**	**Dilution and Diluent**
1	Anti-Inhibin Beta A antibody (ab97705)	Abcam	Western blot	1:1000 in 2.5% NFDM
2	Anti-Inhibin Beta A antibody (ab56057)	Abcam	Western blot	1:1000 in 2.5% BSA
3	Human/Mouse/Rat Activin A Beta A subunit antibody (AF338)	R&D systems	IHC	1:100 in HNS
4	Anti-p63 antibody [4A4] (ab735)	Abcam	Western blot	1:200 in 2.5% NFDM

5	Anti-p63 antibody [4A4] (ab735)	Abcam	IHC	1:200 in GNS
6	p53 antibody (DO-1) (sc-126)	Santacruz	Western blot	1:12000 in 2.5% BSA
7	p53 antibody (DO-1) (sc-126)	Santacruz	IHC	1:100 in GNS
8	Anti-SMAD2 antibody [EP567Y] (ab33875)	Abcam	Western blot	1:1000 in 2.5% BSA
9	Anti-SMAD2 antibody [EP567Y] (ab33875)	Abcam	IF	1:100 in 2.5% BSA
10	Anti-SMAD2 (phosphor S467) antibody (ab53100)	Abcam	Western blot	1:500 in 2.5% BSA
11	SMAD 2/3 antibody sampler kit (12747T)	CST	Western blot	1:1000 in 5% BSA
12	ACTR-II (F-12) (sc390977) antibody	Santacruz	Western blot	1:1000 in 2.5% BSA
13	ACTR-I (H-170) (sc25449)	Santacruz	Western blot	1:500 in 2.5% BSA
14	p44/42 MAPK (ERK1/2) (137F5) antibody (4695)	CST	Western blot	1:1000 in 5% BSA
15	Phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) antibody (9101)	CST	Western blot	1:1000 in 5% BSA
16	p38 MAPK (D13E1) XP antibody (8690)	CST	Western blot	1:1000 in 5% BSA

17	Phospho-p38 MAPK (Thr180/Tyr182) (D3F9) XP antibody (4511)	CST	Western blot	1:1000 in 5% BSA
18	SAPK/JNK antibody (9252)	CST	Western blot	1:1000 in 5% BSA
19	Phospho-SAPK/JNK (Thr183/Tyr185) (81E11) antibody (4668)	CST	Western blot	1:1000 in 5% BSA
21	Goat anti-rabbit HRP conjugated secondary antibody	Abcam	Western blot	1:6000 in 2.5% diluent
22	Goat anti-mouse HRP conjugated secondary antibody	Santacruz	Western blot	1:3000 in 2.5% diluent
23	Horse anti-goat biotinylated secondary antibody	Vectorlabs	IHC	1:200 in HNS
24	Goat anti-mouse biotinylated secondary antibody	Vectorlabs	IHC	1:200 in GNS

Instruments: List of instruments used in the study is as follows:

Table 5: List of Instruments

Instrument	**Model**	**Company**
Vertical Electrophoresis assembly for SDS-PAGE	Mini-PROTEAN Tetra Cell	Bio-Rad, USA
Electroblotting	Trans-Blot Cell	Bio-Rad, USA
Electrophoresis Power packs	EPS-301	Amersham, UK
ELISA Reader	Spectra Max 190	Molecular Devices, USA
Real Time PCR Machine	Quant Studio 12K Flex	Applied Biosystems, USA
PCR Machine	VeriFlex	Applied Biosystems, USA
pH meter	Orion	Thermo Scientific, USA
X-ray developing machine	Optimax	Pro-Tec, Germany
Table top Centrifuge	MIKRO 120	Hettich, Germany
Cooling Centrifuge	Heraeus Multifuge X3R	Thermo Scientific, USA
Fluorescence Confocal Microscope	LSM 780 Meta	Zeiss, Germany
Inverted Microscope	Axio-vert-200M	Zeiss, Germany
Upright Microscope	Axio Imager Z1	Zeiss, Germany
Cryotome	CM1950	Leica. Germany
Spectrophotometer	NanoDrop 1000	Thermo Scientific, USA

4.2. Methods

4.2.1. Cell Culture

Ten cell lines were used in the present study; their names, tissue of origin and culture media are outlined below in Table 6. All cells were grown in respective media containing 10% FBS and antibiotics, and were maintained in a humidified atmosphere in incubator at 37^0C and 5% CO_2.

Table 6: List of Cell Lines

Cell line	**Tissue or Site of Origin**	**Culture Medium**	**Reference**
FBM	Foetal Buccal Mucosa	IMDM	[79]
DOK	Dysplastic Oral Keratinocytes derives from tongue epithelium	DMEM	[80]
SCC9	Tongue squamous cell carcinoma	DMEM-F12	[81]
SCC15	Tongue squamous cell carcinoma	DMEM-F12	[81]
SCC25	Tongue squamous cell carcinoma	DMEM-F12	[81]
SCC029B	Buccal Mucosa squamous cell carcinoma	DMEM	[82]
SCC040	Tongue squamous cell carcinoma	DMEM	[82]
SCC74	Tongue squamous cell carcinoma	DMEM	[82]
AW8507	Tongue squamous cell carcinoma	IMDM	[83]
AW13516	Tongue squamous cell carcinoma	IMDM	[83]

4.2.1.1. Cell Cryopreservation

Mammalian cells to be used in the study were frozen in liquid nitrogen using the following protocol. Cells growing in 90mm culture dishes at 80-90% confluency were washed twice with 1X PBS to remove dead cells and media. 0.25% Trypsin-EDTA in 1X PBS was used to dissociate cells at 37^0C. The activity of trypsin was inactivated using media containing serum and the cells collected in media were subjected to centrifugation at 1200 rpm for 10 minutes. The cell pellets so obtained were resuspended in 3ml of freezing mixture (90% serum and 10% DMSO) and were mixed thoroughly with a pipette to obtain a single cell suspension. 1ml of this suspension was aliquoted into each freezing vial and gradually introduced into liquid nitrogen vapours for few hours, following which the vials were immersed in liquid nitrogen to be stored in cryo-boxes until further use.

4.2.1.2. Cell Revival

Cells were revived for use from the previously frozen vials from liquid nitrogen. The vials were first placed into a water bath heated to 37^0C. Once thawed, these cells in 1ml freezing mixture were added dropwise to 5ml of complete media (containing serum) and mixed thoroughly into a glass tube. Tubes were then centrifuged at 1200 rpm for 10 minutes to obtain cell pellets. Media containing freezing mixture was discarded and cells were resuspended in fresh complete media, mixed thoroughly to generate a single cell suspension, seeded onto a 60mm culture dish and allowed to incubate at 37^0C until adherent and confluent. Cells were passaged at least once after revival before being used for experiments or assays.

4.2.1.3. Cell Passaging

For maintenance, cells were passaged at 80-90% confluency (exponentially growing), unless otherwise stated. For passaging, cells were washed twice with 1X PBS to remove

dead cells and media. 0.25% Trypsin-EDTA was used to dissociate cells at 37^0C, following which trypsin was inactivated using twice the volume of complete media (containing serum) and cells were collected in this media into a glass tube. Cells were then centrifuged at 1200 rpm for 10 minutes to obtain cell pellets. Cell pellets were further resuspended in fresh media and seeded at required density for various assays or for maintenance or cryopreservation.

4.2.1.4. Cell Counting

Cell pellets obtained after trypsinisation were thoroughly resuspended in complete medium to obtain a single cell suspension and placed on ice throughout the process of cell counting so as to maintain viability. Cell suspensions were mixed with Erythrosine B or Trypan Blue dye in 1:1 ratio and loaded onto a Neubauer's chamber (Haemocytometer) slide beneath a coverslip, to be observed under a light microscope. The dye used stains dead cells whereas live cells appear unstained. Cells within the four peripheral WBC chambers were counted to obtain an average number of cells per WBC chamber. Using this average, cell count per microlitre was determined using the formula below:

Total number of cells per µl = Average cell number per WBC chamber X 2 (dilution factor) X 10

4.2.2. DNA Isolation

Genomic DNA isolation was performed using Qiagen DNA Isolation kit and Plasmid DNA isolation was performed using Nucleospin Plasmid DNA Isolation kit as per the manufacturer's protocol. Briefly, cell pellets were suspended in 1X PBS, lysed and the lysates were clarified for debris. Lysates were then loaded onto columns within the kit

that bind DNA. DNA bound silica membranes were washed twice, dried and the DNA was eluted in 25-30µl of elution buffer provided. Genomic or Plasmid DNA so obtained was quantified using NanoDrop 1000, and DNA quality was confirmed from the 260/280 and 260/230 ratios so obtained.

4.2.3. RNA Isolation

Total RNA was extracted from cell pellets using TRIzol reagent (a monophasic solution of phenol guanidine isothiocyanate). Cell pellets were thoroughly suspended in 1ml of TRIzol reagent by pipetting several times. These cell suspensions were incubated at room temperature for 5 minutes to allow complete dissociation of nucleoprotein complexes. 200µl of chloroform was then added to these, and tubes were incubated at room temperature for 10 minutes with intermittent vigorous shaking for 20-30 seconds multiple times. Post incubation, tubes were centrifuged at 15200rpm for 15 minutes for phase separation. The upper aqueous phase that contains total cell RNA was carefully transferred to a fresh tube (avoiding any mix/contamination from the middle hazy and lower phenol phase), and 500µl of 100% isopropanol was added to this aqueous phase so collected. Solution was mixed well by inverting tubes several times and incubated either at room temperature for 10 minutes or at -80^0C overnight, prior to centrifugation at 15200rpm for 10 minutes. Supernatants were discarded and the RNA pellets so obtained were washed twice with 200µl of 70% ethanol made in nuclease-free water. Washed RNA pellets were air-dried for 30 minutes or until the residual ethanol evaporated completely. Dried RNA pellets were thoroughly resuspended in 20-30µl of nuclease-free water, incubated at 550C to resolve any secondary structures, before quantification using NanoDrop 1000 at 260nm. RNA quality was ensured by 260/280 (between 1.8-2.1) and

260/230 (between 2-2.2) ratios. RNA thus obtained was either used to convert into cDNA for downstream applications or stored at -80^{0}C until further use.

4.2.4. Reverse Transcription or cDNA synthesis

cDNA was synthesized from RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) as per manufacturer's protocol. 1µg of RNA was converted to cDNA in a reaction volume of 20µl as described below:

10µl of 2X Master Mix prepared from kit components:

Reaction Component	Volume (in µl)
10X RT Buffer	2
25X dNTP Mix (100mM)	0.8
10X RT Random Hexamer Primers	2
MultiScribe Reverse Transcriptase (enzyme)	1
Nuclease-free Water	4.2
Total Volume (per reaction)	10

10µl of RNA sample:

1µg RNA (volume as per quantification) diluted in nuclease-free water to make a total volume of 10µl.

10µl 2X master mix + 10µl of diluted RNA = Total 20µl of reaction

20µl of reaction so prepared was loaded onto a thermal cycler (VeriFlex PCR machine from Applied Biosystems) with the below reaction protocol:

25^{0}C / 10 mins ➡ 37^{0}C / 120 mins ➡ 85^{0}C / 5 mins ➡ 4^{0}C / hold for ∞

4.2.5. Semi-quantitative PCR:

Semi-quantitative PCR was performed during Chromatin Immunoprecipitation (ChIP) assay to determine the enrichment of Activin promoter and enhancer regions in p63 pull down cells.

The PCR reactions were setup as follows:

Reaction Component	**Volume (in µl)**
Template DNA	4
10X PCR Buffer	1
25X dNTP Mix (100mM)	0.8
25 mM $MgCl_2$	0.6
10pm Forward Primer	0.3
10pm Reverse Primer	0.3
Taq Polymerase (enzyme)	0.5
Nuclease-free Water	2.5
Total Volume (per reaction)	10

The following protocol was used in a thermal cycler to carry out the PCR reaction:

Initial Denaturation	**PCR Stage (X 40 cycles)**			**Final Extension**	**Hold**
95^0C	95^0C	60^0C	72^0C	72^0C	4^0C
5 mins	30 secs	30 secs	30 secs	10 mins	∞

4.2.6. Quantitative Real-time RT-PCR

Quantitative Real-time RT-PCR was performed using 10ng of reverse transcribed cDNA per reaction for determining the expression of all Activin / inhibin subfamily and pathway genes, as well as for microarray validation (primer sequences as listed in Table 3). For detection using Quant Studio 12K Flex Real-time PCR machine, PowerUp SYBR Green Master Mix (Applied Biosystems) was used in the PCR reaction as follows:

Reaction Component	**Volume (in µl)**
2X Power SYBR Green Master Mix	2.5 (1X)
5ng/µl diluted cDNA	2 (10ng)
Gene-specific Forward Primer	0.25
Gene-specific Reverse Primer	0.25
Total Volume (per reaction)	5

The PCR program was setup as follows:

Hold Stage		PCR Stage		Melt Curve Stage	
50^0C	95^0C	95^0C	60^0C	95^0C	60^0C
2 mins	10 mins	15 secs	1 min	15 secs	1 min

For analysis, melt-curve temperature was determined for each gene to ensure purity of the amplified product. Cycle threshold (Ct) values determined from analysis by the Applied Biosystems software were used to calculate Relative Expression ($2^{\wedge-\Delta Ct}$) and Fold Change ($2^{\wedge-\Delta\Delta Ct}$), taking GAPDH and/or ACTB as housekeeping/normalizing genes, wherever indicated.

4.2.7. Agarose Gel Electrophoresis (AGE)

Semi-quantitative PCR products from ChIP experiments were loaded onto 1.5% agarose gels. For this, 1.5% (w/v) of agarose was diluted in 1X TAE buffer and boiled in a microwave for 2 minutes. Once the solution cooled to lukewarm, 0.2µg/ml of Ethidium Bromide (EtBr) was added to it, and the gel was poured into horizontal gel casting tray with comb for solidifying at room temperature for 1 hour. After solidifying, DNA samples were loaded into each individual well alongside a standard marker or DNA ladder (100bp-1kb), and electrophoresed at a constant voltage (60V) for 1 hour or until a desired resolution was achieved. Separated DNA was visualized and imaged under UV light using an UV Transilluminator.

4.2.8. Western Blotting

4.2.8.1. Protein Extraction

Cell pellets obtained from cultured cells were resuspended in appropriate volumes of Proteo JET mammalian cell lysis reagent (with 1X protease inhibitor cocktail and phosphatase inhibitors, where required) by vigorous pipetting and immediately placed on ice for 30 minutes for cell lysis. Tubes were further vigorously agitated on a vortex for 2-3 minutes in cold room and placed back on ice for additional 10 minutes. Post incubation, tubes were centrifuged at 13000rpm for 20 minutes at 4^0C. Supernatants so obtained were transferred into fresh microcentrifuge tubes and these lysates were used for total protein estimation and stored at -80^0C until further use.

4.2.8.2. Protein Estimation

The Bradford assay optimized for a 96-well microtiter plate was used to quantitate total protein in the lysates. For this, 5µl of serially diluted Bovine Serum Albumin (BSA) was added in triplicates to the plate to generate a standard curve (0, 0.0625, 0.125, 0.25, 0.5 and 1µg/µl BSA dilutions respectively). 1µl of the cell lysate (unknown concentration of protein) was added in triplicates to separate wells in the plate. 100µl of 1X Bradford's Reagent was added to each standard and test well, plate was incubated in dark for 5 minutes before measuring the absorbance of samples at 595nm on a microplate reader. The mean absorbance of each blank corrected standard was used to generate a scatter plot, where a straight-line equation was deduced from the trendline using MS-Excel. Absorbance from unknown or test samples was extrapolated to this graph and concentration of these samples was thus calculated from the equation.

4.2.8.3. Protein Separation

4.2.8.3.1. Sample Preparation

Equal amounts of protein samples (20-30µg) were loaded onto polyacrylamide gels for separation. The required amount pf protein (volume as per quantification from Bradford assay) was mixed with equal volume of 2X Laemmli buffer, and a total volume to be loaded onto gel was adjusted with 1X Laemmli buffer. Samples were then boiled on a water bath heated at 100^0C for 5 minutes before loading onto the gel.

4.2.8.3.2. SDS-Polyacrylamide Gel Electrophoresis (PAGE)

The SDS-PAGE gel separates proteins form the lysates based on their molecular weights. Gels of a required pore size are formed by crosslinking monomeric acrylamide with N,N'-methylene Bis-Acrylamide in a concentration dependent manner. Size of the pores decreases with increasing Bis-Acrylamide-Acrylamide ratio. 12% resolving gel composition (as indicated below) was used in this study to resolve proteins with molecular weights between 40-130kDa. 5% stacking gel composition was used to stack proteins before entering into the resolving gel.

The boiled protein samples were loaded in individual wells of stacking gel and electrophoresed at a constant current of 25-30mA at room temperature until the desired resolution was achieved. A pre-stained protein ladder with specific molecular weight markers was simultaneously loaded and separated along with protein samples, in order to reference and determine the mobility and molecular weights of various proteins in the gel.

Composition of Resolving and Stacking SDS-PAGE gel:

Components (for 10ml)	**Vol. (in ml) for 12% Resolving Gel**	**Vol. (in ml) for 5% Stacking Gel**
De-ionized Water	3.3	6.8
30% Acrylamide Mixture (Acrylamide: Bis-Acrylamide: 29:1)	4	1.7
1.5M Tris-Cl (pH 8.8)	2.5	-
1 M Tris-Cl (pH 6.8)	-	1.25
10% SDS	0.1	0.1
10% APS	0.1	0.1
TEMED	0.008	0.008

4.2.8.4. Protein Transfer or Electroblotting

Proteins separated on SDS-PAGE were electro-transferred onto adsorbent Polyvinylidene difluoride (PVDF) membranes to facilitate binding of antibodies. We used the wet electroblotting method where proteins from the gel were transferred to the membrane in the presence of a buffer at a constant voltage of 16V for 16 hours. After electro-transfer, proteins on the membrane were visualized by staining them with Fast green stain for 15 minutes, followed by destaining with distilled water and destainer solution (45% Water : 45% Methanol : 10% Glacial Acetic Acid).

4.2.8.5. Immunoblotting

PVDF Membrane blots with proteins were incubated in blocking solution (5% BSA or 5% Non-Fat Dry Milk, as indicated for each antibody in Table 4) at room temperature for 1 hour with slow rocking. Blots were then incubated with appropriate protein-specific primary antibodies at 4^0C overnight with slow rocking. Post incubation with primary antibody, blots were washed thrice with 1X Tris Buffered Saline with 0.1% Tween-20 (TBST) for 20 minutes each, followed by incubation with horseradish peroxidase (HRP) conjugated secondary anti-mouse or anti-rabbit antibodies at room temperature for 1 hour with slow rocking. Blots were again washed thrice with 1X TBST for 20 minutes each, and an additional wash with 1X TBS for 10 minutes, before protein visualization using ECL chemi-luminescent substrate. Protein bands so detected were quantified by densitometric analysis using Image Lab software from BioRad.

4.2.9. Immunohistochemistry (IHC)

4.2.9.1. Ethics Approval

The use of tissue samples in the study was reviewed and duly approved by the Institutional Ethics Committee (IEC-III), TMC, ACTREC (Project No.: 934). A total of 102 paired oral adjacent normal – tumour tissue samples obtained from Tumour Tissue Repository, ACTREC; and slide sections of 18 normal oral mucosa samples obtained from individuals undergoing minor dental surgeries at KBH Dental College, Nashik were used for IHC staining of Activin A, p53 and p63 proteins in this study.

4.2.9.2. IHC Staining

The tissues were fixed in 10% buffered formalin (v/v in PBS) overnight and mounted in paraffin blocks. Tissue sections of 5µm thickness were obtained from these paraffin-embedded blocks using a cryotome. These formalin-fixed and paraffin embedded tissue sections were then deparaffinized and rehydrated by passing through several grades of xylene and alcohol. The endogenous peroxidases were then blocked by incubating sections in methanol and H_2O_2 at room temperature for 30 minutes in dark. Subsequently, sections were washed in PBS for 10 minutes and heat-induced epitope retrieval was performed using sodium citrate buffer at pH 5.8 by heating in a microwave for 5 minutes. The slides were then allowed to cool and again washed in PBS for 10 minutes. Sections were then incubated with Horse Normal Serum (for primary antibodies raised in goat) or Goat Normal Serum (for primary antibodies raised in mouse) as a blocking agent for 1 hour at room temperature in a humidified chamber. Post incubation, blocking agent was carefully drained off the tissue sections without allowing them to dry. Tissue sections were further incubated with appropriately diluted protein-specific primary antibodies (Activin A, p53 or p63) at 4°C overnight in a humidified chamber. Next day, these slides

were washed with PBS containing 0.1% Tween-20 (PBST) for 10 minutes, followed by a PBS wash for 10 minutes, and incubated with appropriately diluted biotinylated secondary antibodies at room temperature for 1 hour in a humidified chamber. Slides were again washed once with PBST and PBS for 10 minutes each and incubated with Avidin-Biotin Complex (ABC) at room temperature for 1 hour in a humidified chamber. Following washing steps, tissue sections were stained with freshly prepared 3,3'-diaminobenzidine tetrahydrochloride (DAB) and H_2O_2 as the chromogen for suitable time so as to observe specific staining. Staining was terminated by immersing the sections in de-ionized water, and slides were counter stained with Haematoxylin for 1-2 minutes followed by immersing the slides in tap water. Finally, tissue sections were again dehydrated by passing them through several grades of alcohol and xylene (in the reverse order) and mounted using a DPX mountant and air-dried. Slide were imaged using the Axio Imager.z1 Zeiss (upright) microscope. Expression of activin A, p53 and p63 was evaluated using a combined scoring method that accounts for both the staining intensity and the percentage of stained cells. Strong, moderate, weak, and negative staining intensities were scored as 3, 2, 1, and 0, respectively. For each of the intensity scores, the percentage of cells that are stained at the specific level were visually estimated by two independent pathologists. The resulting combined H score on a scale of 0-300 was calculated as the sum of the percentage of stained cells multiplied by the intensity scores.

4.2.10. Immunofluorescence (IF)

Immunofluorescence was used to determine the activation of SMAD2 and its nuclear translocation in order to demonstrate the functionality of Activin signalling pathway in oral cancer cell lines. Briefly, cells grown on coverslips were treated with recombinant

Activin A for 1 hour, with an untreated control panel alongside. Post treatment, cells were gently washed twice with PBS and fixed with 4% (w/v) Paraformaldehyde (PFA) at room temperature for 15 minutes. Following another PBS wash, cells were permeabilized with 0.5% (v/v) Triton-X 100 at room temperature for 10 minutes and again washed with PBS. Next, the cells were incubated with 5% BSA in PBS (blocking solution) at room temperature for 1 hour. Cells were then incubated with appropriate dilution of protein-specific primary antibody (SMAD2) diluted in PBS at 4^0C overnight by inverting the coverslips against a drop of the antibody on a parafilm (cell side facing downwards). Next day, following the washes, cells were incubated with fluorophore-conjugated diluted secondary antibody at room temperature for 1 hour in dark. Cell nuclei were stained with DAPI. Finally, the coverslips were mounted upside down against a drop of Vecta-shield fluorescence anti-quenching mounting medium on acid-washed slides and their boundaries were sealed with nail polish. The slides were stored at 4^0C in dark until image acquisition using a fluorescence microscope.

4.2.11. Chromatin immunoprecipitation (ChIP)

Chromatin immunoprecipitation was done to evaluate the binding of p63 to Activin A promoter and was performed using the Merck-Millipore ChIP Assay kit as per the manufacturer's instructions.

4.2.11.1.DNA Shearing

Cells grown to 90-100% confluence in 90mm culture dishes were cross linked with 1% formaldehyde for 10mins at 37^0C (270µl 37% formaldehyde directly added to 10ml of growth medium). After incubation, media with formaldehyde was removed and cells were scraped in fresh 1ml PBS, collected in a 1.5ml microcentrifuge tube and pelleted at

2000rpm at 4^0C for 4 minutes. 200µl of SDS buffer was added per $1*10^6$ cells and incubated on ice for 10mins for lysis. The lysates so obtained were sonicated using a Bioruptor to shear DNA such that the sheared DNA length ranged between 200-1000bp.

4.2.11.2. Immunoprecipitation

The sonicated samples were centrifuged at 13000rpm for 10 minutes at 4^0C and supernatants were transferred to a fresh 2ml microcentrifuge tube. The sonicated cell supernatants were diluted 10-fold using the ChIP Dilution buffer from the kit with protease inhibitors added to it at 1X concentration (1800µl ChIP dilution buffer added per 200µl sonicated cell supernatant). The 2ml diluted cell supernatant was precleared with 75µl of Protein A Agarose beads at 4^0C for 30 minutes with agitation. Post incubation, the agarose was pelleted down by centrifugation at 1000 rpm at 4^0C for 1 minute. 2% by volume of the supernatant was separated to be loaded as Input. The immunoprecipitating antibody (p63 mouse monoclonal antibody) was added to remaining diluted supernatant fraction and incubated overnight at 4^0C with slow rotation. For the isotype control, control IgG antibody was added to the supernatant fraction and incubated overnight. The next day these supernatants containing respective antibodies were incubated with 75µl of agarose beads on a slow rotating platform for 2 hours. Agarose beads were then pelleted down by centrifugation at 1000rpm at 4^0C for 1 minute, washed once with each of the Low Salt Immune Complex Wash Buffer, High Salt Immune Complex Wash Buffer, LiCl Immune Complex Wash Buffer and two washes of 1X TE Buffer from the kit. One fraction each of the p63 antibody-containing beads and the isotype antibody-containing control beads was processed for western blotting, while a second fraction of each was utilized for DNA extraction. The fraction that was processed for immunoblotting was centrifuged after the last wash at 1000rpm at 4^0C for 1 minute. 25µl of 1X Laemmli buffer was added to these samples, which were boiled for

10 minutes before loading onto a 12% PAGE gel and immunostained for p63, to confirm the pulldown. The fraction that was processed for DNA isolation was used to perform semi-quantitative PCR using Activin A promoter and Enhancer specific primers. Percentage enrichment for presence of activin βA promoter in p63 immunoprecipitated fraction was calculated over IgG isotype control fraction taking Input sample as the reference.

4.2.12. siRNA and shRNA Transfection

Cells were seeded into 24-well or 6-well culture dishes at a density so as to obtain a confluency of about 70% next day, and were grown overnight at 37^0C with 5% CO_2. Next day, the cells were transfected with siRNA using Lipofectamine 3000. Briefly, gene-specific siRNA (SMAD2/3 siRNA from Santacruz Biotech.) or non-targeting control siRNA was diluted at required concentration in incomplete growth medium (without serum). P3000 was added along with the siRNA to these tubes. In a separate microcentrifuge tube, Lipofectamine 3000 reagent was also diluted at required amount in incomplete growth medium. After 5 minutes of incubation, the diluted siRNA was mixed with diluted Lipofectamine 3000, mixed well and incubated again for 20 minutes. Post 20 minutes incubation, this siRNA + transfection reagent mixture was added dropwise to PBS washed cells, and the remaining volume required for the plate was replenished with fresh incomplete media. Cells were incubated at 37^0C and transfection was allowed to take place for 6 hours, after which, transfection media was replaced with fresh complete media. Cells were harvested after 24 or 48 hours of transfection for analysis in downstream assays.

For shRNA transfection (p63 shRNA), similar transfection protocol was followed, except that instead of harvesting after 48 hours, cells were maintained in complete growth media

with appropriate concentration of puromycin as a selection marker for several days until individual colonies of transfected clones started to grow. Each clone was then spot-trypsinized and cultured separately into individual culture plates. Each clone thus regrown was expanded, harvested and analysed for p63 knockdown using western blotting. Two clones each from AW8507 and SCC040 cells with more than 70% p63 knockdown were used in downstream assays further.

4.2.13. Wound healing assay

Wound healing or Scratch assay was used to demonstrate the effect of SMAD2/3 knockdown and that of treatment with EW-7197 Activin signalling inhibitor on oral cancer cell migration. Briefly, cells were grown in 6-well culture plates until they were completely confluent. Fully confluent cell layers were then serum starved overnight (to inhibit proliferation), before making a wound/scratch with the help of a 10µl tip. The cells were then thoroughly washed with 1X PBS in order to get rid of cell debris. Fresh incomplete media (without serum) with or without the inhibitor treatment was then added to these cells; in case of SMAD2/3 knockdown, non-targeting control siRNA and SMAD2/3 siRNA were transfected in cells in these 6-well plates, and wounds were made in them post 24 hours of transfection. Wounds were imaged with the help of a time-lapse microscope, AxioVert 200M, Zeiss for 20 hours. The initial image was taken as 0 hour and the image of healed wound was taken at 20 hours. Area of wound closure was determined using Image J software and percentage wound closure compared to untreated or control was calculated.

4.2.14. Boyden chamber assay

Boyden chamber or Transwell Matrigel assays were performed to determine the effect of SMAD2/3 knockdown, EW-7197 inhibitor treatment and recombinant Activin and Follistatin treatment on the Matrigel invasion of oral cancer cells. Transwell assay without the use of Matrigel were also performed to evaluate the effect of p63 knockdown and recombinant Activin treatment of p63 knockdown clones on their cell migration. Briefly, 30µg Matrigel was loaded onto the Boyden chambers in a volume of 100µl of incomplete media, and incubated at 37^0C for 4 hours. Cells were either transfected with SMAD2/3 or non-targeting control siRNA 24 hours prior to the assay, or they were treated with recombinant proteins or inhibitor 24 hours prior to the assay in incomplete media. Post knockdown or treatment for 24 hours, cells were harvested, washed with incomplete media to remove traces of serum, and again resuspended in incomplete media. Cells were then counted and 50,000 cells were seeded in a volume of 200µl incomplete media onto the upper Boyden chambers already loaded with Matrigel. 700µl of complete media was added to the bottom or lower well of the 24-well plate holding the chambers. Chambers with cells were incubated at 37^0C for 48hours before staining with Calcein AM dye. Calcein AM was added at 2ng/µl to the lower chamber and incubated in dark for 40 minutes. After incubation, uninvaded cells and Matrigel from the upper chamber were thoroughly removed with the help of sterile cotton buds. Invaded cells present on the lower surface of the chamber membrane and stained with Calcein fluorescence were imaged using Axio Vert 200M inverted microscope. Membranes were carefully cut out from the chambers using a surgical blade and loaded into 96-well plate with 200µl media to submerge them completely. Fluorescence intensity was read using BioRad Cytation 5 fluorimeter (excitation 485nm, emission 535nm) and relative fluorescence units (RFU) were plotted against each sample to determine the proportion of cells invading in each

treatment case compared to control. RFU of initial count of cells from control sets was used to normalize the final RFU of invaded cells in the test samples.

4.2.15. Microarray sample preparation, data analysis and validation

P63 knockdown was achieved in AW8507 and SCC040 oral cancer cells using siRNA strategy and these cells were subjected to a microarray analysis outsourced to iLife Discoveries, India. RNA samples for the same were prepared using Masterpure DNA and RNA purification kit. RNA with an excellent 260/280 and 260/230 ratio was used for the microarray hybridization using the Affymetrix platform. Raw data was analysed by Biocos Life Sciences, India. Of the list of DEGs so obtained, Activin / TGF-β pathway genes along with other several pathways were differentially regulated upon p63 knockdown. Some of these deregulated genes were validated using qRT-PCR. Also, their associations with p63 expression in TCGA database were analysed using the cBioPortal tool.

4.2.16. Statistical Analysis

Statistical analysis was done using SPSS 21.0 (SPSS Inc., Chicago, IL, USA). ANOVA was used to compare differences in transcript/protein expression between cell lines and relative expression of various genes within cell lines. Mann-Whitney test was used to assess differences in expression between normal oral mucosa versus adjacent normal or tumor tissues; Wilcoxon signed rank test was used to assess differences in expression between paired adjacent normal-tumor tissues. Median H-score of each protein was used to categorize the cohort into low/high expressors to be used for following analysis. Associations between Activin expression and clinicopathological parameters were tested

using Chi-square tests. Univariate and Multivariate analysis of protein expression with Overall Survival and Recurrence-Free Survival was analyzed using Kaplan-Meier curves, survival probabilities were compared using log rank test, Cox proportional hazard model was used to assess prognostic role of these proteins and effect was quantified using HR and 95% CI. Data is reported as mean/median$\pm$SD; p-value of 0.05 was considered statistically significant.

Chapter 5

Results

5.1. To perform expression analysis of Activin / Inhibin subunits & their receptors in OSCC

5.1.1. Activin βA subunit or monomer is predominantly overexpressed in oral cancer cell lines compared to normal oral mucosa

The relative expression of all subunits or monomers that form the functional Activin/Inhibin dimers was assessed by quantitative real-time reverse transcription PCR (qRT-PCR) using monomer-specific primers. GAPDH was used for normalization of transcript data by the comparative delta Ct method to determine fold change in expression in oral cancer cell lines over that in normal oral mucosa samples. A panel of seven immortalized oral cancer cell lines, one dysplastic oral keratinocyte cell line (DOK) and 12 normal oral mucosa samples obtained from healthy individuals undergoing minor dental surgeries were used for this transcript expression analysis.

Comparison of the relative expression of all subunits together indicated that the βA subunit was predominantly expressed in all cell lines and tissue samples tested, and it was further significantly upregulated in oral cancer cells as well as DOK cell line compared to normal oral mucosa ($p<0.0001$, Figure 7). On the contrary, other β subunits including βB ($p<0.0001$), βC ($p<0.0001$) and βE ($p=0.0012$) were downregulated in oral cancer cell lines compared to normal oral mucosa (Figure 7b). The Inhibin-forming α subunit was also upregulated in OSCC cells ($p=0.0015$); however, its expression relative to all β subunits was significantly low ($p<0.0001$, Figure 7b). This relative expression analysis together suggested that since βA is the only predominantly expressed subunit in

oral cancer cells and the relative expression levels of all other subunits are significantly low compared to βA levels, Activin A (βA-βA homodimer) is likely to be the predominantly formed Activin in OSCC.

Next, we assessed the protein expression of Activin βA subunit in a panel of oral cancer cell lines by western blotting as compared to its expression in Fetal Buccal Mucosa cell line as control. At the protein level also, we could confirm the overexpression of Activin βA subunit in majority of the oral cancer cell lines tested ($p<0.0001$, Figure 8).

Together, the transcript and protein expression analysis of Activin/ Inhibin subunits demonstrated that Activin βA subunit is significantly upregulated in oral cancer cells.

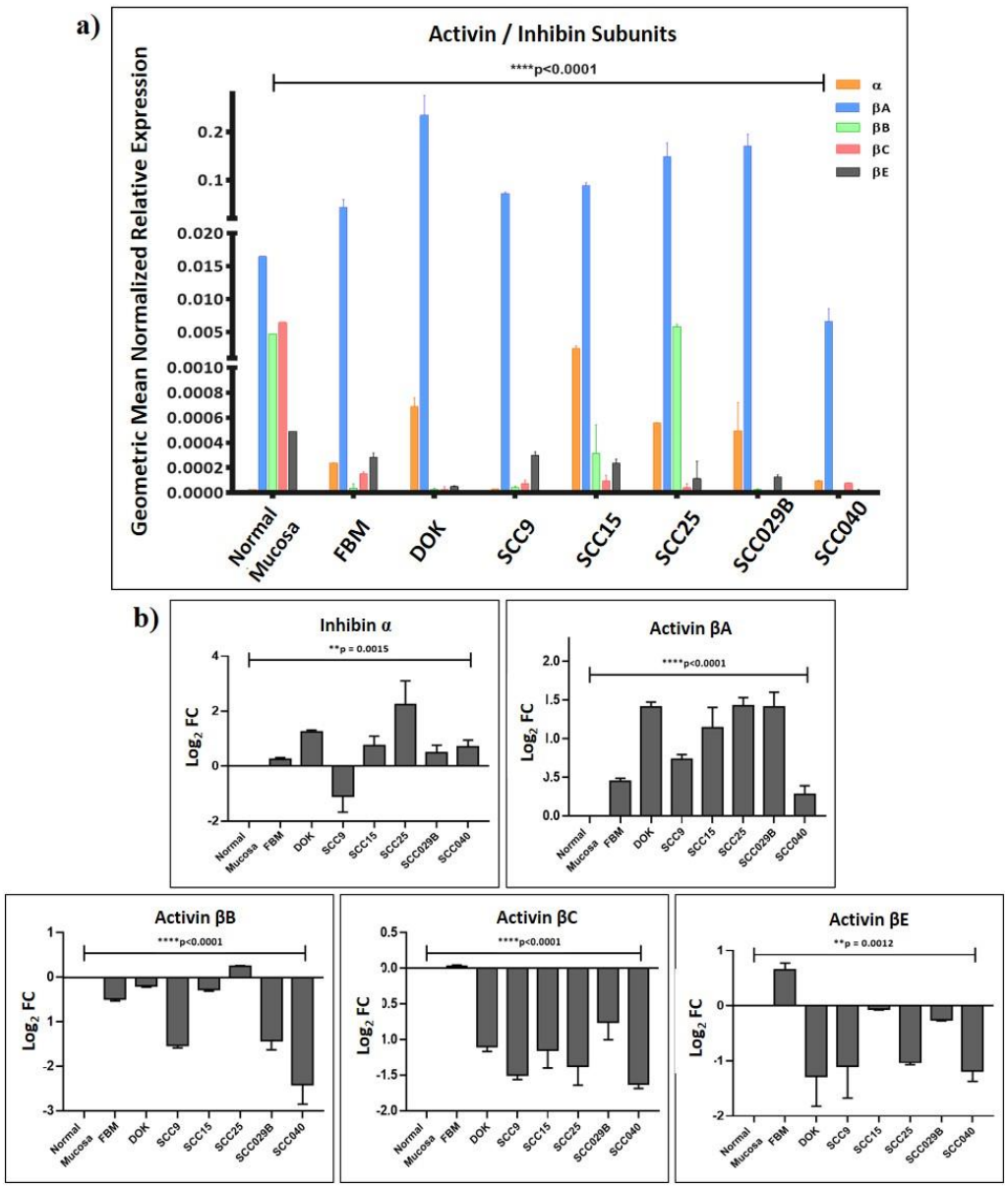

Figure 7: Transcript Expression of Activin Subunits in OSCC cells: a) Relative expression of Activin subunits compared to normal oral mucosa b) Fold change in expression of individual Activin subunits compared to normal oral mucosa

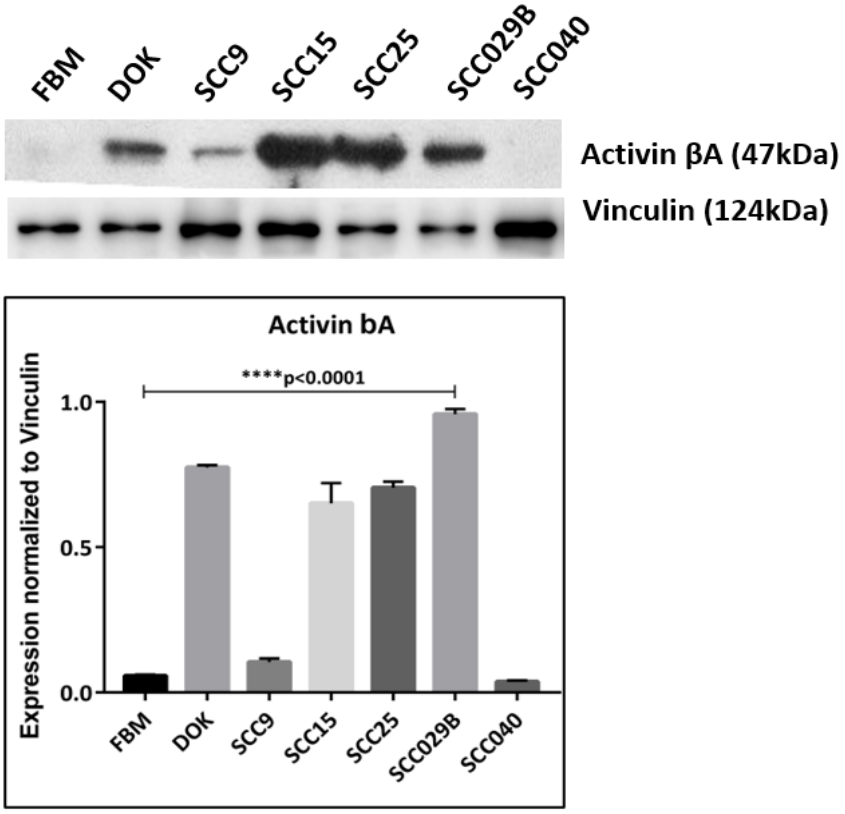

Figure 8: Protein Expression of Activin βA subunit in OSCC cells compared to Fetal Buccal Mucosa

5.1.2. Negative regulators of Activin signaling are differentially expressed in oral cancer cell lines

Activin signaling is negatively regulated by two of the secreted molecules, FST and FLRG, which bind to Activin dimers, rendering them unavailable for binding to their receptors. We assessed the expression of these two molecules in oral cancer cell lines by qRT-PCR. FST transcripts were found to be significantly upregulated in some of the oral cancer cell lines tested compared to normal oral mucosa ($p<0.0001$, Figure 9). FSTL-3 transcripts, on the contrary, were downregulated in these cell lines ($p<0.0001$, Figure 9). Expression of another negative regulator of Activin signaling, Cripto-1, which acts as a transmembrane pseudo-receptor that sequesters Activin dimers, was also tested in oral cancer cells using qRT-PCR. Transcript levels of Cripto-1 were also found to be significantly downregulated in the oral cancer cell lines as compared to normal oral mucosa ($p<0.0001$, Figure 9).

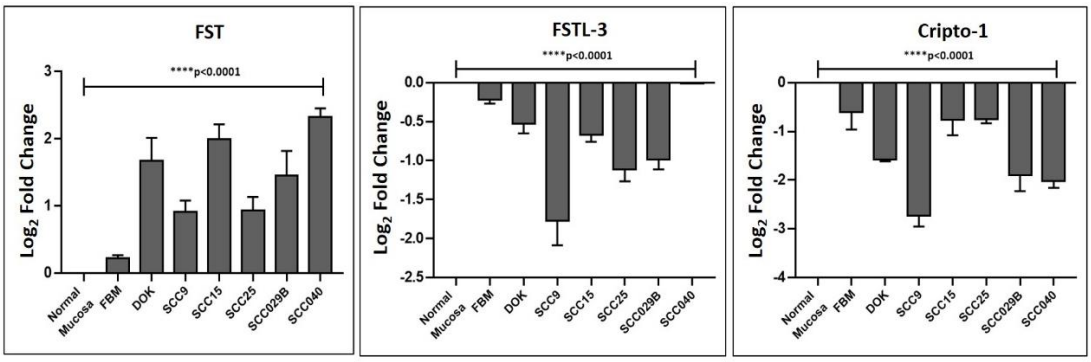

Figure 9: Transcript expression of negative regulators of Activin signaling in OSCC cells

5.1.3. Activin / Inhibin receptors are differentially expressed in oral cancer cell lines

As mentioned earlier, Activins ligands are secreted molecules that function by binding to receptor type II (ACVR2A / ACVR2B) on the cell membrane, that triggers activation of Activin receptor type I (ACVR1B or ALK4), to further initiate the cascade of signaling events. We also assessed the expression of the Activin receptors in oral cancer cell lines by qRT-PCR and western blotting. We observed a differential expression of the two type II receptors of Activin, namely ACVR2A and ACVR2B. ACVR2A receptor transcripts were downregulated as compared to normal oral mucosa ($p=0.005$), while the ACVR2B receptor transcripts were upregulated in all oral cancer cell lines ($p=0.005$) (Figure 10a). At the protein level, ACVR2 (tested using an antibody that detects both ACVR2A and ACVR2B) receptor was expressed at varying levels in our oral cancer cell line panel (Figure 10b). Transcripts of the type I receptor ALK4 were also downregulated in oral cancer cell lines as compared to normal oral mucosa ($p=0.005$, Figure 10a). However, at the protein level, ALK4 was significantly overexpressed in 3/6 oral cancer cell lines while it was downregulated in 3/6 cells ($p<0.001$, Figure 10b). Thus, the expression analysis of Activin receptors demonstrated a differential expression across oral cancer cell lines. However, a downregulation in their expression does not indicate a complete absence of the protein; indicating the possibility of the signaling pathway downstream of Activins through SMAD2/3 proteins to be still functional.

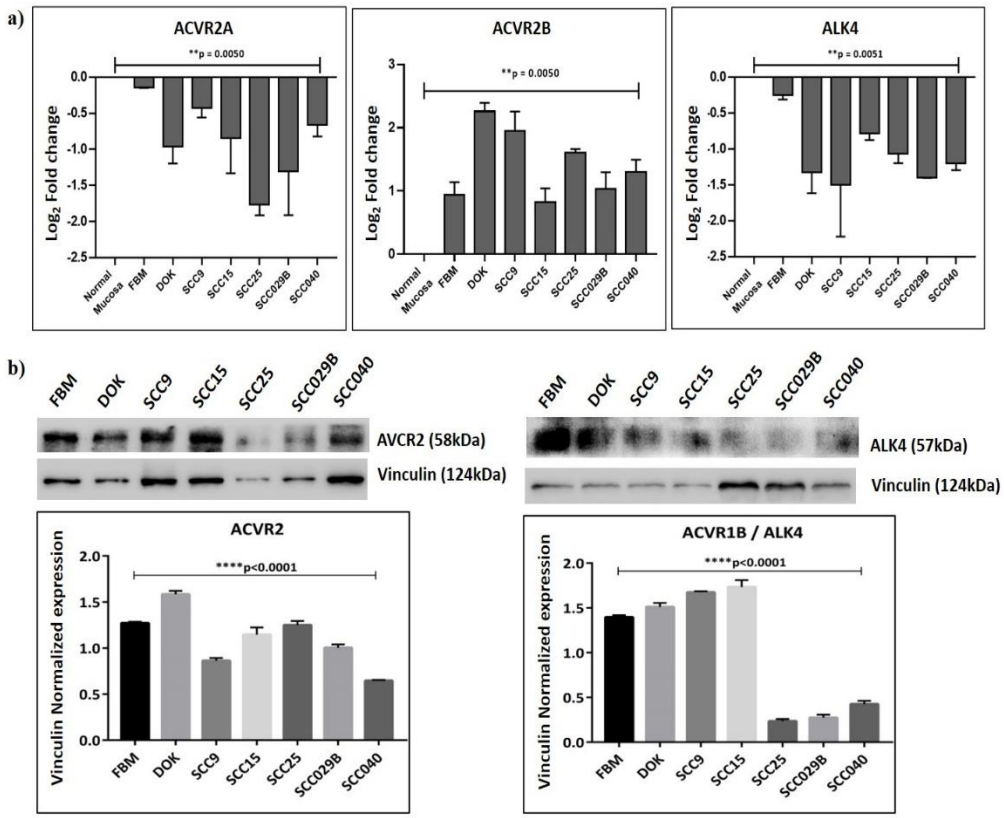

Figure 10: Expression of Activin Receptors in OSCC cells: a) Transcript expression of Activin receptors compared to normal oral mucosa b) Protein expression of Activin receptors compared to normal oral mucosa

5.1.4. Canonical Activin-SMAD pathway is functional in oral cancer cell lines

We next examined the expression of SMAD2 and SMAD3, proteins that are phosphorylated downstream of Activins through their receptor-binding, and transduce the Activin signal to the nucleus. SMAD2/3 proteins were found to be expressed at varying levels in oral cancer cell lines (Figure 11a). Given the differential expression of Activin receptors and downstream signaling molecules (SMAD2/3), we examined whether the canonical Activin-SMAD2/3 signaling pathway is functional in oral cancer cells. For this, we treated AW8507 and SCC029B cells with recombinant Activin A, since Activin A was found to be the predominantly expressed isoform, and checked for the nuclear translocation of SMAD2. Upon treatment with recombinant Activin A, SMAD2 was found to

translocate to the nucleus, indicating that the Activin receptor machinery is functional and that recombinant Activin A could induce activation of the canonical SMAD pathway in these cells (Figure 11b).

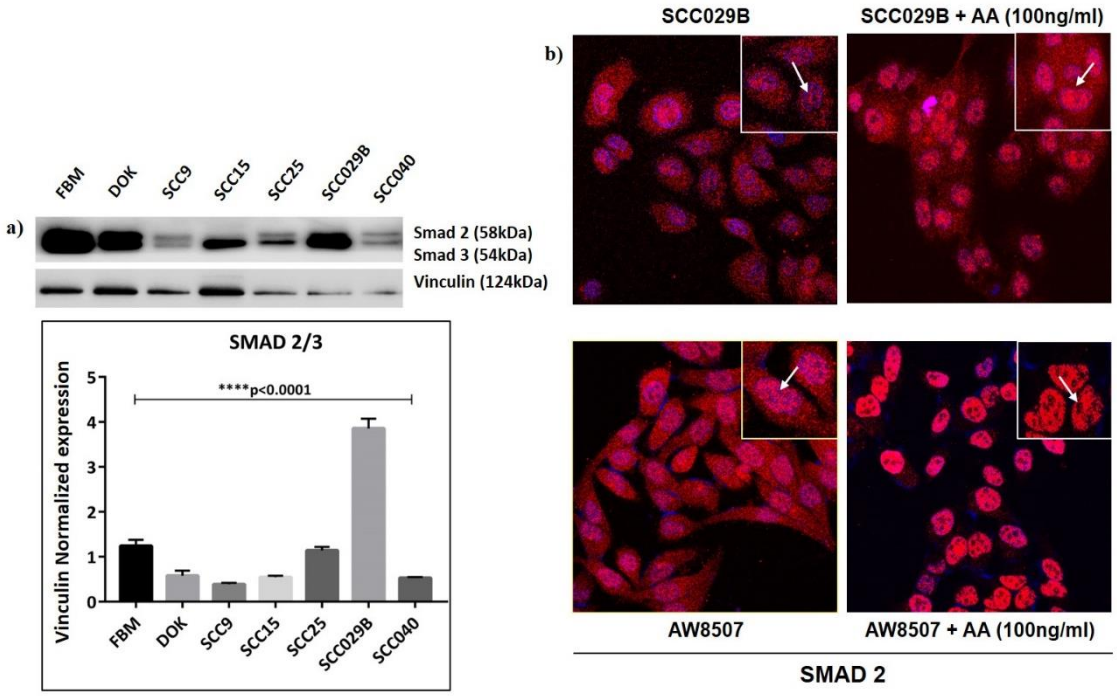

Figure 11: Expression and activation of SMAD2/3 in OSCC cells: a) Protein expression of SMAD2/3 compared to FBM cells b) Nuclear translocation of SMAD2 upon recombinant Activin treatment in oral cancer cell lines

The expression analysis of all the components of Activin signaling pathway in oral cancer cell lines is summarized below in Figure 12.

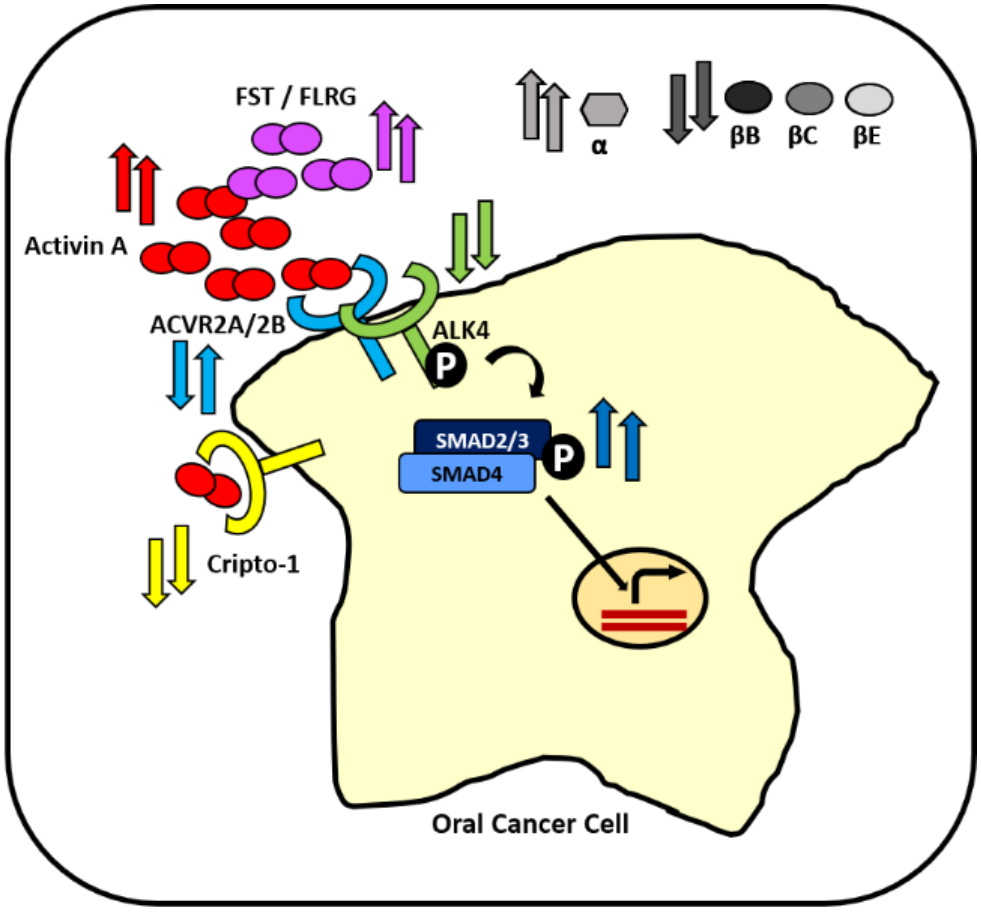

Figure 12: Expression of Activin Signaling Components in Oral Cancers

5.1.5. Activin βA subunit or monomer is significantly upregulated in paired adjacent normal and oral tumor tissues compared to normal oral mucosa

Our comprehensive expression analysis of all Activin / Inhibin forming subunits or monomers in oral cancer cell lines demonstrated that Activin βA is the predominantly expressed subunit and that it is further upregulated in oral cancer cells. We next went on to evaluate the expression of this subunit in oral cancer patient tumor samples. For this, we assessed the protein expression of Activin βA in 102 paired oral adjacent normal – tumor tissue sections by IHC and compared its expression to that in 18 normal oral mucosa samples. A combined H-score (0-300) was then calculated taking into consideration both percent positivity and staining intensity. We found an overexpression of Activin βA in 56% (57/102) of oral tumor tissues examined compared to normal oral mucosa tissues ($p<0.0001$, Figure 13). Interestingly, tumor-matched, adjacent histologically normal tissue sections also showed a significant overexpression of Activin βA ($p<0.0001$, Figure 13) compared to normal oral mucosa. A paired analysis of adjacent normal-tumor tissues showed that adjacent normal tissues express significantly higher Activin βA protein than their paired tumor counterparts ($p=0.007$), indicating that Activin βA upregulation could be an early event in oral carcinogenesis. It is noteworthy to recollect here that such an overexpression of Activin βA molecule was also noted in our cell line expression profile where the dysplastic oral cell line, DOK, which represents a precancerous state, also showed an overexpression of Activin βA (Figure 7).

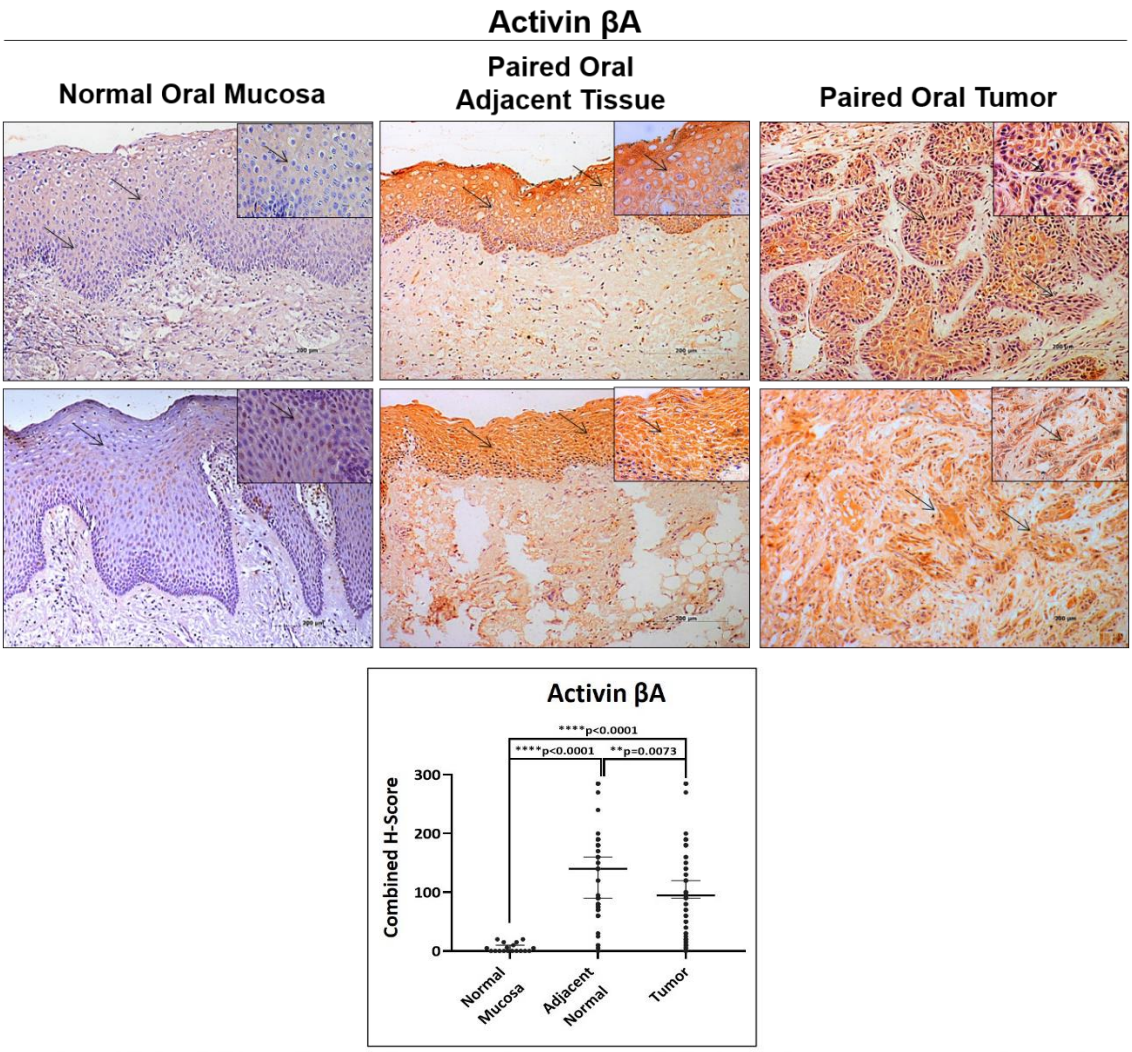

Figure 13: Activin βA protein expression in normal oral mucosa and paired oral adjacent normal-tumor tissue sections

5.1.6. Activin βA overexpression in oral tumors is associated with higher tumor stage, positive lymph node metastases and poor recurrence-free and overall survival in oral cancer patients

To understand the effect of Activin overexpression on oral cancer patient clinicopathological characteristics and survival, we analyzed the association between Activin βA expression and clinicopathological parameters of patient tumors. To this end, we dichotomized the patient tumor cohort into Activin βA low and high expressors based on median H-score in tumor samples (H-score =

95). The two groups were then analyzed for associations between Activin βA expression and clinicopathological characteristics of tumors such as age, gender, tumor stage (T stage), lymph node metastases or positivity (N stage), differentiation status, primary site of tumor and tobacco and alcohol consumption habits, using Chi-square tests. Activin βA overexpression was significantly associated with advanced tumor stage (T3-T4 vs. T1-T2, p=0.021, Table 6) and positive lymph node metastases (N+ vs. N0, p=0.045, Table 7) in our cohort of oral cancer patients. There was no significant association observed between Activin βA overexpression and other tumor/patient characteristics examined. We next analyzed the influence of Activin A overexpression on recurrence and survival in our patient cohort. Further, Activin A overexpression was associated with poor recurrence-free survival (p=0.013) as well as overall survival (p=0.024) in these patients (Figure 14), indicating that this molecular alteration is an indicator of poor prognosis in oral cancer.

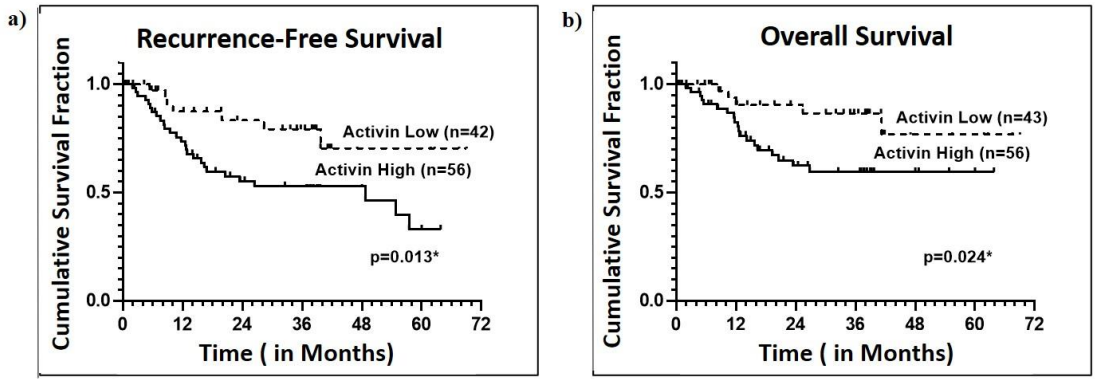

Figure 14: Kaplan-Meier Curves for Activin A overexpression in oral cancer patient cohort (a) Recurrence-Free Survival (b) Overall Survival

Table 7: Association of Activin βA expression with clinicopathological parameters in oral tumors

	Activin A over expression (%)		*P* value
	No	Yes	
Age			
Range	26-80	30-76	0.9205
Mean+/- SD	52.6+/-13.5	53.7+/-11.8	
Gender			
Males	43 (47)	49 (53)	0.1058
Females	2 (20)	8 (80)	
T Stage			
T1-T2	16 (64)	9 (36)	**0.0212***
T3-T4	29 (38)	48 (62)	
N Stage			
N 0	23 (56)	18 (44)	**0.0457***
N +	22 (36)	39 (64)	
Differentiation[a]			
WD	4 (50)	4 (50)	0.8978
MD	29 (43)	39 (57)	
PD	12 (46)	14 (54)	
Primary Site			
Tongue	14 (39)	22 (61)	0.6067
Buccal Mucosa	25 (49)	26 (51)	
Alveolus	6 (40)	9 (60)	
Habits			
Tobacco			
Yes	37 (47)	42 (53)	0.9294
No	1 (50)	1 (50)	
Alcohol			
Yes	3 (34)	6 (66)	0.6576
No	1 (50)	1 (50)	
No Data	7 (34)	14 (66)	
Total	**45**	**57**	

[a]WD: Well Differentiated; MD: Moderately Differentiated; PD: Poorly Differentiated
*Statistically Significant

5.1.7. Activin βA expression negatively correlates with p63 expression in oral tumor tissues

From our cell line expression analysis of Activin βA protein, we observed that all our cancer cell lines showed an upregulation of this molecule, expect two, namely SCC9 and SCC040 (Figure 8). We noted that both these cell lines do not express p53 protein (Figure 15). SCC9 also does not express its related protein, p63; while SCC040 expresses p63 protein. Additionally, SCC15 cells, that also lack p53 protein did show an overexpression of Activin βA. All other cell lines that overexpress Activin βA have mutant p53 protein. Together, these observations suggested a link between expression of Activin βA and that of mutant or inactivated p53 and/or p63.

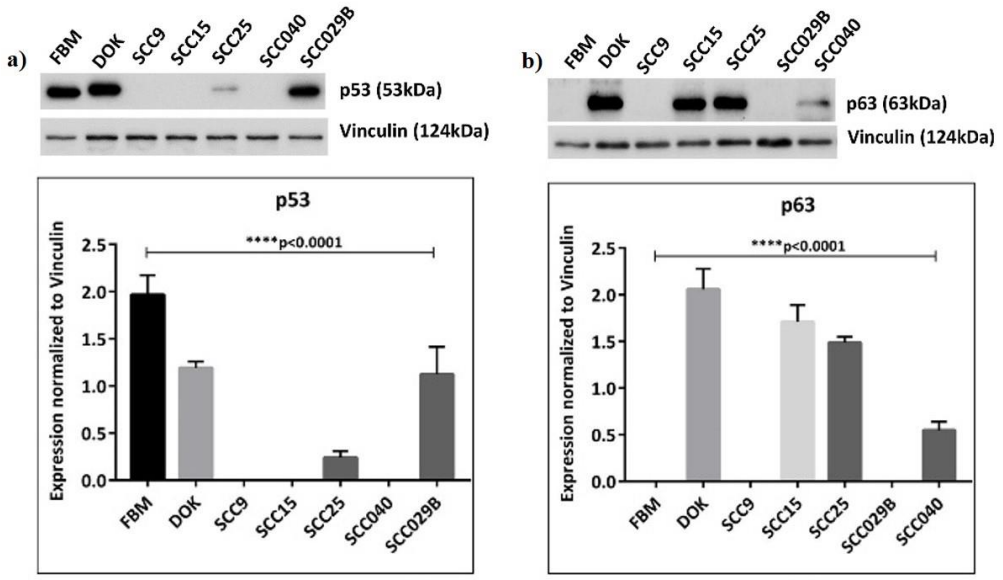

Figure 15: Protein expression of (a) p53 and (b) p63 in oral cancer cell lines compared to FBM cells

To investigate whether there exists any such correlation, we assessed the expression of p53 and p63 proteins by IHC in the same paired oral adjacent normal-tumor tissue samples used earlier for Activin βA expression analysis. Compared to adjacent normal tissues, p63 was found to be overexpressed in 59% (60/101) of oral tumor tissues, while p53 was overexpressed in 47% (48/101) of these. Recollecting here that Activin βA was found to be overexpressed in 56% of this tumor cohort, we analyzed the correlation between expression of these three proteins. We found that Activin βA expression was negatively correlated to p63 expression in 52.5% (53/101) of oral tumors. Although the correlation was weak, it was statistically significant ($r = -0.215$, $p=0.030$, Figure 16). In other words, majority of oral tumors (about 56%) from this cohort overexpressed either Activin βA or p63; however, about 29% (29/101) of oral tumors also exhibited a concomitant overexpression of both these proteins, while remaining 15% tumors (15/101) expressed both proteins at low levels.

A weak negative correlation was also detected between Activin βA and p53 protein expression in 53.5% (54/101) of oral tumors, although this was not statistically significant ($r = -0.146$, $p=0.143$, Figure 16). As mentioned earlier, p53 gene is frequently mutated in oral cancers and mutant p53 is known to highly stabilized. Therefore, high p53 expression detected by IHC in our oral tumor samples could be majorly mutant p53. Given the cell line expression analysis where presence or absence of p53 protein seemed to influence Activin βA expression, we tested whether p53 expression in tumor tissues has an impact on the Activin βA – p63 expression correlation. For this, we dichotomized the cohort

into p53 low and high expressors and reanalyzed the correlation between Activin βA and p63. We observed that the Activin βA-p63 negative correlation was exclusively concentrated strongly and more significantly in the p53 high expressors group (48/101 or 47.5% of the cohort, r = -0.394, p=0.005), while in the p53 low expressing group, there was no such negative correlation seen (53/101 or 52.5% of the cohort, r = 0.025, p=0.849). Cases 1 and 2 (Figure 17) represent p53 high expressing tumors with a strong Activin βA – p63 negative correlation, while cases 3 and 4 (Figure 17) represent p53 low expressing tumors with no negative correlation between Activin βA and p63.

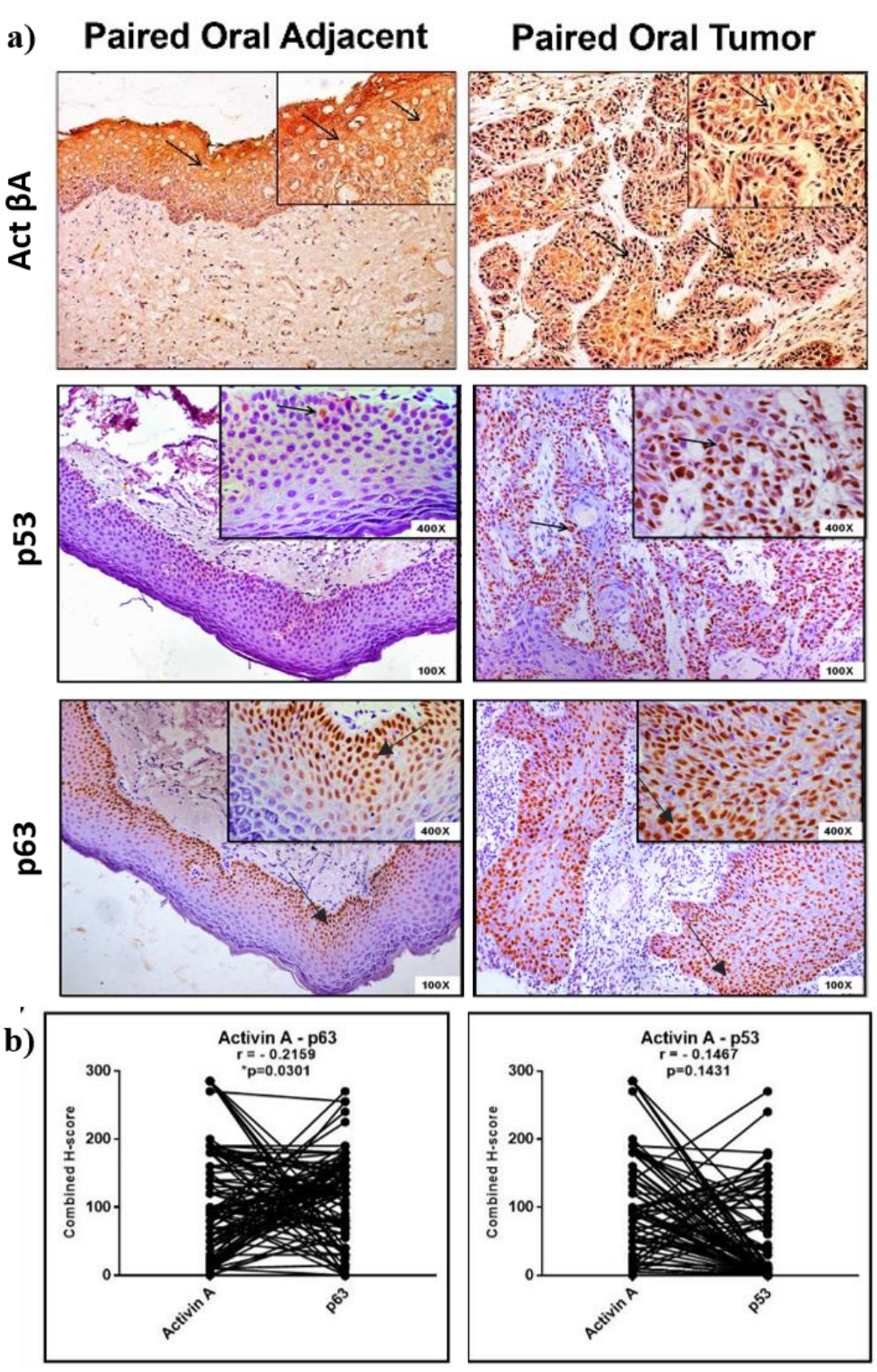

Figure 16: IHC expression of and correlation between Activin A , p53 and p63

(a) Representative IHC images for Activin A, p53 and p63 in paired oral adjacent normal – tumor tissue sections (b) Correlation between Activin A, p63 and p53 expression in oral tumor tissues

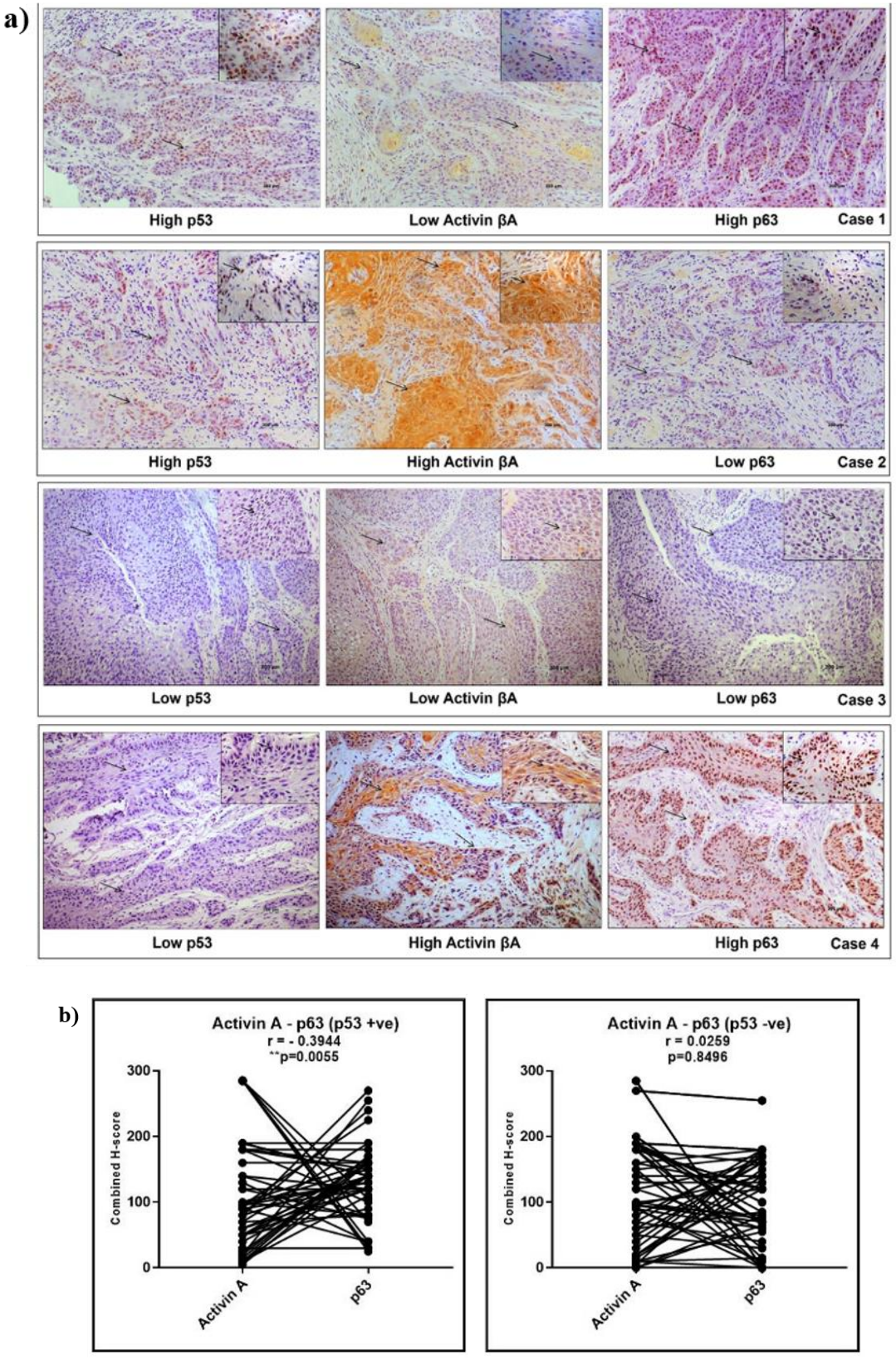

Figure 17: Correlation of Activin A – p63 IHC expression in p53 high/low expressing tumors

(a) Representative IHC images for Activin A-p63 expression correlation in p53 high/low expressing oral tumors (b) Correlation between Activin A-p63 in p53 high/low expressing oral tumors

5.1.8. Activin βA and p63 concomitant overexpression is an indicator of recurrence and strongly predict a poor prognosis in oral cancer patients

As described earlier, Activin βA overexpression was significantly associated with poor recurrence-free and overall survival. However, p63 overexpression alone was not significantly associated with survival, although high p63 expressing tumors exhibited a trend towards poor recurrence-free survival (p=0.075) as well as overall survival (p=0.205). We next analyzed the impact of Activin βA and p63 combined expression on the survival of oral cancer patients; for which we categorized the cohort into three groups: tumors with both Activin βA and p63 low expression, either Activin βA or p63 overexpression and both Activin βA and p63 combined overexpression. The group which expressed both these proteins at low levels exhibited significantly better survival; the one with either of the proteins being overexpressed showed an intermediate survival; while the group of tumors with a concomitant overexpression of both these proteins exhibited the worst recurrence-free survival (p=0.005, Figure 18a) as well as overall survival (p=0.014, Figure 18b).

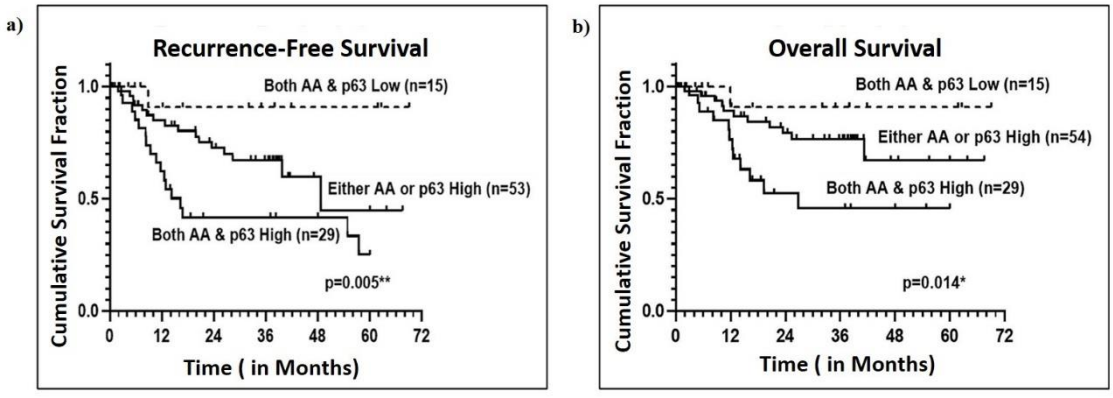

Figure 18: Kaplan-Meier curves for Activin A-p63 combined expression groups

(a) Recurrence-Free Survival for Activin A-p63 combined expression groups

(b) Overall Survival for Activin A-p63 combined expression groups

Further, we performed univariate and multivariate regression analysis using the variables Activin βA overexpression, p63 overexpression, Activin βA and p63 combined overexpression, T stage and N stage; using two or three variables at a time in order to maintain the variable : event ratio. Univariate Cox regression analysis indicated that Activin A overexpression could predict recurrence-free (HR= 2.74, CI: 1.19-6.31) as well as overall survival (HR= 2.96, CI: 1.10-7.95). However, p63 overexpression alone could not predict either (Table 8). Tumor stage alone could predict only recurrence-free survival (HR= 2.94, CI: 1.03-8.37). Interestingly, when a combined expression of both proteins was used for univariate analysis (Table 8), the group with concomitant overexpression of Activin βA and p63 could more significantly predict recurrence-free survival as indicated by a prominent increase in hazard ratio (HR: 10.66, CI: 1.41-80.19). Such a combined overexpression, however, could not significantly predict overall survival (HR: 7.57, CI: 0.98-58.39).

Table 8: Cox regression analysis on RFS and OS in oral tumours

Cox proportional Hazard Models on Recurrence-free Survival				
	Univariate Crude HR (95% CI)	P value	Multivariate adjusted HR (95% CI)	P value
T stage				
T3-T4 vs, T1-T2	**2.94 (1.03 - 8.37)**	**0.044***	2.39 (0.83 - 6.83)	0.104
N stage				
N>0 vs. N=0	1.32 (0.65 - 2.67)	0.437	NA	NA
AA[a] overexpression				
Yes vs. No	**2.74 (1.19 - 6.31)**	**0.017***	**2.83 (1.21 - 6.61)**	**0.016***
p63 overexpression				
Yes vs. No	1.90 (0.92 - 3.90)	0.08	2.03 (0.97 - 4.22)	0.057

Cox proportional Hazard Models on Overall Survival				
	Univariate Crude HR (95% CI)	P value	Multivariate adjusted HR (95% CI)	P value
T stage				
T3-T4 vs, T1-T2	3.86 (0.90 - 16.42)	0.067	3.49 (0.81 - 14.90)	0.091
N stage				
N>0 vs. N=0	2.26 (0.89 - 5.71)	0.083	NA	NA
AA[a] overexpression				
Yes vs. No	**2.96 (1.10 - 7.95)**	**0.031***	**2.74 (1.02 - 7.38)**	**0.045***
p63 overexpression				
Yes vs. No	1.72 (0.73 - 4.02)	0.211	NA	NA

Cox proportional Hazard Models on Recurrence-free Survival for Correlation groups				
	Univariate Crude HR (95% CI)	P value	Multivariate adjusted HR (95% CI)	P value
T stage				
T3-T4 vs, T1-T2	**2.940 (1.032 - 8.375)**	**0.044***	2.369 (0.829 - 6.773)	0.108
Correlation groups				
Either AA/p63 High vs. Both Low	4.880 (0.645 - 36.909)	0.125	4.212 (0.554 - 32.008)	0.165
Both AA & p63 High vs. Both Low	**10.660 (1.417 - 80.198)**	**0.022***	**8.756 (1.154 - 66.463)**	**0.036***
Cox proportional Hazard Models on Overall Survival for Correlation groups				
	Univariate Crude HR (95% CI)	P value	Multivariate adjusted HR (95% CI)	P value
T stage				
T3-T4 vs, T1-T2	3.862 (0.908 - 16.429)	0.067	3.341 (0.781 - 14.290	0.104
Correlation groups				
Either AA/p63 High vs. Both Low	2.943 (0.380 - 22.820)	0.302	2.446 (0.314 - 19.073)	0.393
Both AA & p63 High vs. Both Low	7.577 (0.983 - 58.394)	0.052	6.111 (0.787 - 47.433)	0.083

AA[a]: Activin βA, *Statistically Significant

NA: Not Applicable since variable did not qualify the criteria for Multivariate Analysis

The correlation in expression between Activin A, p53 and p63 proteins in oral tumor tissues and its impact on patient survival is summarized below in Figure 19.

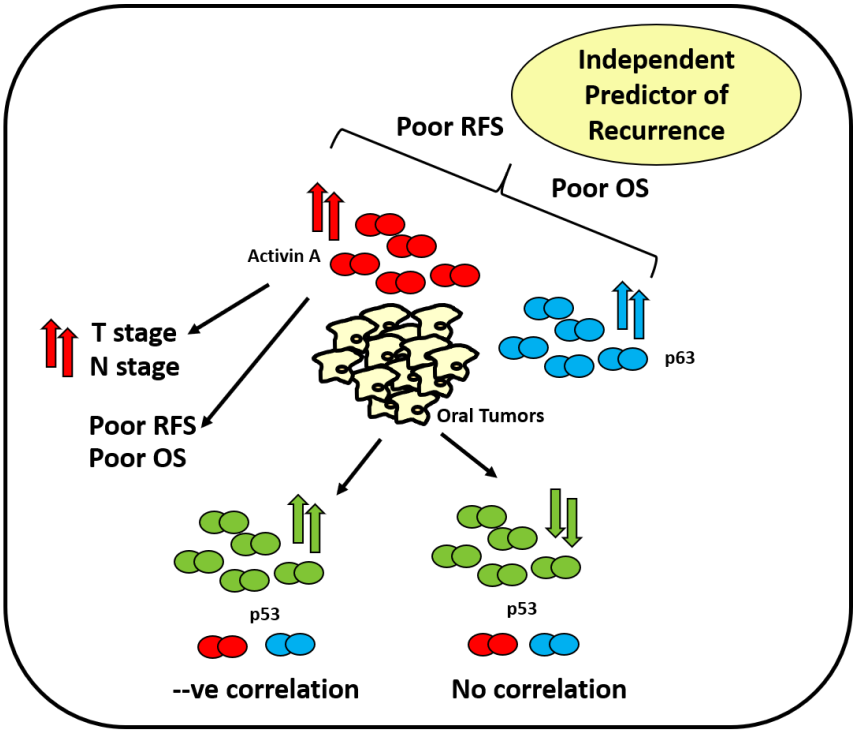

Figure 19: Correlation of Activin A, p53 and p63 in oral tumors and impact on Prognosis

5.1.9. Activin βA expression could be transcriptionally regulated by p63 and mutant p53

From our tissue expression data of the three proteins, we observed and reported that Activin βA and p63 expression was correlated, and that a concomitant overexpression of these two proteins was associated with poor patient prognosis. Further support to this observation came from a parallel study ongoing in the lab, where the role of p63 in oral cancer is being assessed. Under this study, we obtained shRNA-mediated knockdown of ΔNp63, the predominant p63 isoform expressed in oral cancer cells, and subjected these cells to a microarray analysis. We performed this analysis in two of our oral cancer cell lines, AW8507

(expressed R273H mutant p53) and SCC040 (no p53 protein expressed). In the differentially regulated genes (DEGs) so obtained, INHBA (Activin βA) gene was found to be significantly downregulated (log fold change: -1.2; adjusted p value: 0.004) in the p63 knockdown set of SCC040 cell line. Deregulated expression of few other Activin/TGF-β pathway genes was also found in both the cell lines upon p63 knockdown, suggesting that p63 could regulate the expression of the Activin/TGF-β family genes. Further, we validated this finding by qRT-PCR where we observed that knockdown of p63 leads to decreased Activin βA expression in oral cancer cells (Figure 20).

Since p63 is a transcriptional factor that can regulate gene expression transcriptionally by binding to gene promoters using p53 response elements, we hypothesized that p63 was regulating INHBA gene expression at the transcriptional level using p53 consensus sequences. To test this, we analyzed the INHBA gene 5' promoter and 3' enhancer region for p53 response elements using ALGGEN Promo tool.

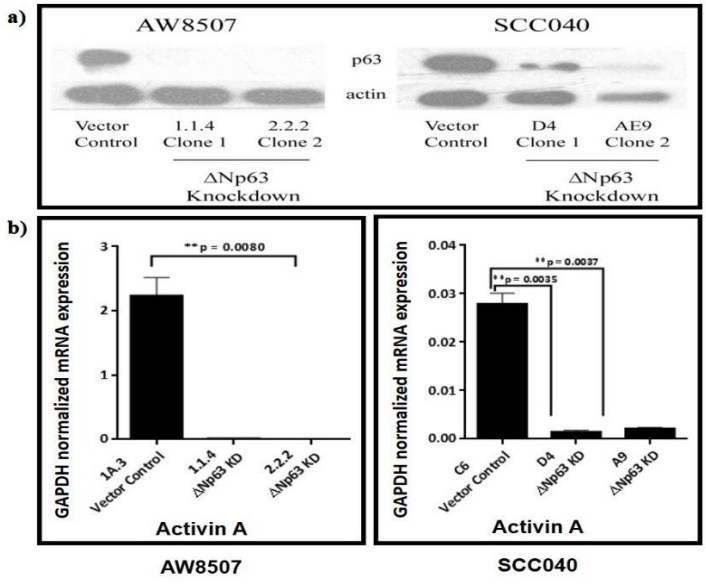

Figure 20: Effect of p63 knockdown on Activin A expression (a) p63 knockdown in AW8507 and SCC040 oral cancer cells (b) Activin A transcript downregulation in p63 the knockdown clones of AW8507 and SCC040

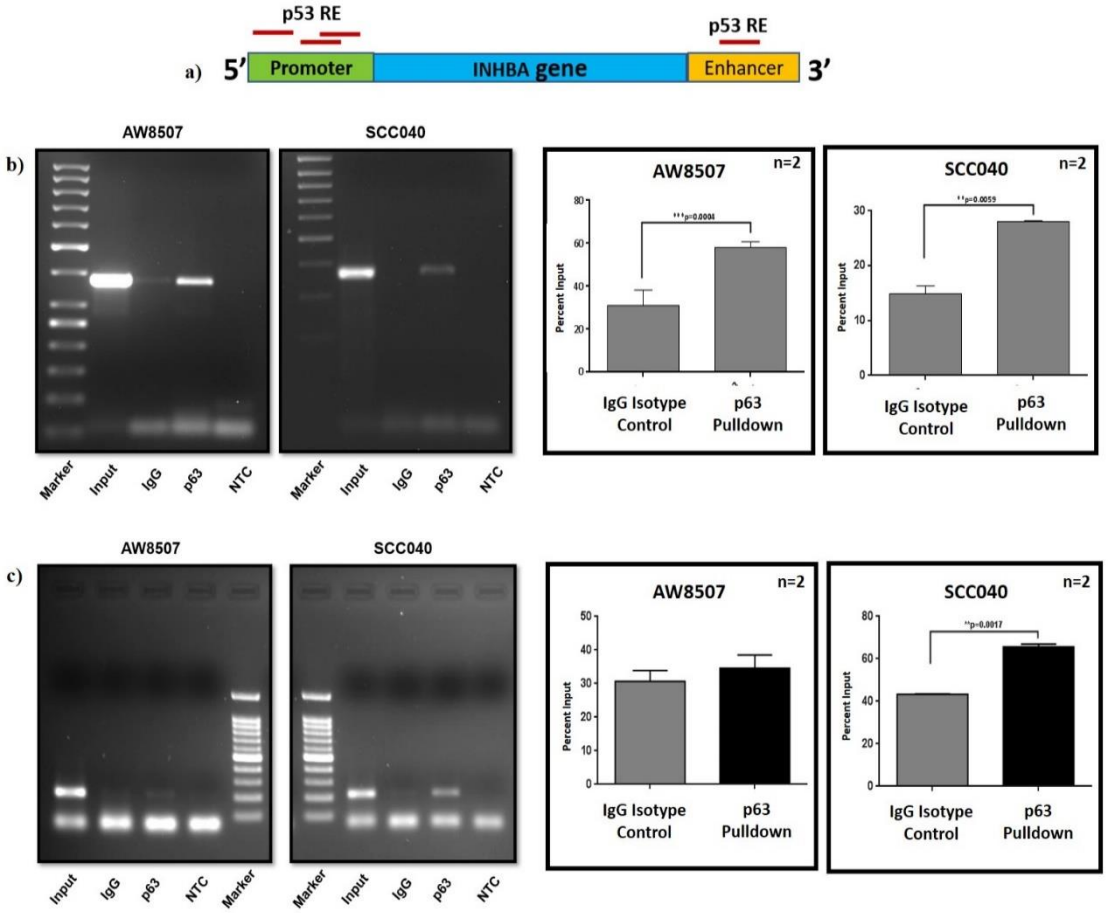

Figure 21: ChIP analysis of Activin A promoter using p63 pulldown (a) Activin A gene (INHBA) promoter schematic depicting p53 response elements (RE) (b) Activin A promoter enrichment in p63 pulldown ChIP assay (c) Activin A enhancer enrichment in p63 pulldown ChIP assay

We designed primers spanning these p53 binding sites for the promoter and enhancer of Activin βA gene, and performed a ChIP assay. For this, we used AW8507 and SCC040 cells and performed a chromatin immunoprecipitation using p63-specific antibody. Using the DNA from pull down cells, we performed semi-quantitative PCR for INHBA promoter and enhancer. We found an enrichment of the INHBA promoter as well as enhancer region in the p63-pull down cells compared to antibody-isotype control (Figure 21), indicating that p63 was bound to the Activin βA gene promoter and enhancer regions spanning p53 consensus binding sequences.

5.1.10. Microarray Validation and network analysis post p63 knockdown

As described earlier, under a parallel study in the lab, we generated p63 knockdown AW8507 and SCC040 oral cancer cells and subjected these cells to a microarray analysis on an Affymetrix GeneChip Human Primerview Array Platform (iLife Discoveries, Gurgaon, India). The purpose of using these two cell lines was that AW8507 expresses mutant p53 R273H, while SCC040 cells do not express p53 protein; however, both cell lines express p63 protein. We aimed to decipher the genes and pathways regulated by p63, and the influence of mutant p53 on the regulation of this repertoire of p63 target genes. The differentially regulated genes (DEGs) and pathways affected by p63 knockdown were analyzed from raw data by Biocos India Pvt. Ltd. This analysis identified 679 DEGs in AW8507, 502 genes of which were downregulated while 177 were upregulated post p63 knockdown. In SCC040, a total of 1532 DEGs were identified, of which, 778 genes were downregulated while 754 were upregulated post p63 knockdown. Comparing the DEGs from both cell lines revealed that 51 genes were commonly downregulated while only 7 genes were commonly upregulated in them,

together representing genes that were similarly affected by p63 knockdown, irrespective of p53 status. However, 57 genes were found to be downregulated in AW8507 but upregulated in SCC040 post p63 knockdown, while 22 other genes were upregulated in AW8507 but downregulated in SCC040 upon p63 knockdown. These represent the genes differentially regulated by p63 in the presence of mutant p53 (unpublished data). We validated some of these commonly and differentially affected genes using qRT-PCR. Network analysis of DEGs was done using Reactome and Gene Ontology (GO) classification of DEGs was also performed. We found the DEGs to be clustered in various pathways related to cancer, cell adhesion, integrin signaling, tight junction networks, angiogenesis, focal adhesion, p53 signaling, and the EGFR and Notch signaling. Since Activin A was downregulated in both cells post p63 knockdown (refer Figure 20), and since the Activin/TGF-β pathway has been implicated in cell migration, we next analyzed the functional significance of the p63-Activin A regulatory axis.

5.2. To study the functional role of Activins / Inhibins & their receptors in OSCC

5.2.1. p63-Activin βA transcriptional regulatory axis has functional relevance in oral cancer cells

From the oral cancer tissue expression data and from our microarray analysis of p63 knockdown oral cancer cells, we found an association between Activin A and p63 expression. Additionally, the ChIP analysis of Activin A promoter and enhancer using p63 antibody for pulldown, also demonstrated a transcriptional regulatory mechanism for Activin A expression by p63 transcription factor. Together, this made us hypothesize that p63 could be an upstream transcriptional regulator of Activin A, and Activin A in turn would be contributing to oncogenic

mechanisms downstream of p63 regulation. Since p63 knockdown in AW8507 and SCC040 cells lead to a decrease in migration along with decrease in expression of Activin A, we tested if this phenotype was mediated by Activin A. For this, we treated the p63 knockdown clones in both cell lines with recombinant Activin A to restore the lost Activin function, and assessed the migration of these cells. We found a significant increase in migration of these p63 knockdown cells, comparable to the extent of migration seen in vector control cells, upon restoration of Activin A functions (Figure 22). This suggested that p63 could regulate cell migration in oral cancer, at least in part, through the Activin A signaling and that Activin A signaling possibly contributes to migration of oral cancer cells.

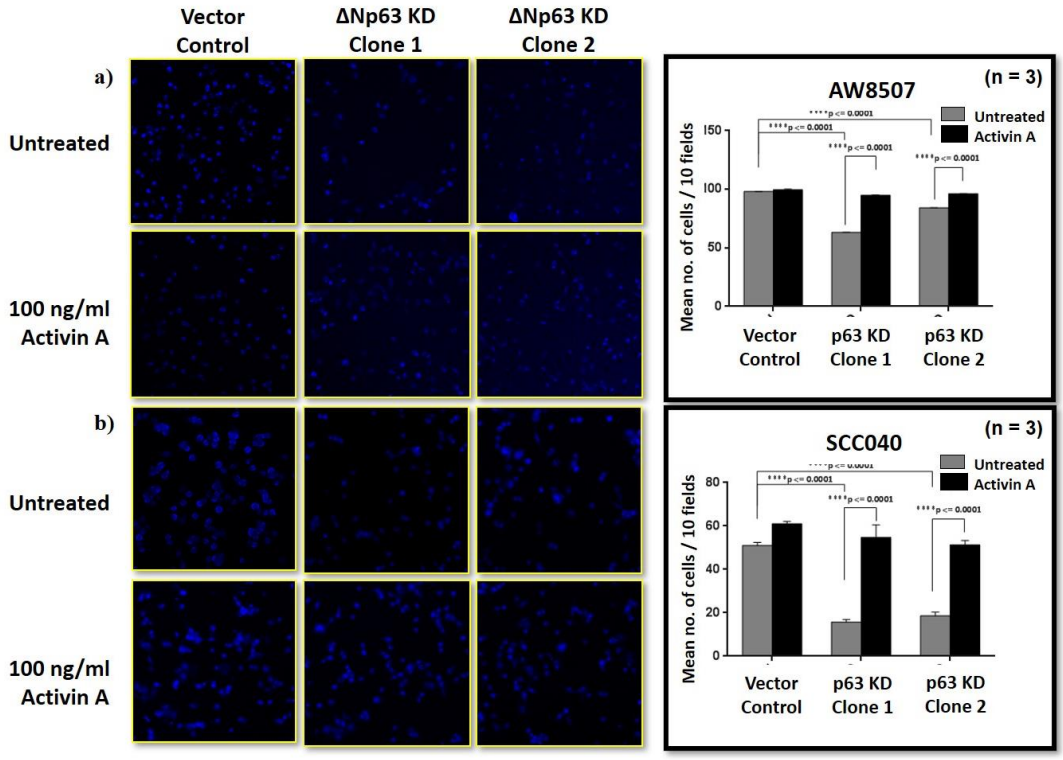

Figure 22: Rescue in migration of p63 knockdown clones of oral cancer cells (a) AW8507 and (b) SCC040 upon addition of recombinant Activin A

5.2.2. Recombinant Activin A promotes EMT, migration and invasion of oral cancer cells

To further test the contribution of Activin A to oral cancer phenotypes, we treated SCC9 oral cancer cells that exhibit very low expression of Activin A, with recombinant Activin A, alone or in combination with its antagonist Follistatin (FST), and assessed its effect on the invasion and migration of these cells. We found that recombinant Activin A treatment led to increased invasion and migration of these cells, which was attenuated by a combined treatment of Activin A and FST, emphasizing its specificity (Figure 23). Cancer cells undergo Epithelial-Mesenchymal Transition (EMT) to acquire the invasive and migratory phenotypes. We therefore tested the effect of recombinant Activin A treatment on EMT in SCC9 cells by assessing the expression of epithelial marker, E-cadherin (E-Cad), and mesenchymal markers, N-cadherin (N-cad) and Vimentin. We found a slight decrease in the E-cadherin expression, while a significant upregulation of both the mesenchymal markers, N-cadherin and Vimentin was observed upon treatment with recombinant Activin A (Figure 24).

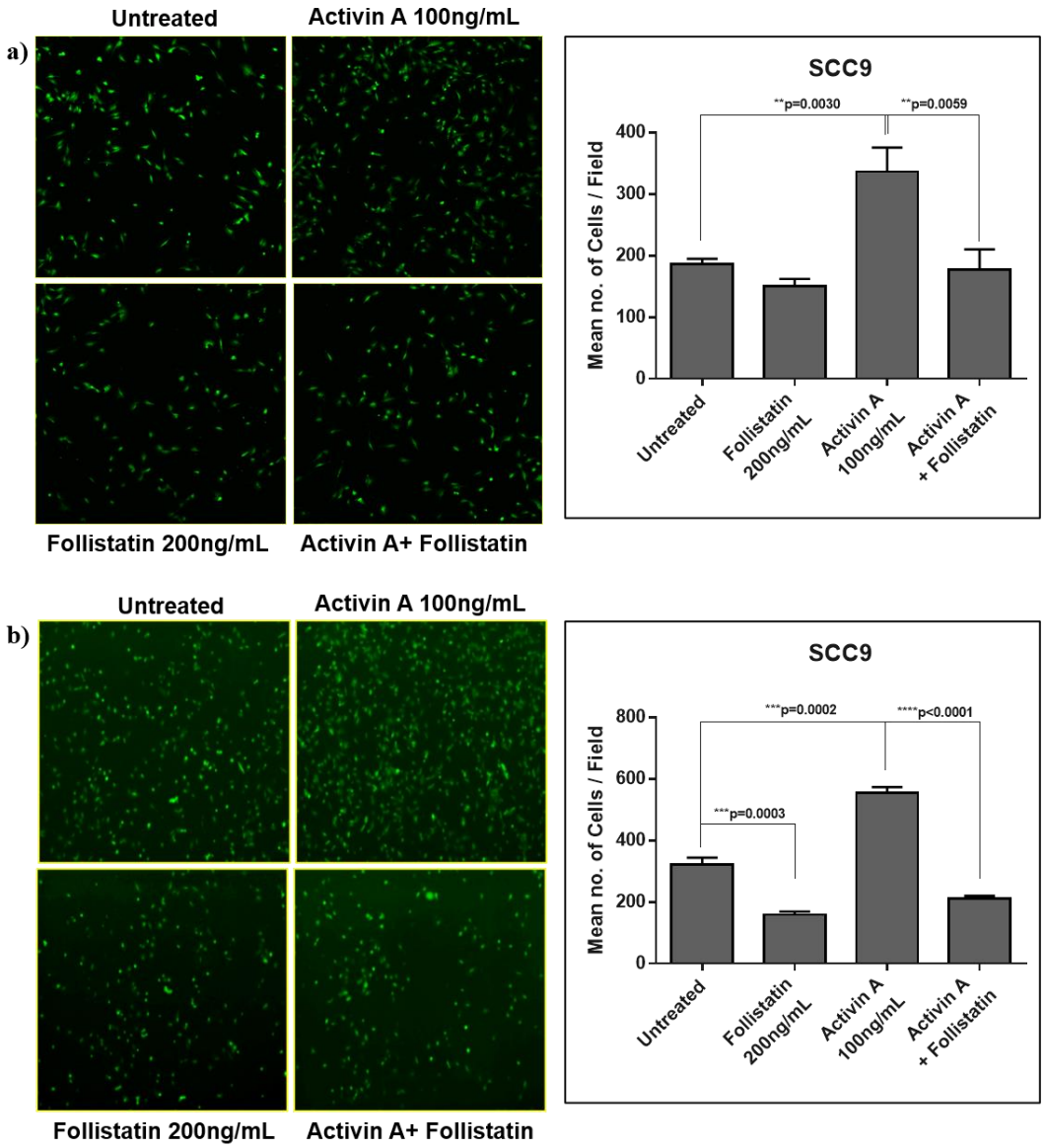

Figure 23: Effect of recombinant Activin A and Follistatin on SCC9 oral cancer cells (a) Effect on cell invasion (b) Effect on SCC9 cell migration

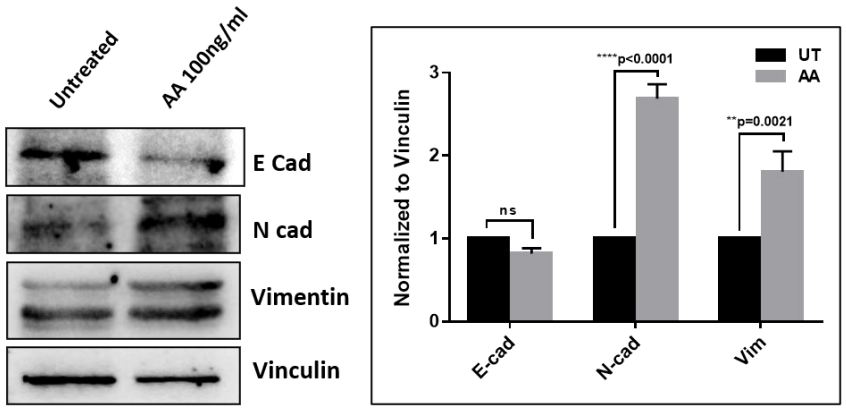

Figure 24: Expression of EMT Markers upon recombinant Activin A treatment

5.2.3. Recombinant Activin A induces canonical SMAD and non-canonical MAPK signaling in oral cancer cells

To assess the mechanisms downstream of Activin A that may be contributing to the phenotypes affected, we analyzed the activation of canonical SMAD2/3 as well as non-canonical MAPK signaling post recombinant Activin A treatment. Recombinant Activin A induced phosphorylation of SMAD2, which was attenuated upon a combined treatment with recombinant FST, indicating an activation of the Activin-SMAD2 canonical pathway (Figure 25a). We also observed a decrease in the phosphorylation of ERK1/2 in a time dependent manner (Figure 25b), while the other two MAPKs, p38 and JNK1/2 were phosphorylated and activated within 15 minutes of Activin A treatment (Figure 25c and 25d). Together, these results indicated that both canonical and non-canonical Activin A signaling mechanisms are functional in oral cancer cells and may be contributing to the cell migration, invasion and EMT phenotypes in these cells.

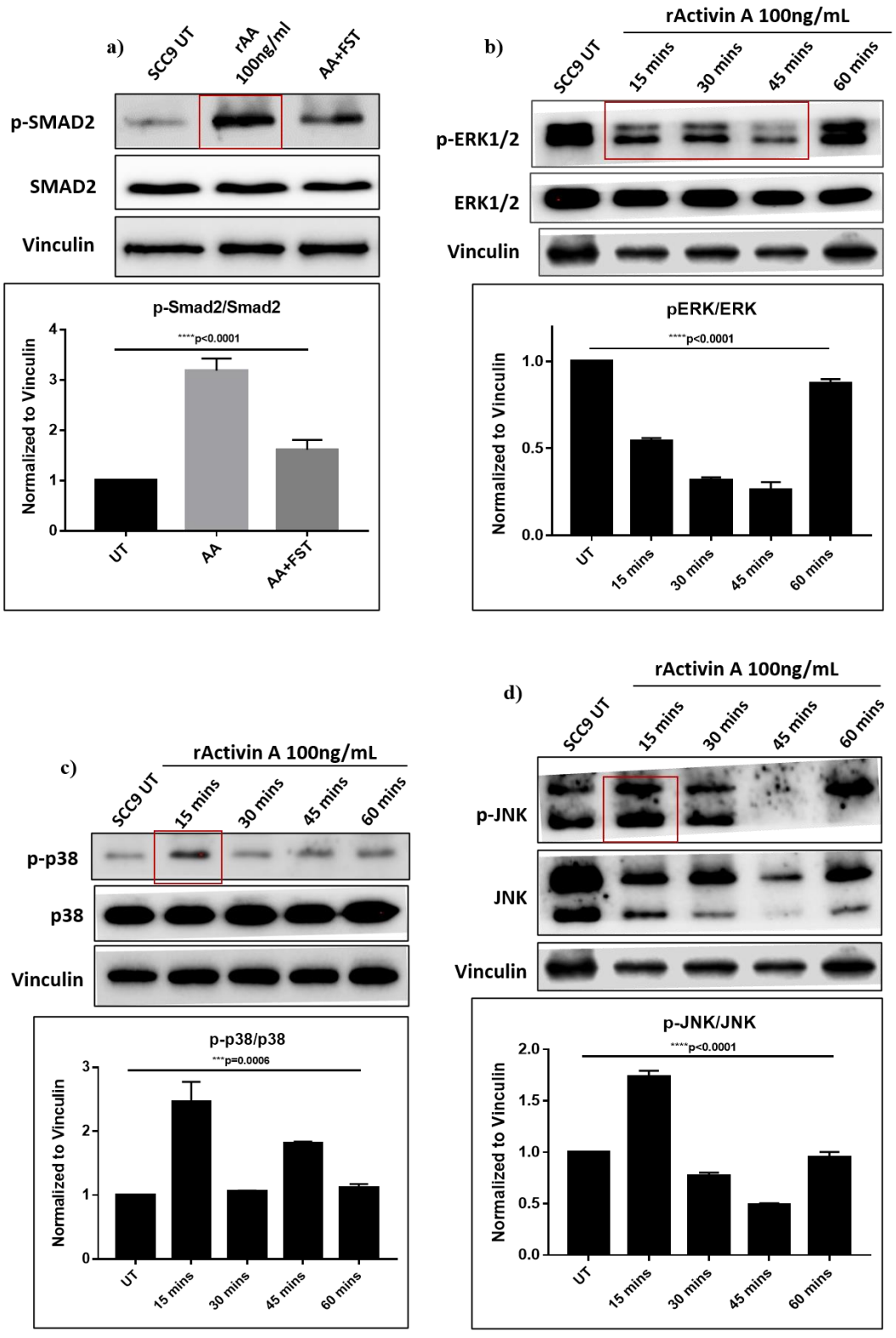

Figure 25: Activation of (a) canonical SMAD signaling and (b, c, d) non-canonical MAPK signaling upon treatment with recombinant Activin A to SCC9 cells

5.2.4. Knockdown of Activin βA leads to decreased migration and invasion of oral cancer cells

We next used the siRNA-mediated knockdown strategy to deplete Activin A from oral cancer cells expressing the same. For this, we used 50nM of Activin A siRNA to obtain Activin A knockdown in AW8507 cells (Figure 26a). Wound healing assay was performed after 48 hours of Activin A knockdown and Transwell Matrigel invasion assay was performed after 24 hours of knockdown to assess the effect of Activin A knockdown on the cell migration and invasion respectively. We could observe a significant decrease in the cell migration capacity (Figure 26b) and cell invasion (Figure 26c) upon knockdown of Activin A, suggesting that Activin A promoted these oncogenic phenotypes in AW8507 cells. Both the assays were performed in duplicates and only in AW8507 cell line. When we tried to replicate the knockdown in AW8507 again, as well as in two other cell lines, SCC15 and SCC029B, we could not achieve the desired knockdown of Activin A. We tried using another lot of siRNA, but with no success. We could always observe a knockdown at the transcript level, but the depletion of protein was insignificant. We then tried using the shRNA strategy to generate a stable knockdown of Activin A. shRNA targeting Activin A was provided as a kind gift from Dr. Paturu Kondaiah, IISc, Bangalore, India. We generated colonies of Activin A knockdown clones and determined the knockdown of Activin A. We could observe about 70-90% transcript knockdown in multiple clones, but the protein level knockdown in these clones was only about 40%. With no success in achieving Activin A knockdown in oral cancer cells after multiple trials in multiples cell lines, we switched to other strategies to study the implications of

inhibiting Activin A signaling on oral cancer cells. We therefore used knockdown of Activin downstream molecules, SMAD2/3 and an inhibitor of Activin receptor, ALK4 in further studies.

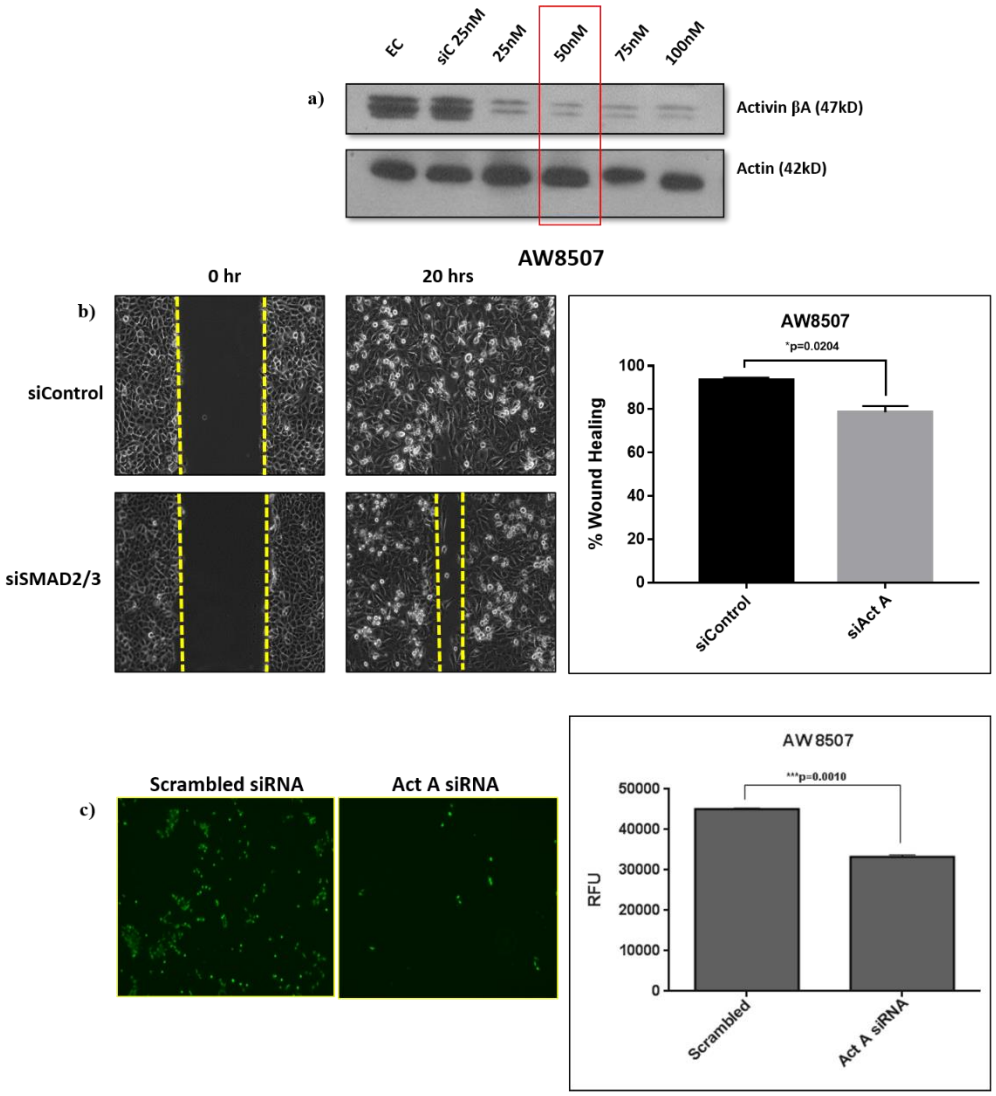

Figure 26: Effect of Activin A knockdown on AW8507 oral cancer cells (a) Knockdown of Act A in AW8507 cells using siRNA (b) effect of Activin A knockdown on migration (c) effect of Activin A knockdown on invasion

5.2.5. Knockdown of SMAD2/3 leads to decreased migration and invasion of oral cancer cells

To examine the contribution of canonical Activin-SMAD signaling to oral cancer phenotypes, we depleted SMAD2/3 using siRNA in AW8507 cells (Figure 27a) and performed wound healing and Matrigel invasion assays. The Matrigel invasion was decreased upon knockdown of SMAD2/3, as indicated by decrease in relative fluorescence units (RFU) post Calcein staining (Figure 27b).We also observed a significant reduction (about 30%) in migratory capacity of these cells upon knockdown of SMAD2/3, as indicated by less wound closure after 20 hours in these knockdown cells (Figure 27c)

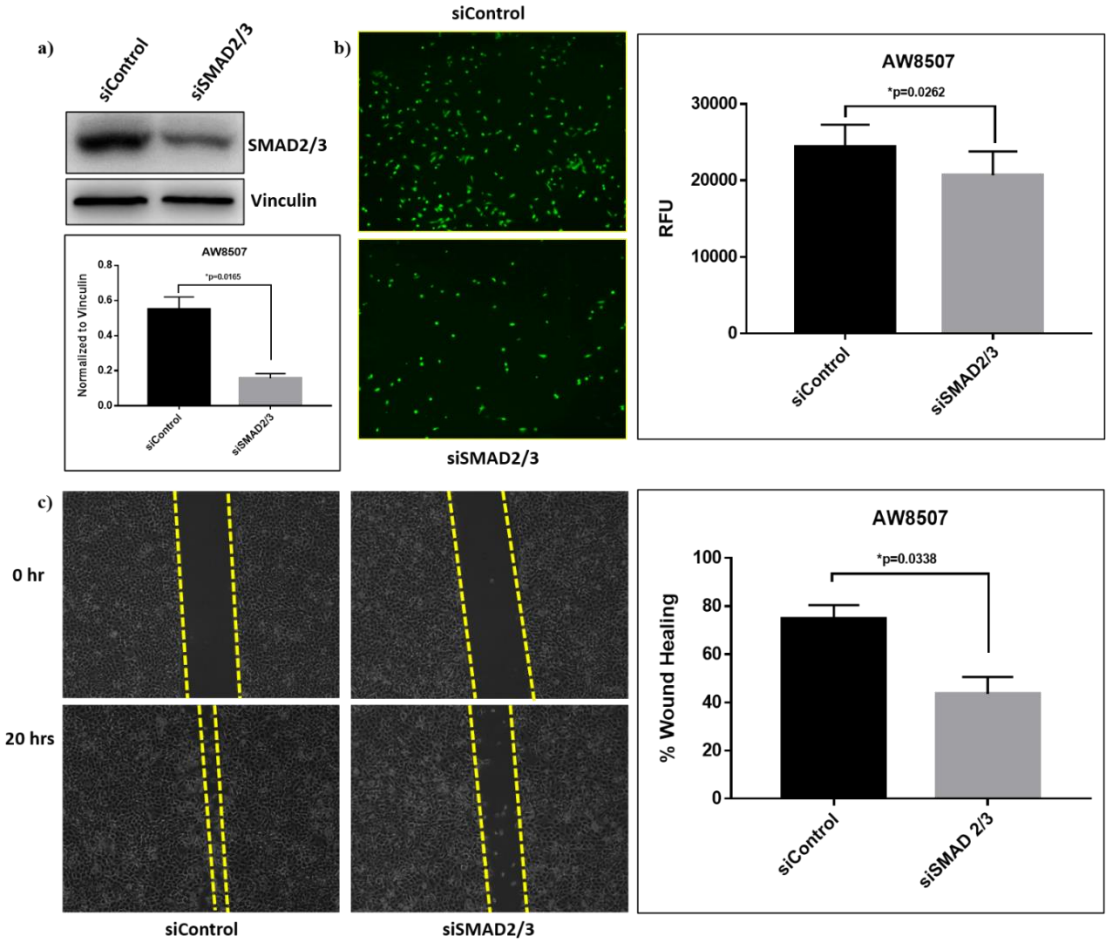

Figure 27: Effect of SMAD2/3 knockdown on AW8507 oral cancer cells (a) SMAD2/3 knockdown in AW8507 cells (b) invasion of AW8507 cells post SMAD2/3 knockdown (b) migration of AW8507 cells post SMAD2/3 knockdown

5.2.6. Blockade of Activin signaling by EW-7197 inhibitor leads to decreased migration and invasion of oral cancer cells

We also used another strategy to block the Activin/TGF-β signaling pathway by treating oral cancer cells AW8507 and SCC029B with an inhibitor, EW-7197 (also called Vactosertib) of this signaling. EW-7197 inhibits the kinase activity of ALK4/5 (Activin and TGF-β type I receptors respectively), thereby blocking the phosphorylation of SMAD2/3 and inhibiting the pathway. Activin ligands are also known to activate certain other non-canonical pathways downstream of

ALK4 activation, such as the MAPK pathway; although such non-canonical pathways could also be activated by Activins independently of the ALK4 activity. We treated the cells with EW-7197 and assessed the effect on migration and invasion of oral cancer cells by wound healing and Matrigel invasion assays respectively. A significant decrease in the percentage of wound healed was observed upon treatment with the inhibitor in both AW8507 (about 25%) and SCC029B (about 12%) cells (Figure 28). Similarly, a decrease in the invasion capacity was also observed in both these cells upon treatment with EW-7197 (Figure 28) as indicated by the relative fluorescence units (RFU) post Calcein staining.

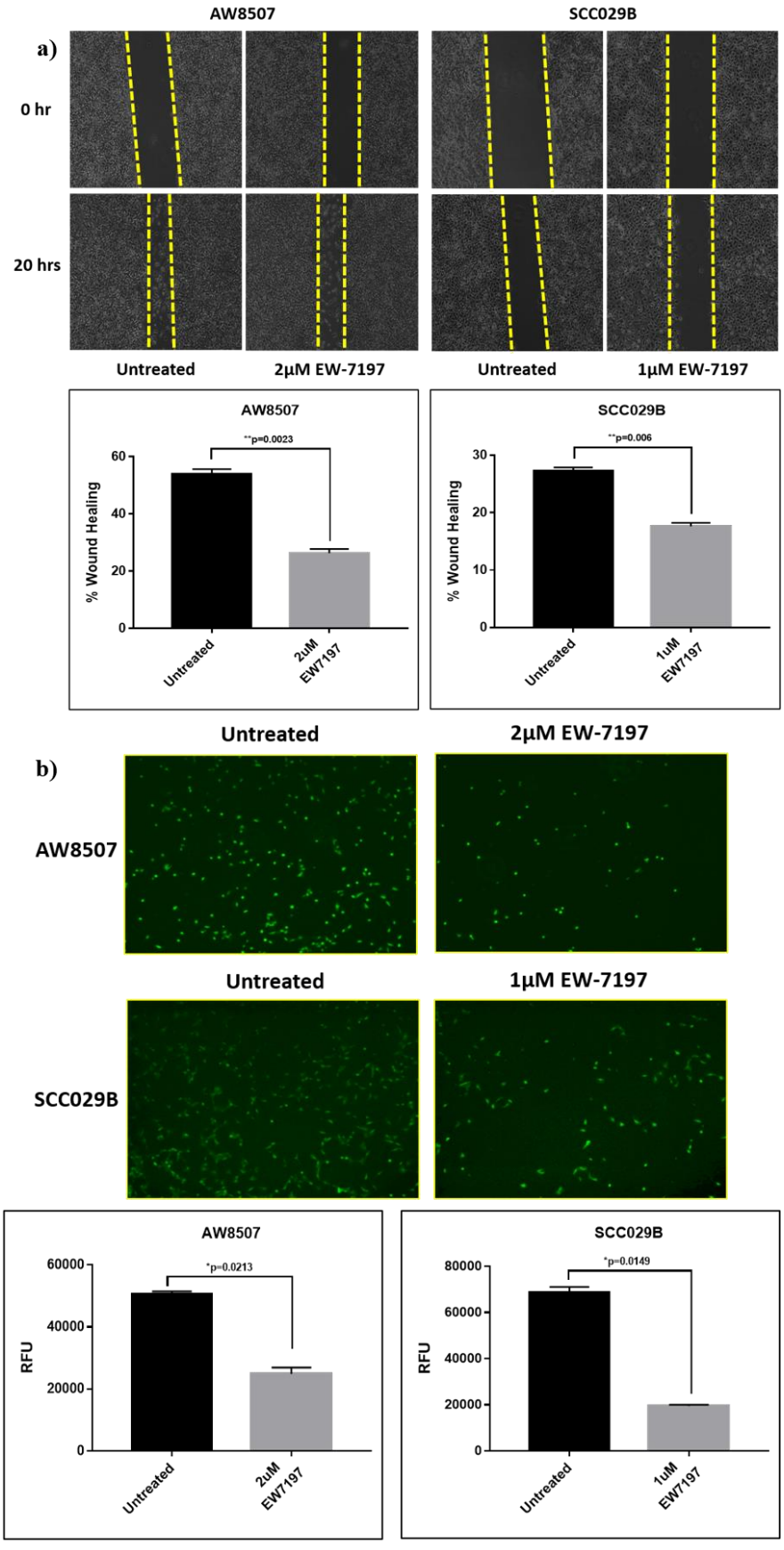

Figure 28: Effect of EW-7197 Inhibitor on AW8507 and SCC029B oral cancer cells (a) Effect of EW-7197 cell migration (b) Effect of EW-7197 cell invasion

The oncogenic role of Activin A and its downstream mechanisms are summarized below in Figure 29.

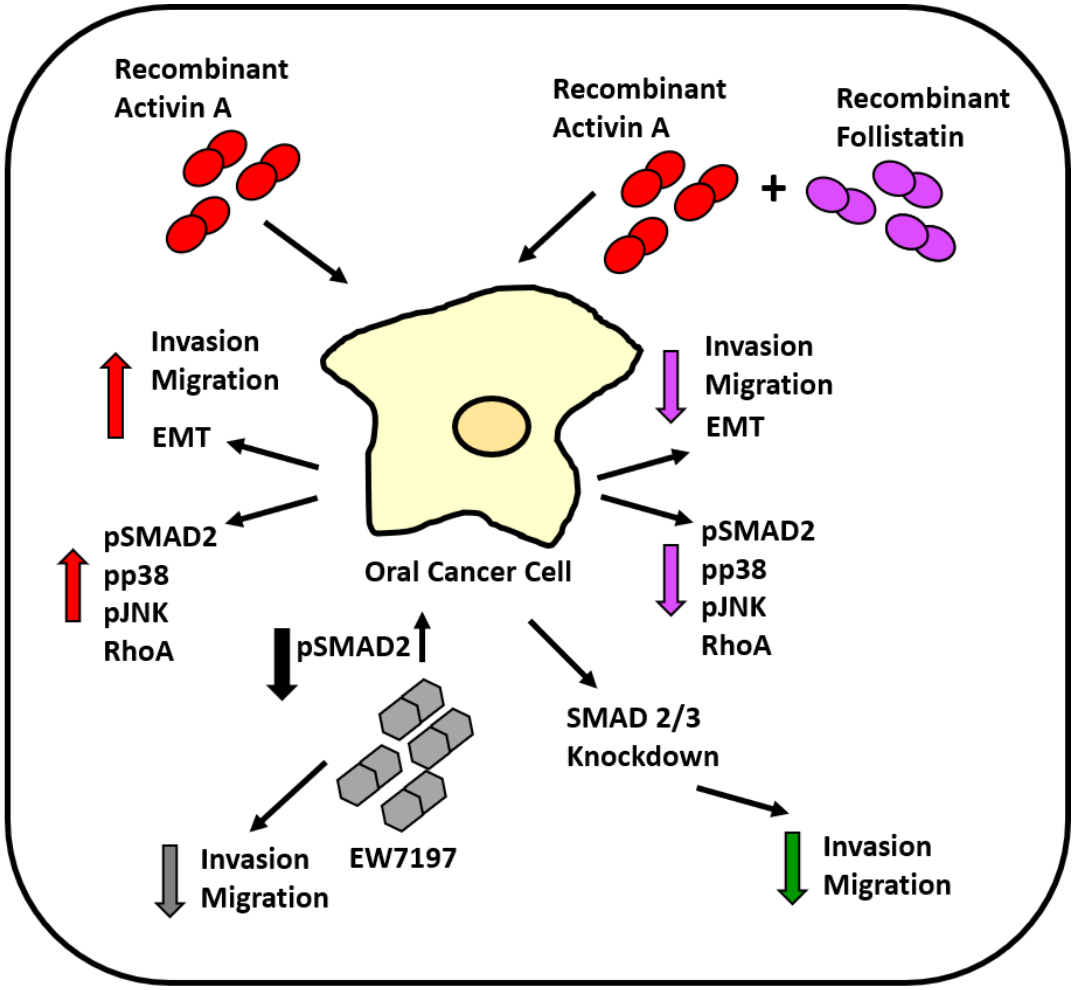

Figure 29: Oncogenic Role of Activin A in oral cancer cells

Chapter 6

Discussion

6.1. Deregulation of expression of Activin pathway components in oral cancer cells

Our study is the first comprehensive expression analysis of all the components of Activin/Inhibin signaling pathway in oral cancer cells. This included the Activin/Inhibin ligands, receptors and negative regulators of this pathway, as well as the downstream signaling molecules, SMADs. We found a prominent overexpression of one of the Activin ligands, Activin A in oral cancer cells compared to normal counterparts, while all other ligand molecules of this pathway were downregulated. This suggested that Activin A is the predominantly formed Activin in oral cancer cells; which may function by binding to its receptors on these cells, the expression of which was also found to be deregulated. Specifically, among the ligand binding receptors that initiate a cascade of signaling downstream of Activin A, namely ACVR2A and ACVR2B, ACVR2B was predominantly expressed in oral cancer cells, while ACVR2A was downregulated. At the protein level, expression of this ligand binding receptor was seen at varying levels. The next receptor that is activated upon ligand binding, namely ALK4, was also found to be expressed at relatively low levels in these cells. When we examined whether the Activin A signaling was functional via these receptors at the given level of expression in these cells, the phosphorylation and nuclear translocation of SMADs upon treatment with recombinant Activin A demonstrated that the canonical Activin A – ACVR2 – ALK4 – SMAD2/3 signaling axis is functional in these cells, despite the deregulated expression of both receptors. Thus, Activin A is found to be predominantly overexpressed and the Activin signaling pathway to be activated in majority of oral cancer cell lines tested. Earlier studies in HNSCC or OSCC have also reported an upregulated Activin βA gene (INHBA) expression, determined by large scale gene expression profiling where INHBA was one of the upregulated genes among the DEGs [58-62]. These reports however did not validate the deregulation in INHBA expression or did not functionally relate this

change in expression to oral carcinogenesis, since these studies were aimed at determining changes in the global gene expression and were not targeted towards determining the expression or role of Activin signaling. The first study that assessed the expression of Activin βA protein in oral cancers and correlated it to tumor characteristics and cellular phenotypes was by Chang et. al. group [63]. They demonstrated that both Activin βA subunit and FST were upregulated in oral tumors. This is in line with our observations of the upregulation of both Activin βA and FST transcripts in oral cancer cells. However, their study assessed the expression of the Activin βA subunit alone, without considering the expression of other subunits that would dimerize with this Activin βA subunit to form functional Activins/Inhibins. Without the knowledge on the relative expression of all subunits, it is not possible to predict which Activins or Inhibins are formed by incorporating the overexpressed Activin βA subunit. In fact, whether this Activin βA subunit that is overexpressed forms Activin dimers that would activate the signaling pathway or Inhibin dimers that would inactivate this pathway can also not be predicted from their study. Therefore, the effect of Activin βA upregulation on oral cancer cells or tumor tissues demonstrated by Chang et. al. group may not necessarily be attributed to a hyperactivated Activin signaling. However, in our present study, we assessed the relative expression of all Activin/Inhibin subunits together in our cell lines, and observed that only the Activin βA subunit is prominently overexpressed, while all other β subunits are rather downregulated; the Inhibin forming α subunit was also overexpressed, but it's expression relative to that of Activin βA was negligible. The possibility of Activin βA-βA homodimerization is thus maximum, and we can conclude that Activin A dimers (Activin βA-βA homodimers) are predominantly formed in oral cancer cells. This also formed the rationale behind our expression analysis in oral tumor samples where we assessed the expression of only the Activin βA subunit further.

Another report in oral cancer suggested that Activin A was rather expressed in and secreted by the myofibroblasts in the tumor microenvironment or stroma, and this secreted Activin A acts in a paracrine manner to promoter tumorigenesis of the associated oral cancer cells [84]. Activins are known to function in both autocrine and paracrine manner; it is possible that the oral cancer cells as well as stromal cancer associated fibroblasts both overexpress and secrete Activin A and these secreted molecules then act on the tumor cells in an autocrine (Activin A secreted from tumor cell acts on the same cell) and paracrine (Activin A secreted from neighbouring stromal cells acts on the tumor cells) manner. In fact, there is also a possibility that Activin A secreted from tumor cells acts on the stromal fibroblasts through its canonical pathway to induce gene transcription via SMADs, that in turn induces Activin A gene expression in these fibroblasts, since Activin A gene exhibits SMAD binding elements. This may then serve as a positive feedback loop for Activin A overexpression between the tumor cells and the associated stromal fibroblasts. This hypothesis is supported by a recent report in ovarian cancer, wherein they demonstrated that Activin A-SMAD2 signaling in tumor cells promotes stromal fibroblast activation [47].

A recent report has also demonstrated the upregulation of Activin βB protein in oral cancer tissues, and described it as a potential biomarker for metastases [8]. Our transcript expression data does not support this observation; indeed, we found the Activin βB subunit to be downregulated in oral cancer cells compared to normal oral mucosa. The contradiction in these results could be attributed to the use of different controls used to compare the expression of Activin βB in the two studies. We used normal oral mucosa samples as controls to compare cell line transcript expression, while their group compared its expression in oral cancer cell lines with human normal oral keratinocytes. With regards to oral cancer tissue samples, they compared the expression of Activin βB

with paired adjacent normal tissue samples. Also, their study did not assess the expression of other Activin/Inhibin subunits simultaneously, it is therefore difficult to predict which type of dimers incorporate this Activin βB subunit that is overexpressed. Another interesting report in HNSCC and esophageal squamous cell carcinoma (ESCC) has demonstrated the loss in expression of ALK4, the type I receptor of Activins [85]. They demonstrate that Activin A behaves as a tumor-suppressor when its type I receptor expression is intact, while it behaves oncogenic when this receptor is downregulated. Their study suggests that the oncogenic functions of Activin A may be independent of ALK4, and may occur mechanistically through non-canonical Activin pathways. Notably, we also found a downregulation of ALK4 receptor, and an oncogenic role of Activin A in OSCC cells. However, our studies with ALK4 inhibitor as well as SMAD2/3 knockdown, as described in further sections, do not rule out the role of canonical SMAD signaling via ALK4 in driving the oncogenic functions of Activin A.

Apart from the reports mentioned above in context of the expression of different Activin signaling components in HNSCC or OSCC, several other cancers also exhibit an upregulation of Activins and Inhibins, specifically Activin A. In ovarian cancer, apart from inducing stromal fibroblast activation [47], PITX-2 mediated activation of Activin A and TGF-β induced invasion of these cells [48]. Alongside the standard ovarian cancer serum marker CA125, serum Inhibin α expression, either as a free subunit or as α-βB dimer, has been widely used in clinical practice for diagnosis of certain ovarian cancer subtypes such as mucinous ovarian carcinoma or granulosa cell tumors [50]. High serum Activin A levels were also reported in women with cervical and endometrial carcinoma [52]. Several reports in breast cancers have also underlined the oncogenic role of Activin signaling. A recent study demonstrated that Activin A promotes invasion, EMT and metastatic growth of breast cancer cells [6]. Activin A and TGF-β were also shown to

promote the migration and invasion of breast cancer cells by inducing expression of miR-181, a microRNA known as a metastamir in breast cancers [57]. Contradictorily, Activin A has also been shown to bring about cell growth arrest in breast cancer cells signaling through the p38 MAPK kinase specifically [16]. However, the widely accepted notion is that Activin signaling promotes oncogenesis of breast cancers as well. A comprehensive study of Activin expression in malignant pleural mesothelioma, a subtype of lung cancers, also demonstrated overexpression and oncogenic function of specifically Activin A in this cancer subtype [86]. Overall, Activin A has been reported to be upregulated in several cancers and its widely accepted role is that it promotes oncogenesis. Our study also supports such an oncogenic role for Activin A in OSCC. In the present study, we explored beyond the overexpression of Activin A in oral cancers, and extended our analysis to the regulation of Activin A gene expression, correlation with regulatory proteins, significance in oral cancer prognosis and its oncogenic mechanisms in oral cancer.

6.2. Activin A overexpression could be an early event in oral cancers

Activin A overexpression observed in oral cancer cell lines was also validated in oral tumor tissues. We compared the immunohistochemical expression of Activin A in paired oral adjacent normal – tumor tissue samples and normal oral mucosa tissues. Supporting our observations in oral cancer cell lines, we found an upregulation of Activin A protein in oral tumor samples as well. Interestingly, when compared to the paired adjacent normal counterparts, the expression of Activin A in oral tumor samples was lesser. However, when compared to the expression within normal oral mucosa samples, which showed negligible expression of Activin A, both adjacent normal and their paired tumor

tissues exhibited significant overexpression of this protein. Thus, the pattern of expression of Activin A stage-wise from normal oral mucosa to adjacent histologically-normal but carcinogen exposed tissue exhibited an increasing trend; the overexpression was still sustained in tumor tissues compared to normal oral mucosa; however, it was lesser than the adjacent normal. This is in contrast to the earlier report [63], where they found Activin βA upregulation in oral tumors compared to adjacent normal tissues. Overall, our data indicated that Activin A was overexpressed in oral tumors and that this overexpression could be an early event occurring during oral carcinogenesis, where the tumor-adjacent histologically normal oral cavity tissue that would also be exposed to tobacco carcinogens, would have rather accumulated molecular alterations such as the overexpression of Activin A. In other words, molecular changes in the early stages of oral cancer occurring due to field cancerization phenomenon could include Activin A overexpression. Such early molecular alterations have been observed or reported in the context of other proteins in oral cancer as well [87,88], including one from our group where we observed an upregulation of two Survivin isoforms, Survivin 2B and Survivin 3α, in oral adjacent normal tissues [89].

6.3. Activin A overexpression as a marker for poor survival and independent predictor of prognosis in oral cancer patients

We tested the association of Activin A overexpression with the clinicopathological features of oral cancer patient tumors, including age, gender, tumor stage (T stage), lymph node metastases (N stage), differentiation grade of tumor, primary subsite of tumor, and tobacco & alcohol consumption habits. A significant association was found between Activin A overexpression and T stage; tumors which overexpressed Activin A

had a higher tumor stage (T3-T4). Activin A overexpression was also found to be significantly associated with positive lymph node metastases (N+ stage), suggesting that Activin A could be involved in regional metastases. No other patient characteristic tested was found to be associated with Activin A expression. Overall, the association of Activin A overexpression with higher T stage and positive N stage suggested it to be a promoter of oncogenesis in oral cancers. This inference was also reflected in the survival of these patients. Activin A overexpression was found to be associated with poor recurrence-free as well as overall survival; more the expression of Activin A, less was the time to relapse and less was the overall 5-year survival. Thus, Activin A was determined to be a poor prognostic factor in oral cancers. When analysed by Cox regression, Activin A overexpression could independently predict both recurrence-free and overall survival, making it an independent predictor of poor prognosis of oral cancer patients. In other words, Activin A overexpression alone was sufficient to predict early relapse and poor survival in our oral cancer patient cohort. Our results on the association of Activin A overexpression with tumor characteristics and patient survival are in concordance with earlier reports in oral cancer [63,64], where they also observed Activin βA overexpression to be associated with higher tumor grade and shorter disease-free and overall survival.

6.4. Correlation between Activin A and p63 and its clinical significance in oral cancer

From the cell line expression analysis, we observed that most of our oral cancer cells, except SCC9 and SCC040, overexpressed Activin A. A careful consideration of the cell lines used in this analysis revealed that SCC9 and SCC040 cells were ones that lack the

p53 protein, while majority of other cell lines tested express a mutant p53 protein. SCC15 was an exception since it also did not express p53, however, did express its related protein, p63; SCC9 did not express p63 either, while SCC040 expresses only p63. Overall, this suggested a possible correlation between the expression of Activin A and p53 and/or p63. There are reports that link p63 and/or p53 with the different TGF-β subfamilies including BMP signaling [66,68-78]. Additional hints that such a correlation could exist came from our microarray analysis post p63 knockdown done under a parallel study in the lab. Here, we found that Activin A was downregulated upon p63 knockdown in oral cancer cells, AW8507 (mutant p53 R273H) as well as in SCC040 (no p53 protein), suggesting that p63 could be regulating Activin A independently of p53. Since these are only few cell lines representing few oral cancer patients, we correlated the protein expression of the three molecules Activin A, p53 and p63 in oral tumor tissues from our patient cohort to generate confidence in our hypothesis of these molecules being correlated. We found that Activin A expression in oral tumors was negatively correlated to p63 and p53 expression, although the latter was not statistically significant. However, a closer analysis revealed that the negative correlation between Activin A and p63 was concentrated in tumors with high or stabilized p53, while no correlation existed between these two proteins in tumors with low p53 expression. We have earlier demonstrated that high or stabilized p53 detected by IHC is majorly (about 92%) contributed by mutant p53 [12]. Thus, it is possible that mutant p53 may influence the correlation between Activin A and p63; studies aimed at deciphering these regulatory mechanisms by mutant p53 and p63 are currently ongoing in the lab. Also, the negative correlation between Activin A and p63 was observed in half of the oral tumors tested, while the rest half exhibited either a positive correlation or no significant correlation. We therefore categorized the tumor samples based on combined expression of Activin A and p63 into both Activin A-p63

high & both Activin A-p63 low (together representing positively correlated samples) and either Activin A or p63 high (representing negatively correlated samples), and analysed their survival. The class of tumors that overexpressed both proteins, Activin a and p63, exhibited a poor recurrence-free as well as overall survival, indicating that a concomitant overexpression of both these proteins was an indicator of poor prognosis in oral cancer patients. Cox regression analysis further revealed that this concomitant overexpression of Activin A and p63 could independently predict poor recurrence-free survival, more strongly than that predicted by Activin A overexpression alone, in our patient cohort. Thus, Activin A and p63 concomitant overexpression was sufficient to predict relapse in these patients. The concomitant overexpression, however, could not predict overall survival. Together, this suggested that Activin A and p63 could together be involved in tumor recurrence or relapse, possibly by promoting loco-regional metastases (as indicated by the association of Activin A overexpression with positive node status). To the best of our knowledge, this is the first study that determined a correlation between Activin A and p63 in any cancer, and that these proteins could together act as poor prognostic markers in oral cancers.

6.5. Transcriptional Regulation of Activin A expression by p63 and its functional significance

Given the correlation between Activin A and p63 in oral tumor tissues and cell lines, and the fact that p63 acts as a transcription factor to regulate gene expression and transregulate p53 target genes, we explored the possibility of transcriptional regulation of Activin A gene by p63. In silico analysis revealed that the Activin A promoter and enhancer regions exhibit p53 consensus binding sites. ChIP assays performed using

primers spanning these consensus sequences and using p63 antibody for immunoprecipitation indicated that p63 could bind to the promoter and enhancer regions of Activin A gene, suggesting a possible transcriptional regulatory mechanism. A study done in cardiomyocytes had earlier demonstrated that the TAp63 isoform could associate with the Activin A gene (INHBA) enhancer region and regulate the same [69]. However, the isoform we are studying here is ΔNp63, since this isoform is predominantly expressed over the full-length TAp63 isoform in our cell lines tested (unpublished data). It is noteworthy to recall here that p63 knockdown led to decrease in Activin A transcript levels as determined by microarray hybridization and qRT-PCR. Further experiments to demonstrate such a regulatory mechanism may include luciferase assays using ectopic p63 expression in SCC9 cells that lack p63, co-transfected with Activin A promoter cloned in the pGL3 vector. Since SCC9 also lack p53 protein, this would serve as an ideal model system to test the influence of p53 on the regulation of Activin A gene by p63, where p53 could also be ectopically overexpressed in p63 overexpression background. Another way by which Activin A gene expression could be upregulated in oral cancers was reported by Bufalino's group, where they demonstrated that low expression of miR143/145 cluster leads to upregulation of INHBA gene in oral cancers [64]. We demonstrate here that INHBA gene could be a transcriptional target of ΔNp63, overexpression of which in oral cancers may lead to overexpression of the former.

Since p63 knockdown in AW8507 and SCC040 led to a decrease in Activin A levels as well as a decrease in migration of these oral cancer cells, and because Activin A could regulate cellular processes undergoing loco-regional metastases including cell migration, we postulated that p63 overexpression observed in oral cancers could lead to Activin A overexpression via transcriptional regulation, and Activin A in turn could promote the migration of oral cancer cells. Indeed, we could rescue the loss in migration of p63

knockdown clones upon their treatment with recombinant Activin A protein, suggesting our hypothesis to be true. Thus, p63 could transcriptionally regulate Activin A expression that in turn mediates the oncogenic function of p63 in promoting cell migration in oral cancer. The mechanisms further downstream of Activin A that could regulate cell migration may include both canonical SMAD signaling [7] and non-canonical MAPK signaling [5,16,40,43,45,46], and need to be explored in further comprehensive assays.

6.6. Oncogenic role of Activin A signaling in oral cancers and downstream mechanisms

We studied the role of Activin A in regulating EMT, migration and invasion of oral cancer cells, processes that directly contribute to loco-regional and distant metastases. Recombinant Activin A treatment of SCC9 cells, that endogenously express very low levels of Activin A, induced EMT, migration and invasion in these cells, supporting the oncogenic role of Activin A in oral cancers. This effect was attenuated with simultaneous Follistatin treatment highlighting the specificity of Activin A effect. Further analysis of Activin A treated SCC9 cells revealed that Activin A could stimulate both the SMAD2/3 pathway as well as the MAPK pathways, specifically p38-MAPK and JNK activation. These results suggested that Activin A could regulate oncogenic phenotypes in oral cancer cells through both canonical and non-canonical pathways. The precise contribution of each of these pathways could be tested by knockdown of downstream pathway components or by using inhibitors for each pathway, alone or in combination. Earlier reports in cancers other than oral origin have demonstrated the activation of p38-MAPK signaling pathway [16] and the AKT/GSK signaling pathway [5] downstream of Activin A in breast and ovarian cancer respectively. However, Activin downstream

signaling played contradictory roles in these cancers, inhibiting or promoting tumor growth in a cell-type specific manner. The mechanisms of Activin downstream signaling in oral cancers are yet to be determined. Our results suggest that both SMAD and MAPK pathways may contribute to the oncogenic roles of Activin A; the possibility of other mechanisms cannot be ruled out.

6.7. Targeting the Activin signaling in oral cancers

We used two strategies to inhibit the Activin downstream signaling in oral cancer cells and studied its effect on cell phenotypes. Firstly, we knocked down SMAD2/3 to inhibit the canonical signaling pathway of Activin A; we observed a significant decrease in the migration and invasion of the knockdown oral cancer cells, suggesting that Activin A regulates these properties, at least in part, by the canonical SMAD signaling. Next, we treated oral cancer cells with an inhibitor of Activin receptors, EW-7197. Also called Vactosertib, EW-7197 is an orally bio-available inhibitor, currently in phase I trials for advanced stage solid tumors. Another reason to select this inhibitor was that it blocks both the Activin and the TGF-β type I receptor activities, thus providing a broader inhibition of these related oncogenic pathways. Treatment of oral cancer cells with this inhibitor resulted in a significant decrease in migration and invasion of these cells, underlying the oncogenic role of Activin A in oral cancers and its possible targeting. Future experiments to assess the changes in activation of SMAD2/3 and MAPKs downstream of EW-7197 treatment are needed to understand the mechanisms of Activin A oncogenic functions. Also, combined use of EW-7197 along with MAPK inhibitors could be tested in order to achieve better inhibition of the Activin A oncogenic signaling in oral cancers. Earlier report by Chang et. al. used the siRNA-mediated depletion of

Activin βA subunit to demonstrate its oncogenic role in oral cancer, where they found that Activin βA promotes oral cancer cell proliferation, migration and invasion [63]. A study done in breast cancer using EW-7197 inhibitor has also demonstrated similar effects in attenuating oncogenic phenotypes and metastases to lungs [90]. Our present results are in concordance with both these reports where blockage of Activin downstream signaling by either depletion of SMAD2/3 or using EW-7197 inhibitor attenuates the migration and invasion of oral cancer cells. Further detailed studies using combination of inhibitors that inhibit multiple oncogenic pathways parallelly in oral cancer cells will help decipher the mechanisms downstream of Activins and may advance therapeutics.

Chapter 7

Summary and Conclusions

This study was designed to comprehensively analyse the expression of all components of Activin/Inhibin signaling pathway with an aim to determine the Activins/Inhibins that are differentially expressed in oral cancers and their contribution to oral oncogenesis. We demonstrate here that Activin A ligand from this molecular subfamily is significantly overexpressed in oral cancer cell lines and tissue samples; and that the hyperactivated Activin A signaling exhibits oncogenic functions in oral cancer by promoting epithelial-mesenchymal transition, cell migration and invasion, phenotypes that influence cancer metastases. In oral cancer patients, this reflects on the clinicopathological characteristics of their tumors as well as on patient survival. Patients with higher expression of Activin A exhibit tumors with higher T stage, positive lymph node metastases and a shorter recurrence-free and overall survival. Activin A overexpression in these tumors serves as an independent predictor of recurrence and survival of patients.

Our study is also the first to demonstrate a correlation between Activin A and p63 expression in oral tumors and a direct transcriptional regulation of the Activin A gene by p63 transcription factor in oral cancer cells. Notably, the correlation between Activin A and p63 was p53 dependent; tumors with high or stabilized p53 expression exhibited a negative correlation between Activin A and p63, while the tumors with low p53 expression did not exhibit such a correlation. This correlation between Activin A and p63 was associated with survival in patients; tumors concomitantly overexpressing Activin A and p63 were associated with shorter recurrence-free and overall survival. When taken together, the combined overexpression of these two proteins could independently predict recurrence in oral cancer patients, more efficiently than Activin A overexpression alone. Thus, we postulate that Activin A and p63 combined expression can be used as a marker for relapse and poor prognosis in oral cancers.

We also analysed the effect of inhibiting the signaling pathway downstream of Activin A on the phenotypes of oral cancer cells. Knockdown of the canonical pathway component, SMAD2/3 as well as inhibition of the activity of Activin A receptor, ALK4 using EW-7197 inhibitor, both resulted in attenuation of cell migration and invasion. A reverse effect on these phenotypes was observed when oral cancer cells lacking Activin A expression were treated with recombinant Activin A. We could also demonstrate that Activin A signaling stimulates the phosphorylation of SMAD2/3, p38-MAPK and JNK, thus activating both canonical and non-canonical downstream signaling pathways in oral cancer. Overall, we conclude that Activin A signaling, through its canonical SMAD pathway and non-canonical MAPK pathways, promotes oncogenic functions in oral cancers. Attenuating the action of Activin A in this cancer subtype using orally bioavailable inhibitors such as the one used in this study may aid in achieving loco-regional control of cancer, a long-known hindering factor in treatment success and one of the significant contributors of therapy failure in oral cancers.

The observations and inferences of this study are summarized in Figure 30 as below.

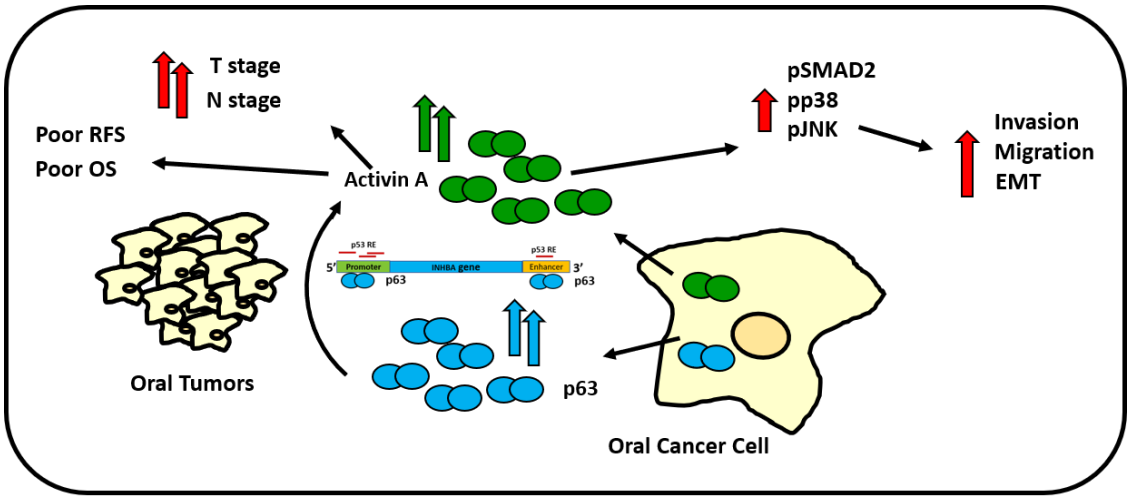

Figure 30: Role of Activin A signaling in Oral Cancers

Chapter 8

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Chapter 9

Appendix

Following are the Buffer/Media/ Reagent compositions used in this study:

1X Phosphate Buffered Saline (PBS)

NaCl	137mM
Na2HPO4	10mM
NaH2PO4	10mM
pH	7.4

1X Phosphate Buffered Saline containing Tween-20 (PBST)

Add 0.1% (v/v) Tween-20 to 1X PBS to prepare 1X PBST

30% Acrylamide Solution

Acryamide	29.2g
Bis-Acrylamide	0.8g
Distilled Water	Make up volume to 100ml

10X Electrophoresis Buffer for SDS-PAGE

Tris Base	30g
Glycine	144g
SDS	10g
Distilled Water	Make up volume to 1000ml

Transfer/Electroblotting Buffer

Tris Base	9g
Glycine	39.5g
Methanol	600ml
10% SDS	1ml
Distilled Water	Make up volume to 3000ml

1X Tris Buffered Saline (TBS)

Tris Base	50mM
NaCl	150mM
pH	7.4

1X Tris Buffered Saline containing Tween-20 (TBST)

Add 0.1% (v/v) Tween-20 to 1X PBS to prepare 1X TBST

Stripping Buffer

B-Mercaptoethanol	1.63g
10% SDS	40ml
1M Tris	12.5ml
Distilled Water	Make up volume to 200ml

10X Sodium Citrate Buffer for IHC

Tri Sodium Citrate	2.94g
pH	5.8 – 6
Distilled Water	Make up volume to 1000ml

Destaining/Fixing Solution

50% (v/v) Methanol

10% (v/v) Glacial Acetic Acid

Fast Green Staining Solution

Fast Green satin powder	0.1g
10% (v/v) Glacial Acetic Acid	100ml

2X Gel Loading Dye

1M Tris (pH 6.8)	0.625ml
β-Mercaptoethanol (add fresh)	0.5ml
Glycerol	1ml
SDS	0.23g
1% Bromophenol Blue	0.5ml
Distilled Water	Make up volume to 10ml

Coomassie Blue Staining Solution

CBB R-250	0.05g
Methanol	50ml
Glacial Acetic Acid	10ml
Distilled Water	Make up volume to 100ml

Incomplete IMDM Media

IMDM	(Iscove's Modified Dulbecco's Medium)
NaHCO3	3.024g/litre

Incomplete DMEM Media

DMEM	(Dulbecco's Minimal Essential Medium)
NaHCO3	3.7g/litre

Incomplete DMEM/F12 Media

DMEM/F12	(DMEM + Ham's F12 Medium 1:1)
NaHCO3	3.024g/litre

Complete IMDM/DMEM Media

Incomplete IMDM/DMEM	90ml
Fetal Bovine Serum (FBS)	10ml
100X Antibiotic-Antimycotic	1ml

Complete DMEM/F-12 Media (100ml)

Incomplete DMEM/F-12	90ml
Fetal Bovine Serum (FBS)	10ml
Hydrocortisone	40µg/ml
100X Antibiotic-Antimycotic	1ml

Trypsin-EDTA (1000ml)

EDTA Sodium salt	0.1g
D-glucose	1g
KCl	0.4g
NaCl	8g
Phenol Red	10g
NaHCO3	0.58g
Trypsin	0.25g

Erythrosine B dye

0.4g Erythrosine B powder dissolved in 100ml of 1X PBS

5X Tris Borate EDTA Buffer (TBE)

Tris Base	54g
Boric Acid	27.5g
0.5M EDTA pH 8	20ml
Distilled Water	Make up volume to 100ml

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ORIGINAL ARTICLE

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Concomitant overexpression of Activin A and p63 is associated with poor outcome in oral cancer patients

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Abstract

Background: The present study aims to comprehensively analyze expression of Activin signaling components in oral cancer and to determine the predominant Activin expressed and its influence on prognosis. As our preliminary studies indicated regulation of Activin gene by p63, we also propose to assess its correlation with p63/p53 in oral tumors and its impact on outcome.

Methods: Expression of Activin subunits, receptors, and regulators was assessed by qRT-PCR and Western blotting. Correlation between Activin A and p63/p53 expression was evaluated in oral tumors by immunohistochemistry and their association with clinical outcome was determined by Kaplan-Meier curves and Cox regression.

Results: Activin βA transcripts were upregulated ($P = .013$) in oral dysplastic and cancer cells compared with normal oral mucosa. Expression of Activin receptors and regulators was also altered. Activin βA protein was significantly upregulated in oral tumors and adjacent normal tissues compared with normal oral mucosa ($P < .0001$). Expression of Activin βA and p63 significantly correlated in oral tumors, correlation being stronger in tumors with high p53 ($r = -.394$, $P = .005$). Activin βA overexpression was associated with advanced tumor stage ($P = .021$), positive nodes ($P = .045$), poor recurrence-free survival ($P = .013$), and overall survival ($P = .024$), while its concomitant overexpression with p63 was a better predictor of recurrence-free survival (HR = 10.66, CI: 1.41-80.19).

Conclusions: Activin A overexpression is an early event in oral cancer pathogenesis and can independently predict survival. Moreover, in combination with p63 overexpression, it served as a better marker for poor prognosis. Activin A could thus be a promising target for improved outcome in oral cancer patients.

KEYWORDS

Activins, oral cancer, p53, p63, prognosis

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1 | INTRODUCTION

Oral cancers in India are associated with chronic tobacco-chewing habit exposing entire oral cavity to tobacco carcinogens for several years, resulting in genetically altered mucosa or oral field cancerization.[1] This process contributes to premalignant/malignant disease progression, multiple primary tumors, higher recurrences, and lymph node metastases leading to poor outcome. Identifying molecules that promote loco-regional metastases and associate with poor prognosis in oral cancers would aid in identifying therapeutic targets and improve patient outcomes.

The TGF-β superfamily proteins have been implicated in cancer progression and metastases,[2] and include the secreted proteins Activins/Inhibins. Activins are homo- or heterodimers of β subunits βA, βB, βC, and βE while Inhibins are heterodimers of α-β subunits. Activins function as ligands that bind to type II receptors ACV2A/2B on cell membrane, activating type I receptor ACVR1B/ALK4, further activating SMAD2/3 signaling cascade to regulate transcription of target genes. Inhibins also bind type II receptors but do not transduce any signal downstream, thereby inhibiting the Activin-SMAD signaling.[3] Importantly, relative abundance of these subunits dictates which dimer/s will be predominantly formed.[4] This makes it crucial to assess expression of all these subunits together to predict the functional dimeric proteins formed and resultant signaling. Other regulatory molecules modulating Activin signals include Follistatin (FST) and Follistatin-like 3 (FSTL3) that bind to Activins to prevent their receptor binding, and Cripto1 that acts as pseudo-receptor for Activins, all thereby negatively regulating Activin signaling.[5] It is important to evaluate expression of these molecules along with Activin ligands to determine their impact on Activin signaling. Although there are discrete studies suggesting altered expression of individual Activin signaling components in oral cancer,[6-8] a comprehensive expression analysis of all Activin/Inhibin subunits, their receptors, and regulators together has not been described till date. Since Activin expression is known to be altered in various cancers, it is also important to decipher molecules that regulate its expression. In this context, preliminary studies from our laboratory have suggested a role for p63, a p53 family member, in regulating Activin gene expression. Whether there exists any correlation between expression of p63/p53 and Activins and their contribution to oral cancer prognosis has not been assessed.

In this study, we evaluated expression of all Activin/Inhibin signaling components and regulatory molecules in oral cancer cell lines. Expression of the predominant Activin identified was validated in paired adjacent normal-tumor oral tissues and normal oral mucosa. Activin expression in oral tumors was correlated to p63 and p53 expression and with clinicopathological parameters. Finally, the prognostic value of Activin and p63/p53 in oral tumors was assessed by comparing outcome between patients with differential expression of these proteins alone or in combination.

2 | MATERIALS AND METHODS

2.1 | Cell lines

Oral squamous cell carcinoma (OSCC) cell lines SCC9, SCC15, SCC25, SCC029B, and SCC040, dysplastic oral cells DOK, and immortalized oral cells FBM[9] were used in the study. Cells were cultured in recommended medium supplemented with 10% fetal bovine serum, standard antibiotic mixture (additionally, 0.4 mg/mL hydrocortisone for SCC9, SCC15, and SCC25) in a humidified atmosphere with 5% CO_2 at 37°C.

2.2 | Tissue samples

All tissue samples used in the study were approved by the Institutional Ethics Committee-III (IEC-III), ACTREC. 102 paired adjacent normal-tumor oral tissue samples were collected during ongoing surgeries of patients recruited at the Tata Memorial Centre, after taking informed consent. Cases with histologically proven OSCC including buccal mucosa, tongue, and alveolus sites as confirmed by the pathologist (PG) and histologically representative tissues with tumor content ≥70% were used in the study for IHC. Age- and gender-matched normal oral mucosa samples (buccal mucosa and gingiva) derived from minor dental surgeries conducted at Nair Dental College, Mumbai, and KBH Dental College, Nashik, on patients with no history of smoking/chewing tobacco were included as controls in the study. 12 of these normal samples were collected in TRIzol for RNA extraction while 18 tissue samples were collected in buffered formalin for preparation of FFPE blocks and subsequent sections for IHC. Mean expression in these normal tissue samples was used as control, and the IHC scoring and analysis were done independently by PG and SP, who were blinded for patient outcomes. Details of the clinicopathological parameters and IHC scores of the cohort are included in Table S3.

2.3 | Quantitative RT-PCR

Expression of Activin/Inhibin signaling components was determined in oral cancer cell lines by real-time RT-PCR using Power SYBR Green (ABI Biosystems) with gene-specific primers (Table S1) as described previously.[10] Geometric mean of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and ribosomal protein S13 (RPS13) was used for normalization[11]; relative quantification by ΔΔCt method was used for analysis; log fold change was determined over mean expression in normal oral mucosa.

2.4 | Western blotting

Western blotting was performed as described previously.[11] Briefly, membranes with proteins were blocked with 5% bovine serum

albumin/non-fat dry milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 hour prior to incubation with primary antibodies (Table S2) for 16 hours at 4°C. Anti-rabbit or anti-mouse HRP-tagged secondary antibodies were used for detection using Advansta WesternBright ECL substrate. Vinculin was used as loading control; band intensities were quantified using ImageJ 1.x.

2.5 | Immunohistochemistry

Immunohistochemistry (IHC) was performed as described previously.[12] Heat-induced epitope retrieval was performed in sodium citrate buffer (pH 5.8). Sections blocked with normal serum were incubated with primary antibodies (Table S2) followed by biotinylated secondary antibodies and avidin-biotin complex (ABC). 3,3′-diaminobenzidine tetrahydrochloride (D5637-54, Sigma) was used as chromogen and hematoxylin as counterstain. Expression of Activin, p63, and p53 was scored independently by two researchers (PG and SP) blinded to patient outcomes. A combined scoring method was used: strong, moderate, weak, and negative staining intensities were scored as 3, 2, 1, and 0, respectively. Sum of multiplication of each intensity score with its percentage positivity was calculated to determine combined H score (0-300).[6]

2.6 | Statistical analysis

Statistical analysis was done using SPSS 21.0 (SPSS Inc, Chicago). ANOVA was used to compare differences in transcript/protein expression between cell lines and relative expression of various genes within cell lines. Mann-Whitney test was used to assess differences in expression between normal oral mucosa versus adjacent normal or tumor tissues; Wilcoxon's signed-rank test was used to assess differences in expression between paired adjacent normal-tumor tissues. Median H-score of each protein was used to categorize the cohort into low/high expressors to be used for following analysis. Associations between activin expression and clinicopathological parameters were tested using chi-square tests. Univariate and multivariate analysis of protein expression with overall survival and recurrence-free survival was analyzed using Kaplan-Meier curves, survival probabilities were compared using logrank test, Cox proportional hazard model was used to assess prognostic role of these proteins, and effect was quantified using HR and 95% CI. Data are reported as mean/median ± SD; *P*-value of .05 was considered statistically significant.

3 | RESULTS

3.1 | Activin signaling components are altered in oral cancer cell lines

Transcript-level expression analysis revealed that Activin βA subunit was predominantly expressed in oral cells compared with all other subunits (Figure 1A). It was further overexpressed in dysplastic oral cells, DOK and OSCC cell lines compared with normal oral mucosa ($P < .0001$) while a significant downregulation was observed in the expression of other β subunits, βB ($P < .0001$), βC ($P < .0001$), and βE ($P = .0012$, Figure 1A). Although the Inhibin-forming α subunit was also upregulated in OSCC cells ($P = .0015$, Figure 1A), its expression relative to all β subunits was significantly low ($P < .0001$, Figure 1A), together suggesting that Activin A (βA-βA homodimer) is likely to be the predominantly formed Activin in OSCC. Alongside Activin βA overexpression, a concomitant increase in expression of follistatin was also observed in these cells ($P < .0001$), whereas expression of Follistatin-like 3 and Cripto-1 decreased ($P < .0001$, Figure 1B). Receptors ACVR2A/2B and ALK4 were expressed in all cells; although a decrease in ALK4 ($P = .005$) and ACVR2A ($P = .005$) expression was seen in cancer cells compared with normal oral mucosa, expression of ACVR2B was significantly increased ($P = .005$, Figure 1C). At the protein level also, Activin βA subunit was found to be overexpressed in DOK and 3/5 oral cancer cell lines compared with immortalized oral cells, FBM ($P < .0001$, Figure 2A). Activin signaling proteins SMAD2/3 and receptor proteins ACVR2A/B and ALK4 were expressed in all cell lines at varying levels (Figure 2B,C).

3.2 | Activin A is upregulated in oral tumors and is associated with advanced stage

Since Activin βA was found to be predominantly expressed in OSCC cells, we assessed its protein expression in 18 normal oral mucosa and 102 paired adjacent normal-tumor oral tissue sections by IHC. Overexpression of Activin βA was seen in 56% of tumor tissues ($P < .0001$) compared with normal oral mucosa (Figure 3A); its overexpression was also seen in adjacent normal ($P < .0001$) compared with normal oral mucosa. Notably, majority of adjacent normal tissues were found to express high Activin βA compared with their paired tumors ($P = .007$). High expression in adjacent normal tissues could possibly be attributed to field cancerization phenomenon, well-documented in chewing-tobacco-associated oral cancers, and justifies the inclusion of normal oral mucosa tissues as controls in this study. Association of Activin A expression with clinicopathological parameters in 102 patient tumors was determined. Activin A overexpression was significantly associated with advanced tumor stage (T3-T4, $P = .021$) and positive lymph nodes (N+, $P = .045$ Table 1), highlighting the clinical significance of its overexpression in oral cancers.

3.3 | Expression of Activin A and p63 is correlated in oral tumors

Our preliminary experiments to understand the regulation of Activin A expression demonstrated that p63 could associate with Activin A gene promoter in oral cancer cells expressing mutant p53, suggesting a role for p63 in Activin A gene regulation (Data

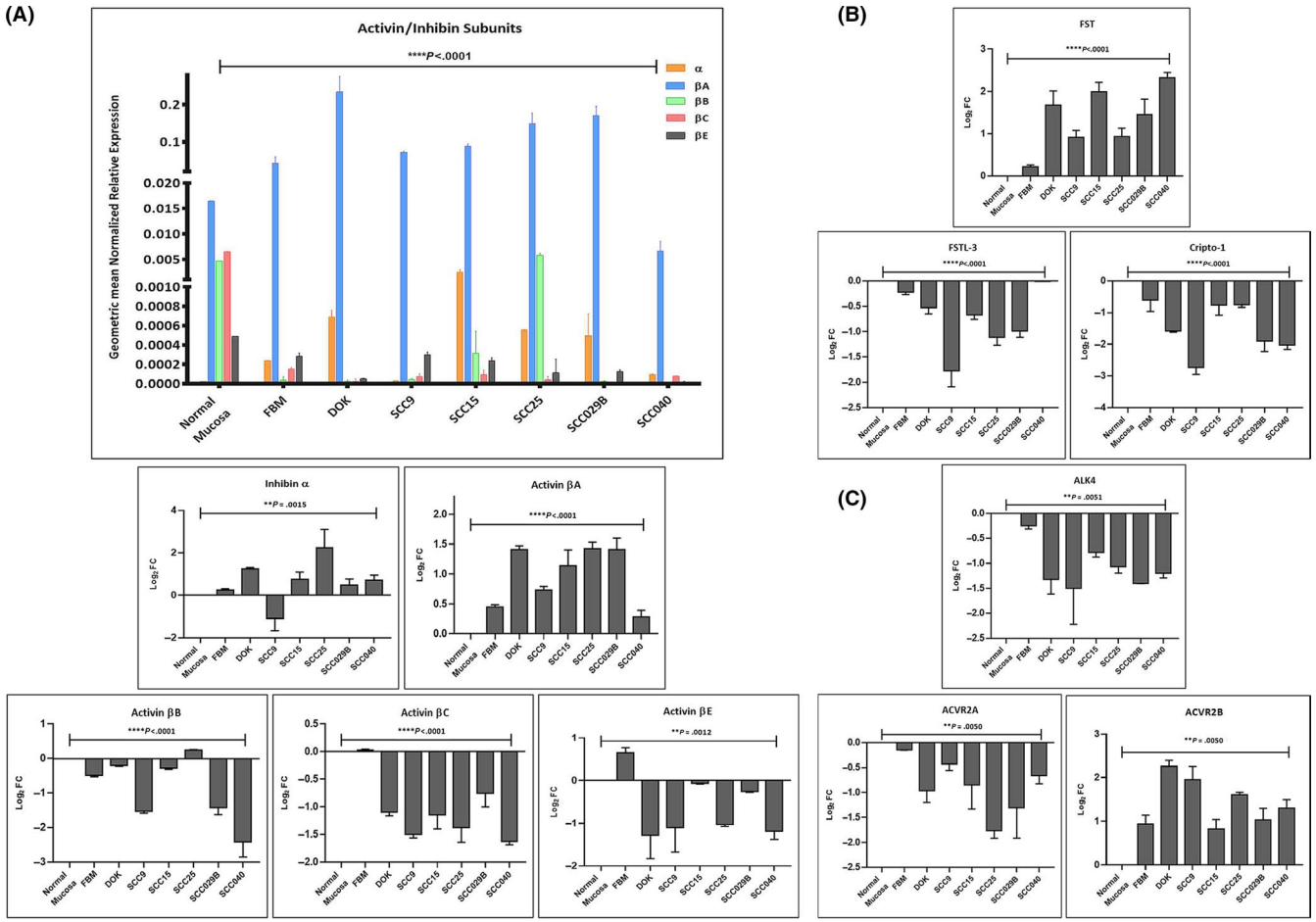

FIGURE 1 Transcript expression of Activin pathway genes normalized to geometric mean of GAPDH and RPS13 expression in oral cancer cells: (A) relative expression of Activin subunits compared with normal oral mucosa, (B) expression of Activin signaling regulators compared with normal oral mucosa, and (C) expression of Activin receptors compared with normal oral mucosa. Graphs represent two independent experiments each performed in duplicates; error bars represent standard deviation

S1). We thus checked the expression of p63 and p53 in our OSCC cell line panel in which Activin A expression was determined. p63 was expressed in DOK, SCC15, SCC25, and SCC040 (Figure 3B). SCC9, SCC15, and SCC040 showed no p53 expression, while remaining cells expressed p53 protein (Figure 3B). To determine whether these proteins are co-expressed in oral cancer, IHC staining for p63 and p53 was performed in tissue samples used for Activin A IHC staining. p63 was found to be overexpressed in 59% of tumors while p53 was overexpressed in 47% of them. Activin A expression negatively correlated with p63 expression in 52.5% of our cohort; correlation being weak but statistically significant (r = −.215, P = .030, Figure 3C). A weak negative correlation was also seen with p53 expression in 53.5% of the cohort, although not statistically significant (r = −.146, P = .143, Figure 3C). Interestingly, when the tumor samples were segregated into groups of low/high p53 expressors, Activin A-p63 negative correlation was predominant and relatively stronger in the p53 high expressors group (47.5% of the cohort, r = −.394, P = .005, Figure 3C: Case 1&2), while p53 low expressors (52.5% of the cohort) did not exhibit such correlation (r = .025, P = .849, Figure 3C: Case 3&4).

3.4 | Activin A and p63 expressions are associated with poor prognosis in OSCC

High Activin A expression was found to be associated with poor recurrence-free survival (P = .013) and overall survival (P = .024, Figure 4A) in our patient cohort, indicating that Activin A overexpression is a poor prognostic marker in OSCC. However, high p63 expression was not significantly associated with survival, although tumors with high p63 exhibited a trend toward poor recurrence-free (P = .075) and overall survival (P = .205). Interestingly, when patients were sub-grouped based on combined Activin A and p63 expression, the group with overexpression of both proteins together showed the worst recurrence-free (P = .005) and overall survival (P = .014, Figure 4B). Univariate Cox regression analysis indicated that Activin A overexpression could predict recurrence-free (HR = 2.74, CI: 1.19-6.31) and overall survival (HR = 2.96, CI: 1.10-7.95). p63 overexpression alone could predict neither recurrence-free nor overall survival. When a combined expression of Activin A and p63 was considered, the subgroup with high expression of both proteins together predicted recurrence-free survival more significantly as indicated by hazard ratio (HR = 10.66, CI: 1.41-80.19) compared with

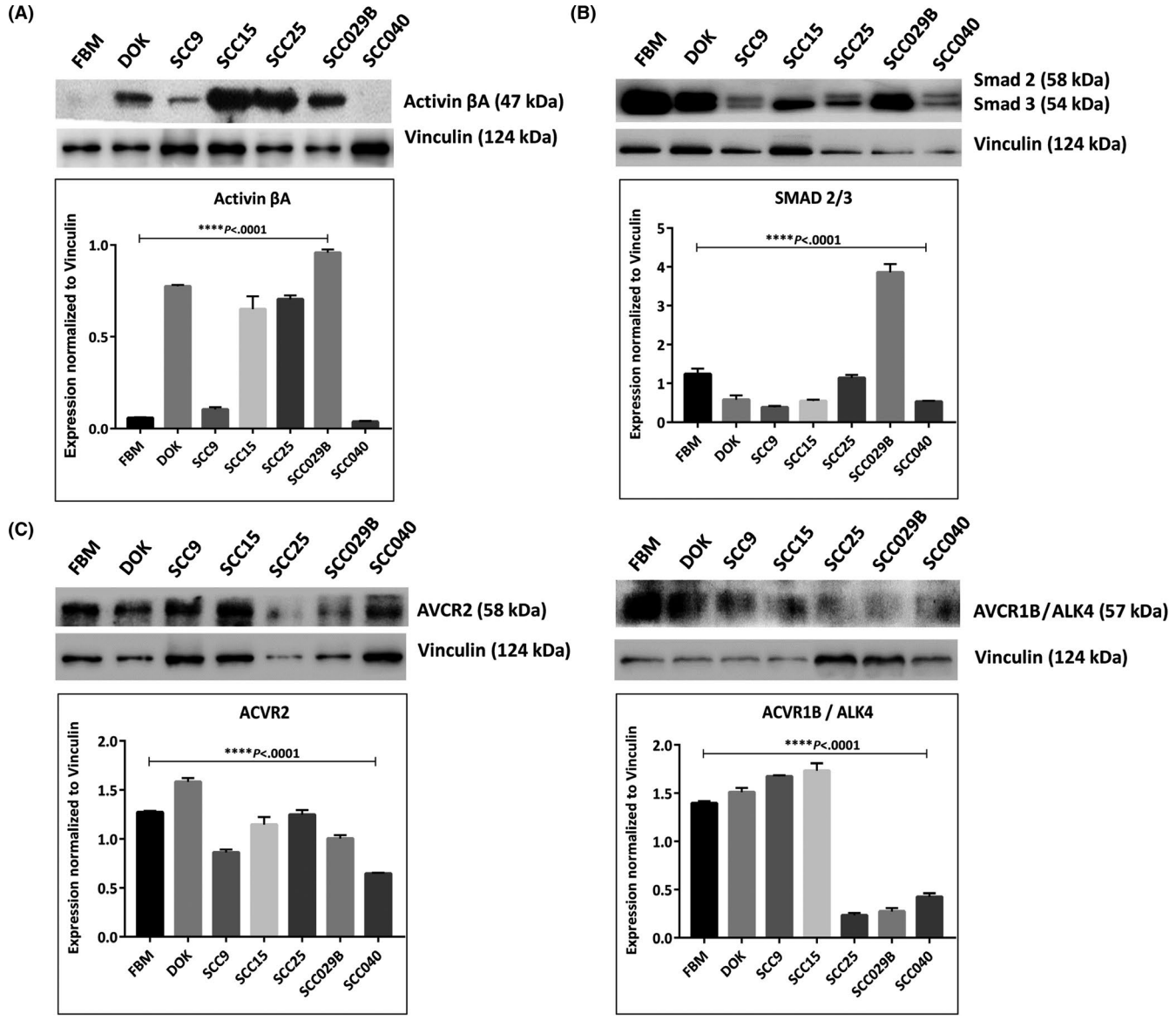

FIGURE 2 Protein expression of Activin signaling components normalized to Vinculin expression in oral cancer cells: (A) expression of Activin βA subunit compared with oral immortalized cells, (B) expression of SMAD 2/3 compared with oral immortalized cells, and (C) expression of Activin receptors ACVR2A/B and ALK4 compared with oral immortalized cells. Error bars represent standard deviation

Activin A expression alone (HR = 2.74, CI: 1.19-6.31); this subgroup, however, could not significantly predict overall survival (HR = 7.57, CI: 0.98-58.39, Table 2). Multivariate Cox regression analysis for overall survival was performed using the variables Activin A expression, Activin A and p63 combined expression, and tumor stage, taking two variables at a time (to maintain the variable: event ratio of 1:10). This indicated that Activin A overexpression could independently predict overall survival (HR = 2.74, CI: 1.02-7.38) whereas combined overexpression of the two proteins, although not significant, exhibited a trend to influence overall survival (HR = 6.11, CI: 0.78-47.43, Table 2). Multivariate analysis for recurrence-free survival was performed using the variables Activin A expression, p63 expression, and tumor stage. This analysis revealed that Activin A overexpression could independently predict recurrence-free survival (HR = 2.83, CI: 1.21-6.61). Notable, a combined overexpression of Activin A and p63 was a better independent predictor of poor recurrence-free survival as indicated by hazard ratio (HR = 8.75, CI: 1.15-66.46) than Activin A overexpression alone (Table 2). Taken together, survival and Cox regression analysis suggested that Activin A overexpression is a poor prognostic marker with respect to both recurrence-free and overall survival, and when considered together with p63 overexpression, Activin A serves as a better predictor of poor recurrence-free survival in oral cancer.

4 | DISCUSSION

Our study is the first comprehensive expression analysis of all components of Activin subfamily, including Activin subunits, receptors, and regulatory molecules in oral cancer cells. Our transcript expression

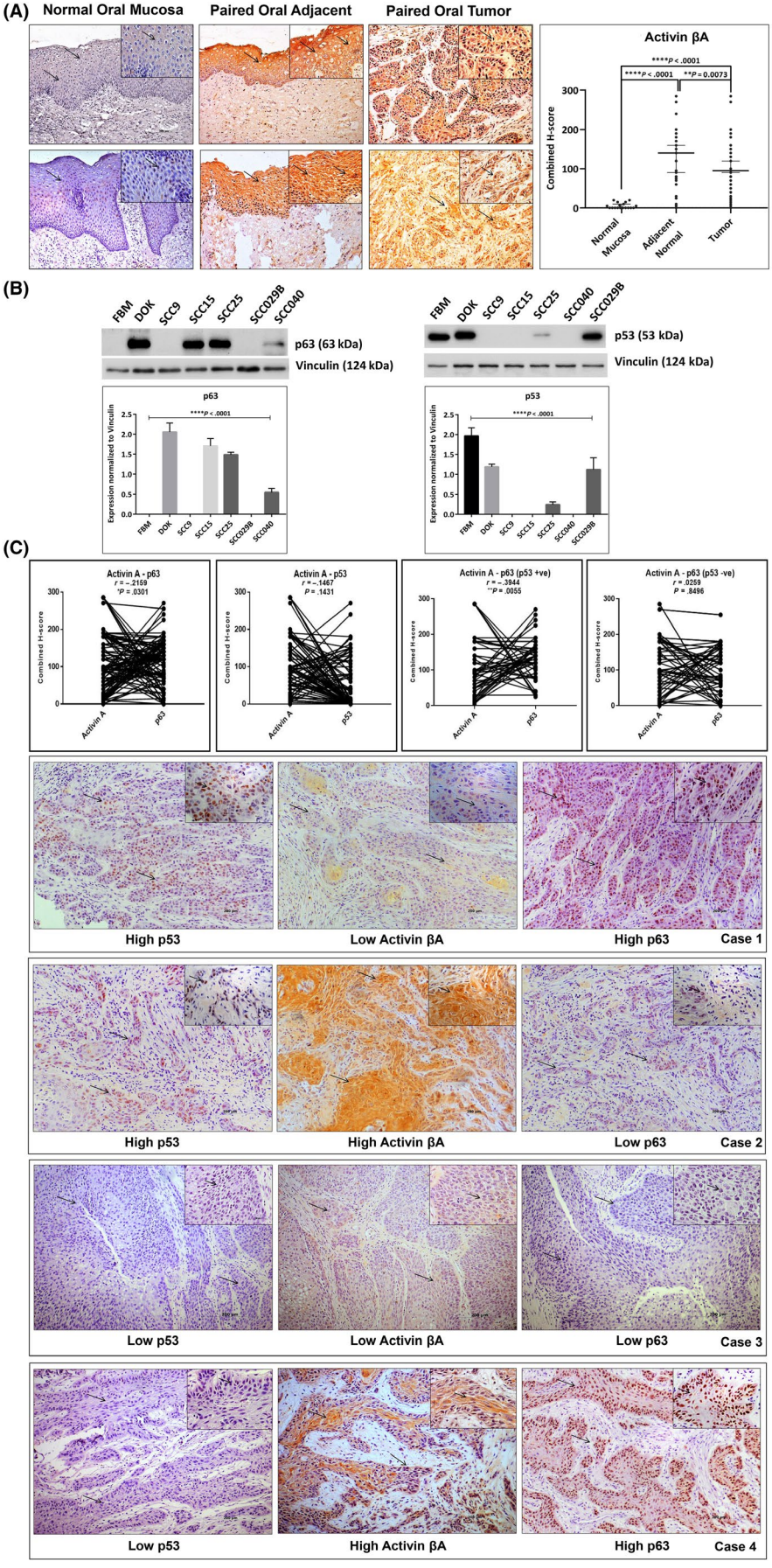
(A)
Normal Oral Mucosa
Paired Oral Adjacent
Paired Oral Tumor
Activin βA
****P < .0001
****P < .0001
**P = 0.0073
Combined H-score
300
200
100
0
Normal Mucosa
Adjacent Normal
Tumor
(B)
FBM
DOK
SCC9
SCC15
SCC25
SCC029B
SCC040
p63 (63 kDa)
Vinculin (124 kDa)
p53 (53 kDa)
p63
p53
****P < .0001
Expression normalized to Vinculin
(C)
Activin A - p63
r = −.2159
*P = .0301
Activin A - p53
r = −.1467
P = .1431
Activin A - p63 (p53 +ve)
r = −.3944
**P = .0055
Activin A - p63 (p53 -ve)
r = .0259
P = .8496
Activin A
p63
p53
High p53
Low Activin βA
High p63
Case 1
High p53
High Activin βA
Low p63
Case 2
Low p53
Low Activin βA
Low p63
Case 3
Low p53
High Activin βA
High p63
Case 4

FIGURE 3 Protein expression of Activin βA, p63, and p53 in oral tissues/cells: (A) representative images of Activin βA IHC staining and dot plot representing median IHC scores for Activin βA expression in normal oral mucosa, paired adjacent normal, and oral tumor tissues, (B) expression of p63 and p53 in oral cell lines, and (C) symbol-line plots and representative IHC images showing correlation of Activin βA with p63, with p53, and with p63 with respect to differential p53 expression. Magnification of the IHC image is 100× and that of inset is 400× with scale bars representing 200 μm and 50 μm, respectively

analysis revealed that Activin βA is the predominantly expressed subunit; while the expression of all other subunits relative to Activin βA was insignificant. At the protein level also, overexpression of Activin βA was observed in OSCC cell lines, further supporting our transcript data. Upregulation of Activin βA has been reported earlier in certain cancers including breast,[13] lung,[14] esophageal,[15] head and neck,[16] and pancreatic cancer.[17] An upregulation and oncogenic role of Activin βA[6,18] and Activin βB[7] have been previously reported in oral cancers. However, our study revealed an upregulation of Activin βA in oral cancer cells and tissues but downregulation of Activin βB in these cells. Our study is also the first to report the expression of Activin βC and βE in oral cancer where we observed their downregulation over normal oral mucosa. Earlier, Vejda et.al. have shown that Activin βC and βE expression is drastically reduced or lost in hepatocellular carcinoma cell line, Hep3B.[19] Given that these are single subunits of the Activin ligand family, and their dimerization with themselves or other subunits to form functional proteins is influenced by their relative proportions,[4] it was important to assess the expression of all Activin subunits together. The present study compared the expression of all these subunits together in oral cancer cells; the relative expression of these indicated that expression levels of Inhibin-forming α subunit and Activin/Inhibin-forming βB, βC, and βE subunits were insignificant compared with βA, suggesting that Activin A (βA-βA) homo-dimers may be predominantly formed in oral cancer.

TABLE 1 Association of Activin βA with clinicopathological parameters in oral tumors

	Activin A over expression (%)		
	No	Yes	*P* value
Age			
Range	26-80	30-76	.9205
Mean ± SD	52.6 ± 13.5	53.7 ± 11.8	
Gender			
Males	43 (47)	49 (53)	.1058
Females	2 (20)	8 (80)	
T stage			
T1-T2	16 (64)	9 (36)	.0212*
T3-T4	29 (38)	48 (62)	
N stage			
N0	23 (56)	18 (44)	.0457*
N+	22 (36)	39 (64)	
Differentiation			
WD	4 (50)	4 (50)	.8978
MD	29 (43)	39 (57)	
PD	12 (46)	14 (54)	
Primary site			
Tongue	14 (39)	22 (61)	.6067
Buccal mucosa	25 (49)	26 (51)	
Alveolus	6 (40)	9 (60)	
Habits			
Tobacco			
Yes	37 (47)	42 (53)	.9294
No	1 (50)	1 (50)	
Alcohol			
Yes	3 (34)	6 (66)	.6576
No	1 (50)	1 (50)	
No data	7 (34)	14 (66)	
Total	45	57	

Abbreviations: MD, moderately differentiated; PD, poorly differentiated; WD, well differentiated.

*Statistically significant

We could further demonstrate that Activin βA protein is upregulated in OSCC tumors compared to normal oral mucosa. However, unlike a study published earlier,[6] we do not see its overexpression in tumors when compared to their paired adjacent normal tissues. Contradictorily, our results indicate that expression of Activin βA is more prominent in adjacent normal tissues than tumors, suggesting that this molecular alteration could be an early event during progression from normal oral mucosa to oral cancer where chronic exposure of oral mucosa to tobacco carcinogens is common. Such early molecular changes have been previously reported by others[20,21] and by our group where we reported an upregulation of survivin 2B and survivin 3α isoforms in adjacent normal versus oral cancer tissues.[10] Our study revealed an association of Activin βA with prognosis in oral cancer, as high Activin βA expression was associated with advanced tumor stage, regional lymph node metastases, and poor recurrence-free and overall survival, supporting its oncogenic role in oral cancer. This is in concordance with findings from the previously mentioned study,[6] where they also demonstrated an association of high Activin A expression with positive nodal status and poor survival.

Expression of Activin receptors was also altered in our cell lines. Though we observed a transcript-level downregulation of receptors, ALK4 and ACVR2A, we could detect the protein expression of ALK4 and ACVR2 at varying levels in these cells. A report in head and neck and esophageal carcinoma cells had earlier suggested loss of ALK4 receptor, with SMAD2 phosphorylation being carried out through non-canonical pathways downstream of Activins.[8] We also examined expression of negative regulators of Activin signaling; while FSTL-3 and Cripto-1 were downregulated in oral cancer cells, Follistatin was upregulated concomitantly with Activin βA. This is in concordance with an earlier report which

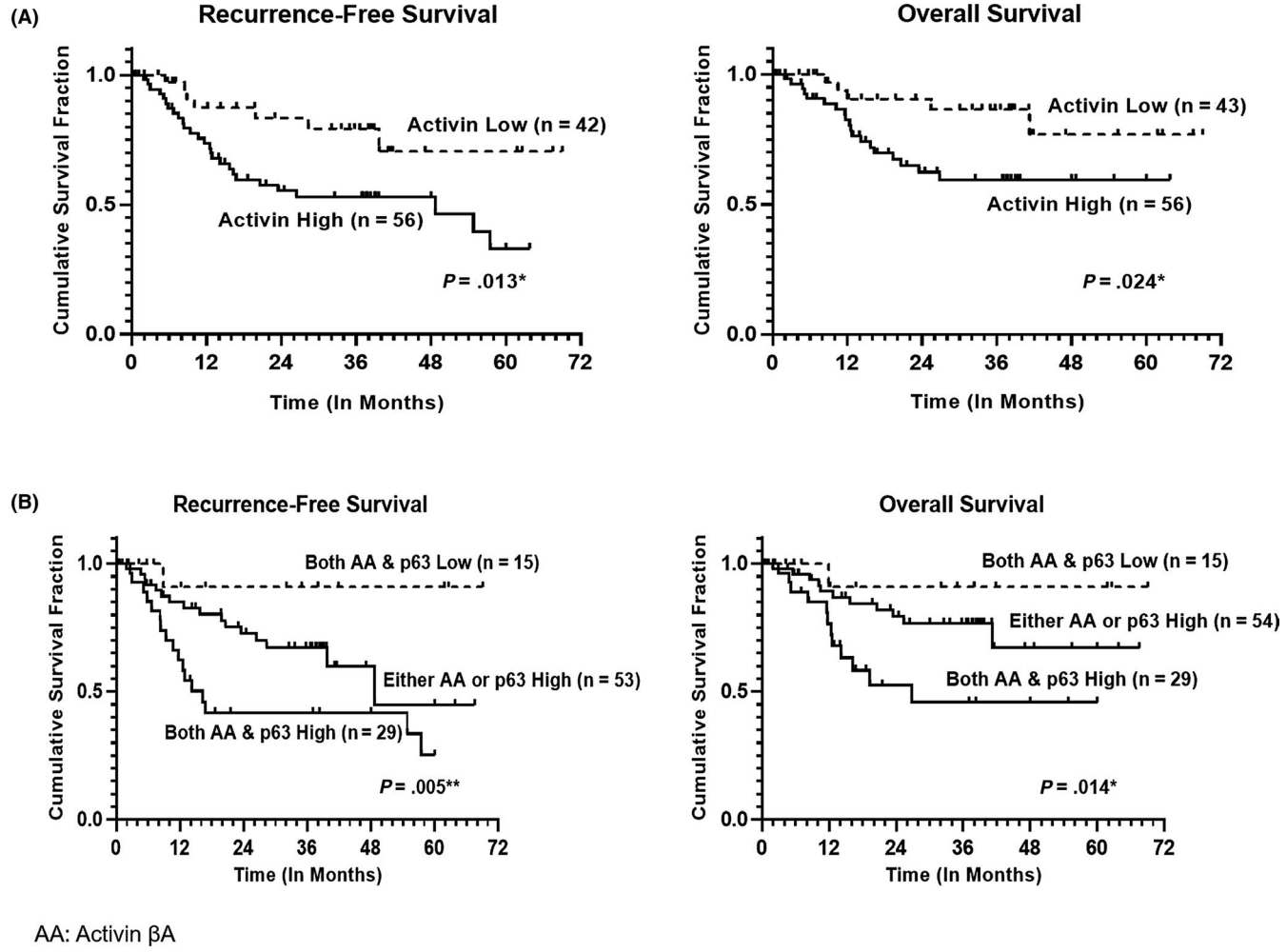

FIGURE 4 Survival of oral cancer patients with respect to differential expression of Activin βA, p63, and p53: (A) recurrence-free and overall survival, respectively, of patients with low and high Activin βA expression and (B) recurrence-free and overall survival, respectively, of patients with positive and negative correlation of Activin βA and p63

demonstrated upregulation of both Activin A and Follistatin in OSCC tissues[6] and could be the cell's compensatory mechanism to regulate Activin signaling.

Our study revealed a negative correlation between Activin βA and p63 expression in 52.5% of patients; this correlation was more predominant in tumors expressing high p53. A study had earlier suggested a role for TAp63, full-length isoform of p63, in regulating Activin βA gene by binding to its 3′ enhancer.[22] Contradictorily, another report demonstrated that ΔNp63, N-terminally truncated isoform of p63, can transcriptionally suppress Activin A in a p53-dependent manner.[23] We and others have earlier observed that ΔNp63 is the predominant isoform over TAp63 in OSCC cell lines and tissue samples [24] and that p63 overexpression is associated with a poor prognosis in oral cancer.[25] In vitro studies from our laboratory have also shown that p63 binds to Activin βA promoter in oral cancer cells expressing mutant-p53, which is known to be highly stable over wild-type p53 and can differentially regulate wild-type p53 target genes, partly by modulating DNA binding ability of co-transcription factors such as p63.[26] Our study suggests a possible regulation of Activin βA gene by ΔNp63 and that this could depend on presence of stabilized or mutant p53. Further in vitro studies will be needed to establish such a role for ΔNp63 or mutant p53 in regulating Activin βA expression.

We also studied the association of Activin βA and/or p63 expression with survival in our patient cohort. Activin βA overexpression associated with poor recurrence-free and overall survival and is a bad prognostic marker in OSCC. Moreover, combined overexpression of Activin A and p63 could more efficiently predict poor recurrence-free survival and also exhibited a trend towards poor overall survival in these patients.

In summary, our comprehensive expression analysis of all components of Activin signaling pathway in oral cancer cell lines and tissues indicated that overexpression of Activin A may be an early event and is associated with poor outcome in oral cancer. This is also the first report on the correlation of Activin A protein expression with p63/p53 expression in any cancer and highlights their prognostic value in predicting survival, thus suggesting Activin A to be a potential therapeutic target in OSCC. An ALK5/4 receptor-kinase inhibitor that targets the TGF-β/Activin signaling, EW-7197, has been tested in phase-I clinical trials for advanced stage solid tumors.[27] Targeting

TABLE 2 Cox regression analysis on RFS and OS in oral tumours

Cox proportional hazard models on recurrence-free survival				
	Univariate crude HR (95% CI)	***P* value**	**Multivariate adjusted HR (95% CI)**	***P* value**
T stage				
T3-T4 vs T1-T2	2.94 (1.03-8.37)	.044*	2.39 (0.83-6.83)	.104
N stage				
N > 0 vs N = 0	1.32 (0.65-2.67)	.437	NA	NA
AA[a] overexpression				
Yes vs no	2.74 (1.19-6.31)	.017*	2.83 (1.21-6.61)	.016*
p63 overexpression				
Yes vs no	1.90 (0.92-3.90)	.08	2.03 (0.97-4.22)	.057
Cox proportional hazard models on overall survival				
	Univariate crude HR (95% CI)	***P* value**	**Multivariate adjusted HR (95% CI)**	***P* value**
T stage				
T3-T4 vs T1-T2	3.86 (0.90-16.42)	.067	3.49 (0.81-14.90)	.091
N stage				
N > 0 vs N = 0	2.26 (0.89-5.71)	.083	NA	NA
AA[a] overexpression				
Yes vs no	2.96 (1.10-7.95)	.031*	2.74 (1.02-7.38)	.045*
p63 overexpression				
Yes vs no	1.72 (0.73-4.02)	.211	NA	NA
Cox proportional hazard models on recurrence-free survival for correlation groups				
	Univariate crude HR (95% CI)	***P* value**	**Multivariate adjusted HR (95% CI)**	***P* value**
T stage				
T3-T4 vs T1-T2	2.940 (1.032-8.375)	.044*	2.369 (0.829-6.773)	.108
Correlation groups				
Either AA/p63 high vs both low	4.880 (0.645-36.909)	.125	4.212 (0.554-32.008)	.165
Both AA and p63 high vs both low	10.660 (1.417-80.198)	.022*	8.756 (1.154-66.463)	.036*
Cox proportional hazard models on overall survival for correlation groups				
	Univariate crude HR (95% CI)	***P* value**	**Multivariate adjusted HR (95% CI)**	***P* value**
T stage				
T3-T4 vs T1-T2	3.862 (0.908-16.429)	.067	3.341 (0.781-14.290	.104
Correlation groups				
Either AA/p63 high vs both low	2.943 (0.380-22.820)	.302	2.446 (0.314-19.073)	.393
Both AA and p63 high vs both low	7.577 (0.983-58.394)	.052	6.111 (0.787-47.433)	.083

Note: NA: not applicable since variable did not qualify the criteria for multivariate analysis.

Abbreviation: AA[a], Activin βA.

*Statistically Significant.

Activin signaling with inhibitors such as EW-7197 may improve patient outcomes in OSCC.

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AUTHOR CONTRIBUTION

Dhanashree Mundhe: Conceptualization; Formal analysis; Investigation; Methodology; Project administration; Validation; Writing-original draft; Writing-review & editing. **Rohit Waghole:** Data curation; Investigation; Project administration; Resources; Writing-review & editing. **Sagar Pawar:** Data curation; Funding acquisition; Project administration; Resources; Writing-review & editing. **Rupa Mishra:** Data curation; Funding acquisition; Investigation; Methodology; Project administration; Resources; Writing-review & editing. **Arusha Shetty:** Investigation; Methodology; Writing-review & editing. **Poonam Gera:** Formal analysis; Visualization; Writing-review & editing. **Sadhana Kannan:** Formal analysis; Software; Writing-review & editing. **Tanuja Teni:** Conceptualization; Funding acquisition; Project administration; Supervision; Writing-review & editing.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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Thesis Abstract

Name : Dhanashree Mundhe

Enrollment Number: LIFE09201304008

Thesis Title: Assessing the Role of Activins / Inhibins in Human Oral Cancers

Oral cancers are one of the most prevalent cancers in the Indian population, and a leading cause of mortality due to cancers. Frequent loco-regional metastases are seen at diagnosis and is one of the important factors contributing to the poor prognosis associated with this disease. Cellular processes such as epithelial-mesenchymal transition (EMT), cell migration and invasion underly this process of regional metastases and are important in determining the spread of cancers. TGF-β signaling pathway plays a crucial role in these cell processes. The role of TGF-β subfamily proteins, Activins / Inhibins, in oral cancers was however, unexplored. This study aimed to evaluate the expression of Activin / Inhibin signaling components and determine the role of this signaling in oral cancers, specifically in the context of its oncogenic functions and mechanisms.

Using real-time PCR, western blotting and Immunohistochemistry, we performed a comprehensive expression analysis of all components of the Activin / Inhibin signaling pathway in oral cancer cell lines &/or patient tumor tissue samples, where we found aberrant expression of these proteins in oral cancer. Particularly, Activin A ligand was found to be highly overexpressed in oral cancers and its overexpression was associated with higher tumor stage, lymph node metastases and poor survival. The expression of Activin A in these oral tumors was correlated to that of p63, a p53 family transcription factor that is also overexpressed in oral cancers. Tumors concomitantly overexpressing Activin A and p63 were associated with shorter recurrence-free and overall survival; the overexpression of these two proteins together could independently predict relapse in these patients. Hence, this study determined new prognostic markers in oral cancers that are linked to a poor outcome in patients. We also studied the regulation of Activin gene expression by p63 and demonstrated that p63 is capable of transcriptionally regulating Activin A gene expression by binding to its promoter and enhancer. Further, recombinant Activin A rescued the loss in migration capacity of oral cancer cells upon p63 knockdown. Thus, p63 is overexpressed in oral cancers, underlining one of the probable ways by which overexpression of Activin A is achieved, which further mediates the oncogenic functions of p63 such as cell migration.

We assessed the functional implications of Activin A overexpression in oral cancer cells. Recombinant Activin A could promote EMT, cell migration and invasion in these cells, demonstrating an oncogenic role. Mechanistically, Activin A induced phosphorylation (activation) of SMAD 2/3, the canonical pathway of Activins. It also activated the non-canonical MAPK pathways, particularly p38 kinase, in oral cancer cells. Blockade of the Activin signaling, by knockdown of the downstream signal transducers, SMAD 2/3 or by using Vactosertib, an upstream inhibitor of Activin signaling, both attenuated these oncogenic phenotypes of Activin A. Our study identifies new molecular mechanism that contributes to migratory & invasive properties of oral cancer cells, phenotypes that may eventually lead to loco-regional metastases in oral cancer patients, and deciphers the probable underlying mechanisms. The results of this study suggest the testing of Vactosertib inhibitor to assess its effectiveness in containing loco-regional metastases in oral cancers.

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Thesis Highlights

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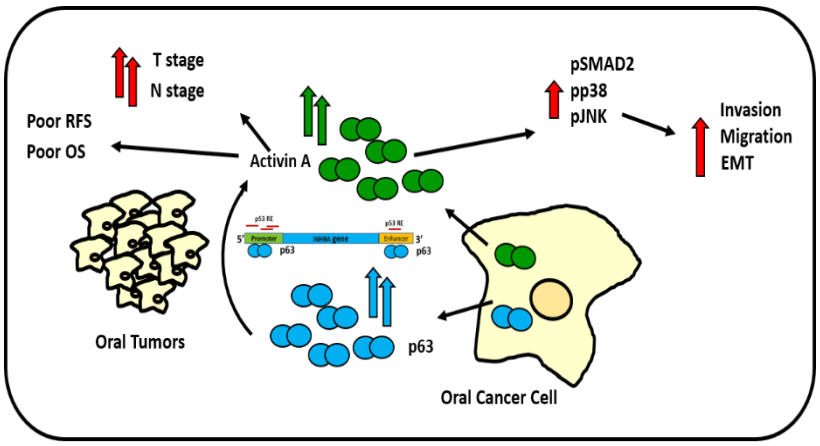
Thesis Title: Assessing the Role of Activins / Inhibins in Human Oral Cancers

Discipline: Life Sciences **Sub Discipline:** Cancer Biology **Date of viva voce**: 10.05.2021

Oral cancers are associated with poor prognosis owing to the high prevalence of loco-regional metastases at diagnosis. This study aimed to evaluate the expression of Activin / Inhibin signaling components and determine the role of this signaling in oral cancers, specifically in the context of its oncogenic functions that contribute to loco-regional metastases and the mechanisms underlying them.

Our comprehensive expression analysis of all components of the Activin / Inhibin signaling pathway using oral cancer cell lines as well as patient tumor tissues revealed an aberrant expression of these proteins in oral cancer. Particularly, Activin A ligand was found to be highly overexpressed in oral cancers and was associated with higher tumor stage, lymph node metastases and poor survival. We also found a correlation between the expression of Activin A and p63, a transcription factor that is also overexpressed in oral cancers. Concomitant overexpression of Activin A and p63 in oral tumors was associated with shorter recurrence-free survival and poor prognosis; the overexpression of these could independently predict relapse and prognosis in these patients, rendering these two molecules as poor prognostic markers in oral cancers. We further demonstrated that Activin A is a downstream transcriptional target of p63 and mediates the oncogenic functions such as cell migration of the latter.



We assessed the functional implications of Activin A overexpression in oral cancer cells. Activin A was found to promote EMT, cell migration and invasion in these cells. Mechanistically, Activin A could activate both canonical SMAD 2/3 pathway as well as non-canonical MAPK pathways, particularly p38 kinase, in oral cancer cells. Blockade of the Activin signaling in oral cancer cells attenuated these oncogenic functions of Activin A. The study thus concludes that Activin signaling is hyperactivated in oral cancers and contributes to its oncogenic phenotypes. This study also provides a basis for future studies that interrogate the effect of blocking Activin signaling in oral cancers in order to contain their loco-regional or distant spread.