Loss of ACVRIB-dependent Activin A signaling induces esophageal and head and neck carcinoma aggressiveness

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This is dedicated to my family (Brian, Patti, and Jason Loomans) and friends for enduring 30 years of me, and also to Peter Kropp, for loving and supporting me even when it has been difficult.

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PREFACE

In this dissertation, I discuss the role the Activin A signaling cascade plays in the initiation and progression of esophageal and head and neck squamous cell carcinomas. The works described in Chapters 1 through 4 have been published in scientific peer-reviewed journals and are presented in the format of their publication. I introduce Activin A signaling and Activin receptor-like kinases, followed by a discussion, specifically, regarding the initial actions of the ligand Activin A as a member of the tumor microenvironment and its interaction with esophageal squamous cells (dysplastic and squamous cell carcinoma) (Chapter 3-4). Next, I examine the contribution of Activin receptor type IB/Activin receptor-like kinase 4 (ACVRIB/ALK4) in this oncogenic phenotype (Chapter 4-5). Chapter 5 is being prepared for publication and is presented in the format of its pending submission. Though the focus of my dissertation has been on the role of Activin A signaling in squamous tissues, further work has been conducted investigating this pathway in an additional histotype of esophageal carcinoma: adenocarcinoma. This work, which has also been published, is presented in Appendix A.

Chapter 1, titled “Intertwining of Activin A and TGFβ signaling: dual roles in cancer progression and cancer cell invasion” provides an introduction to the currently known functions of Activin A in development and cancer. Activin A has been studied substantially and is well defined in a variety of contexts, including cell differentiation and wound healing; however, its role becomes much more controversial in the context of cancer. In some cases, Activin A signaling functions as a tumor suppressor, while in others it operates in an oncogenic manner. Chapter 2 adds further to the complexity of this family of signaling pathways by discussing the role that the type I receptors, the Activin receptor-like kinases, play in initiating and

disseminating a collection of signals to induce an array of downstream activities. Taken together, Chapters 1 and 2 offer a comprehensive study of the ligand-receptor interactions of the TGFβ superfamily.

Chapter 3, entitled “Activin A balance regulates epithelial invasiveness and tumorigenesis”, discusses the ability of Activin A to induce downstream Smad signaling, which controls or contributes epithelial cell invasion. In this work, crosstalk between esophageal squamous epithelial cells and stromal fibroblasts was necessary to observe epithelial cell invasion into an underlying three-dimensional stroma. In this context, Activin A participates in autocrine and paracrine signaling to induce such an effect. Treatment with Activin A antagonists further demonstrated the necessity for Activin A signaling in this process.

Chapter 4, entitled “Esophageal squamous cell carcinoma invasion is inhibited by Activin A in ACVRIB-positive cells”, furthers the work presented in Chapter 3. An array of tissue samples from esophageal squamous cell carcinoma (ESCC) patients indicated that individuals with high stromal Activin A had decreased expression of epithelial ACVRIB. Experimentally, stromal-derived Activin A inhibits ESCC migration and invasion when ACVRIB is intact, however in cell lines, such as the ESCC cell line TE-11, where ACVRIB is lost Activin A is unable to exert this effect. These results denote a novel role of the Activin A-ACVRIB signaling complex in ESCC.

Chapter 5, entitled “Loss of ACVRIB leads to increased squamous cell carcinoma aggressiveness through alterations in adhesion proteins” focuses more specifically on the importance of ACVRIB in mediating cell migration and invasion. Using small interfering RNA and CRISPR/Cas9 systems to knockdown or knockout ACVRIB expression, respectively, we found that loss of ACVRIB in ESCC or head and neck squamous cell carcinoma (HNSCC)

results in increased proliferation, migration, and invasion in both two- and three-dimensional systems. ACVRIB appears to influence the structure of the actin cytoskeleton and, therefore, expression of cell surface receptors. In particular, proteins involved in cell-cell and cell-extracellular matrix adhesion were altered in cells with loss of ACVRIB.

Appendix A, titled “Activin A signaling regulates cell invasion and proliferation in esophageal adenocarcinoma”, describes the actions of Activin A in a closely related, yet distinct cancer: esophageal adenocarcinoma. Esophageal adenocarcinoma occurs along a spectrum, beginning with dysplasia, progressing to Barrett’s esophagus and, eventually, adenocarcinoma. Using cell lines representative of each of these steps, we found that the impact of Activin A in this context occurred in a cell line-dependent manner. From our panel, Activin A effected cell lines with mesenchymal characteristics in an oncogenic manner, while Activin A acted as a tumor suppressor in those cell lines that retained epithelial characteristics. Overall, we showed a role for autocrine Activin A signaling in the regulation of colony formation, cell migration and invasion in the progression from Barrett's esophagus to tumorigenesis.

The summary of this work provides a comprehensive description and characterization of the Activin A signaling pathway, particularly the actions of Activin A through the type I receptor, ACVRIB.

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LIST OF ABBREVIATIONS

Act A	Activin A
ACVL1	Activin A receptor like type I/ALK1
ActRIIA	Activin receptor, type IIA/ACVR2/ACVR2A
ACVRIB	Activin receptor, type IB/ALK4
ACVRII	Activin receptor, type II/ACVR2
ACVRIIB	Activin receptor, type IIB/ACVR2B
ALK	Activin receptor-like kinase
AMH	Anti-Müllerian hormone
AMPK	AMP-activated protein kinase
APC	Adenomatous polyposis coli
BAMBI	BMP and Activin membrane-bound inhibitor
bFGF	Basic fibroblast growth factor
BMP	Bone morphogenic protein
BMPRII	Bone morphogenic protein receptor II/BMPR2
CK8	Cytokeratin 8
CK14	Cytokeratin 14
CK19	Cytokeratin 19
CML	Chronic myeloid leukemia
CRC	Colorectal cancer
DIPG	Diffuse intrinsic pontine glioma
Dll4	Delta-like ligand 4
E	Wild-type E-cadherin keratinocytes
EAC	Esophageal adenocarcinoma
EC	Mutant E-cadherin dominant-negative keratinocytes
ECdnT	Mutant E-cadherin/TβRII dominant-negative keratinocytes
ECM	Extracellular matrix
EdU	Ethynyl-2’-deoxyuridine
EGM	Endothelial growth media
EMT	Epithelial-to-mesenchymal transition
EPCAM	Epithelial cell adhesion molecule/CD326

ESCC	Esophageal squamous cell carcinoma
FGF-2	Fibroblast growth factor-2
Fibro-ActA	Fibroblast-derived Activin A
FOP	Fibrodysplasia ossicans progressiva
FoxP3	Forkhead box P3
FST	Follistatin
GDF	Growth and differentiation factor
hESC	Human embryonic stem cells
hPSC	Human pluoripotent stem cells
HNSCC	Head and neck squamous cell carcinoma
HTT2	Hereditary hemorrhagic telangiectasia type 2
HUVEC	Human umbilical vein endothelial cells
IFNα	Interferon α
IL2RA	Interleukin-2 receptor alpha/CD25
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-13	Interlekin-13
IL-32	Interleukin-32
INHA	Inhibin α
INHBA	Inhibin βA
JPS	Juvenile polyposis syndrome
LAP	Latency-associated protein
Lefty1	Left-right determination factor 1
LTBP1	Latent activating binding protein 1
M2	Alternative macrophage
MAPK/ERK	Mitogen-activated protein kinase/extracellular-signal related kinase
MIS	Müllerian inhibitory substance
MISRII	Müllerian inhibitory substance receptor II
MMP	Matrix metalloproteinase
MS	Microsatellite
NCAM1	Neural cell adhesion molecule 1/CD56
Oct4	Octamer-binding transcription factor 4
OSCC	Oral squamous cell carcinoma
OTC	Organotypic culture

PAI-1	Plasminogen activator inhibitor-1
PBS	Phosphate-buffered saline
PDGFα	Platelet-derived growth factor α
PI	Propridium iodide
Rb	Retinoblastoma
Slug	Snail family zinc finger 2
Sox2	Sex determining region Y-box 2
StAR	Steroidogenic acute regulatory protein
STIP1	Stress-induced phosphoprotein 1
TAM	Tumor-associated macrophages
TβRI	Transforming growth factor β receptor type I/TGFBR1
TBRIII	Transforming growth factor β receptor type III/endoglin
TGFβ	Transforming growth factor β
TGFβRII	Transforming growth factor β receptor type II/TGFBR2/TβRII
Th-1	T-helper cell type 1
Th-2	T-helper cell type 2
Th-17	T-helper cell type 17
THBS1	Thrombospondin-1
Treg	T regulatory cell
VEGF	Vascular endothelial growth factor
VEGF-A	Vascular endothelial growth factor-A
VEGFR	Vascular endothelial growth factor receptor
VEGFR2	Vascular endothelial growth factor receptor 2
Wnt3	Wnt family member 3
ZEB1	Zinc finger E-box binding homeobox 1

CHAPTER I

AN INTRODUCTION TO ACTIVIN A SIGNALING

Intertwining of Activin A and TGFβ signaling: dual role in cancer progression and cancer cell invasion

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Abstract

In recent years, a significant amount of research has examined the controversial role of Activin A in cancer. Activin A, a member of the transforming growth factor β (TGFβ) superfamily, is best characterized for its function during embryogenesis in mesoderm cell fate, differentiation, and reproduction. During embryogenesis, TGFβ superfamily ligands (TGFβs, bone morphogenic proteins (BMPs) and Activins) act as potent morphogens. Similar to TGFβs and BMPs, Activin A is a protein that is highly systemically expressed during early embryogenesis; however, post-natal expression is overall reduced and remains under strict spatiotemporal regulation. Of importance, normal post-natal expression of Activin A has been implicated in the migration and invasive properties of various immune cell types, as well as endometrial cells. Aberrant Activin A signaling during development results in significant morphological defects and premature mortality. Interestingly, Activin A has been found to have both oncogenic and tumor suppressive roles in cancer. Investigations into the role of Activin A in prostate and

breast cancer has demonstrated tumor suppressive effects, while in lung and head and neck squamous cell carcinoma it has been consistently shown that Activin A expression is correlated with increased proliferation, invasion, and poor patient prognosis. Activin A signaling is highly context-dependent, which is demonstrated in studies of epithelial cell tumors and the microenvironment.

Introduction

As a growing body of research has unraveled the functional consequences of transforming growth factor β (TGFβ) superfamily signaling, it has also revealed the complexity of these signaling networks and their pleiotropic effects. This family of ligands consists of TGFβ, bone morphogenic proteins (BMPs), Activins and anti-Müllerian hormone (AMH) proteins. Of these proteins, TGFβ and BMPs have been well characterized for their roles in development, angiogenesis and epithelial-to-mesenchymal transition (EMT) during cancer, particularly regarding cell migration and invasion (1-7). Conversely, Activin A signaling is less well understood. Activins are homo- or heterodimers of Activin β subunits. Currently, there are three known bioactive Activin dimers: Activins A ($\beta_A\beta_A$), B ($\beta_B\beta_B$) and AB ($\beta_A\beta_B$) (8-15). Activin A is best understood for its function in embryogenesis and reproduction; however, its role during cancer progression is still not well documented. This review focuses on the current understanding of normal Activin A signaling, the functional similarities and differences between Activin A and TGFβ and how this signaling pathway becomes dysregulated during cancer progression, influencing cell migration and invasion. Understanding the regulatory mechanism of Activin A in cell migration and invasion will allow for better insight into its role in cancer initiation and progression.

Signaling regulation

Activin A and TGFβ signaling

Though Activin A and TGFβ show structural similarity, Activin A is secreted as an active protein, whereas TGFβ is secreted as an inactive precursor that requires activation (16). Multiple proteins have been identified that can activate latent TGFβ, including proteolytic processing by plasmin and cathepsin D, as well as nonproteolytic processing by heat and detergents (17). Not only do TGFβ superfamily ligands share structural similarity (they share six to nine cysteine residues that form disulfide bonds), the receptor complexes often overlap ligand specificity and downstream signal transduction (18). TGFβ receptor complexes are heteromeric complexes that consist of a type I and type II receptor homodimer (19). Type II and I receptors are distinguished by their sequence. Type II receptors are constitutively active transmembrane serine/threonine kinases (20). These receptors initially bind a TGFβ superfamily ligand to recruit a type I receptor and begin the signal transduction cascade. The number of known type II receptors is limited: transforming growth factor β receptor II (TGFβRII) preferentially binds TGFβ; bone morphogenic protein receptor II (BMPRII) is known to bind multiple ligands, including inhibin A; Activin receptor type II and IIB (ActRII/IIB) bind several ligands, of particular interest Activin A, inhibin A/B, and Nodal; and the Müllerian inhibitory substance type II receptor (MISRII), which is only known to bind AMH (summarized in (21)). Type I receptors, commonly termed Activin receptor-like kinases (ALKs), contain highly-conserved kinase domains and a glycine-serine rich juxtamembrane domain, a necessary component for their phosphorylation and activation (22,23). To date, seven ALKs (ALK1-7) are known and have been characterized. These receptors have been succinctly summarized elsewhere (21). TGFβ has been shown to preferentially signal through ALK5 (TβRI), while

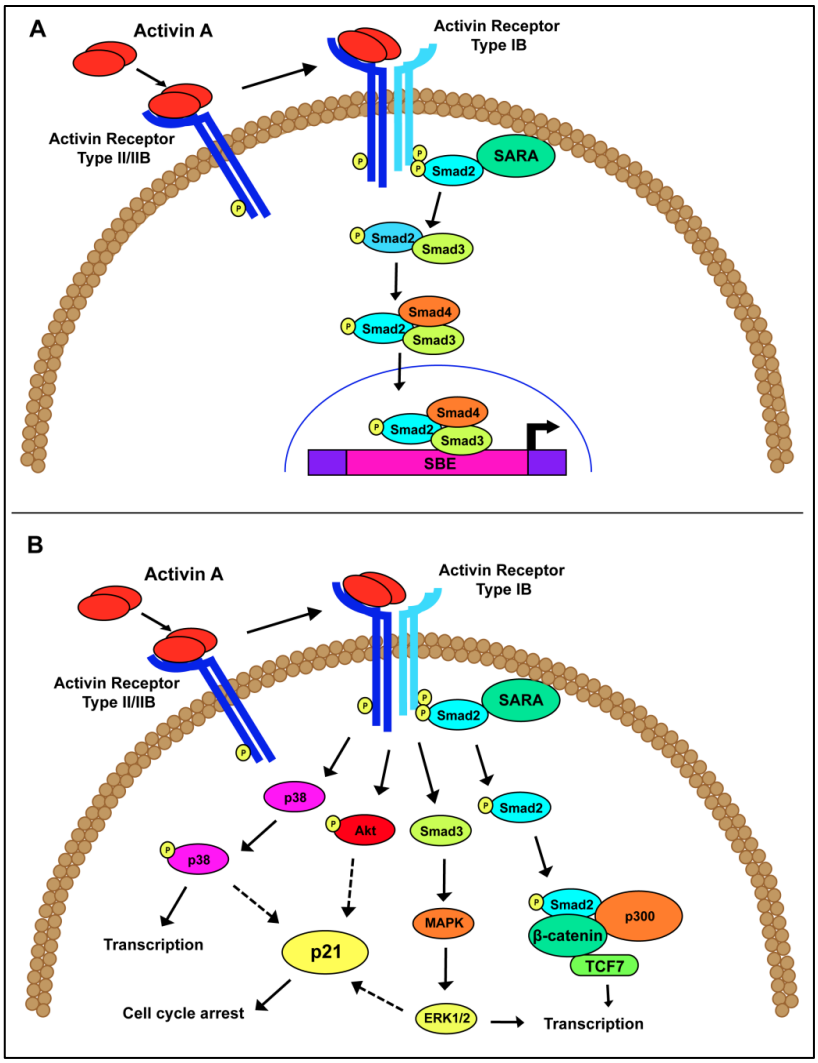

Figure 1-1. Schematic of Activin A signaling. (A) Canonical Activin A signaling occurs through the phosphorylation and activation of the Smad proteins, leading to downstream gene transcription. (B) Non-canonical Activin A signaling has been postulated through a variety of pathways, including PI3K/Akt, MAPK/ERK and β-catenin/p300.

Activin A signals primarily via ALK4 (ACVRIB) (24). Interestingly, ALK5 and ALK4 show almost identical kinase domains; however, they dimerize with different type II receptors (25).

Focusing specifically on Activin A signaling, the signaling pathway begins with an active Activin A ligand secreted from the cell. Activin A binds to ActRII/IIB, which recruits a type I receptor, preferentially ALK4, to form a signal transducing heterodimer (9,26-28). In a similar mechanism to TGFβ signaling, Smad2/3 is recruited and phosphorylated by ALK4. Active Smad2/3 is released into the cytoplasm and complexes with the co-Smad, Smad4. This complex translocates to the nucleus, where it binds to Smad binding elements, with the consensus sequence CAGA, to drive transcription of downstream effectors (Figure 1-1A). In addition to this canonical Activin A pathway, non-canonical signaling, such as Akt/PI3K, MAPK/ERK and Wnt/β-catenin, have been associated with Activin A function independently of Smad activation (Figure 1-1B) (29,30). Despite their overlapping Smad-dependent or non-

canonical pathways, Activin A and TGFβ operate through common, as well as distinct, downstream transcriptional targets, resulting in different functional consequences (31-35).

Due to the overlapping signal transduction pathway of Activin A and TGFβ, it is difficult to untangle specific downstream transcriptional targets for the respective pathways. The best-defined differential downstream targets of Activin A and TGFβ signaling are in the context of human embryonic stem cells (hESCs). In hESCs, Activin A drives downstream transcription of Nanog, whereas TGFβ signaling in this context induces the transcription of SRY-box 2 (Sox2) and octamer binding protein-4 (Oct4) to induce self-renewal and differentiation, which is negatively regulated through the activation of BMP signaling (36-39). In adult tissues, discerning between Activin A and TGFβ signaling is more difficult. Both Activin A and TGFβ, via the Smad2/3/4 complex, have been shown to regulate various cell cycle and extracellular matrix proteins, such as p15, plasminogen activator-1 (PAI-1) and collagen I (40). With the advent of new methodologies, future research should focus on unweaving differential Activin A and TGFβ signaling in post-natal tissues.

Mechanisms of Activin A regulation

Activin A expression is tightly regulated. Regulators of the Activin A signaling cascade can be found in the extracellular matrix, at the cell membrane and intracellularly (Figure 2A-B) (41-43). As there are numerous mechanisms of Activin A regulation, we have focused specifically on the most studied, best understood mechanisms of Activin A regulation, follistatin and inhibin A, endogenous inhibitors found at the cell membrane and in circulation (44-47). Follistatin is expressed in three forms, follistatin-288, follistatin-303 and follistatin-315 (48,49), with different modes of action (Figure 1-2A). Two follistatin proteins surround and inhibit Activin A by

blocking access to both the Activin receptor type I and II binding sites (45,50,51). Follistatin-288, which is mainly found at the cell membrane, sequesters Activin A, resulting in endocytosis and lysosomal degradation (1-7,51-54). Follistatin-288 function itself can be regulated through cleavage at the cell surface by heparin and function in circulation (8-15,51,54,55). Follistatin-303 is produced in relatively low abundance compared to the other follistatin isoforms and has moderate affinity for cell surface proteoglycans, but can bind Activin A in circulation, as well as at the cell membrane (1,9,26-28,37,49). The effects of Activin A signaling can also be counteracted by a structurally-related

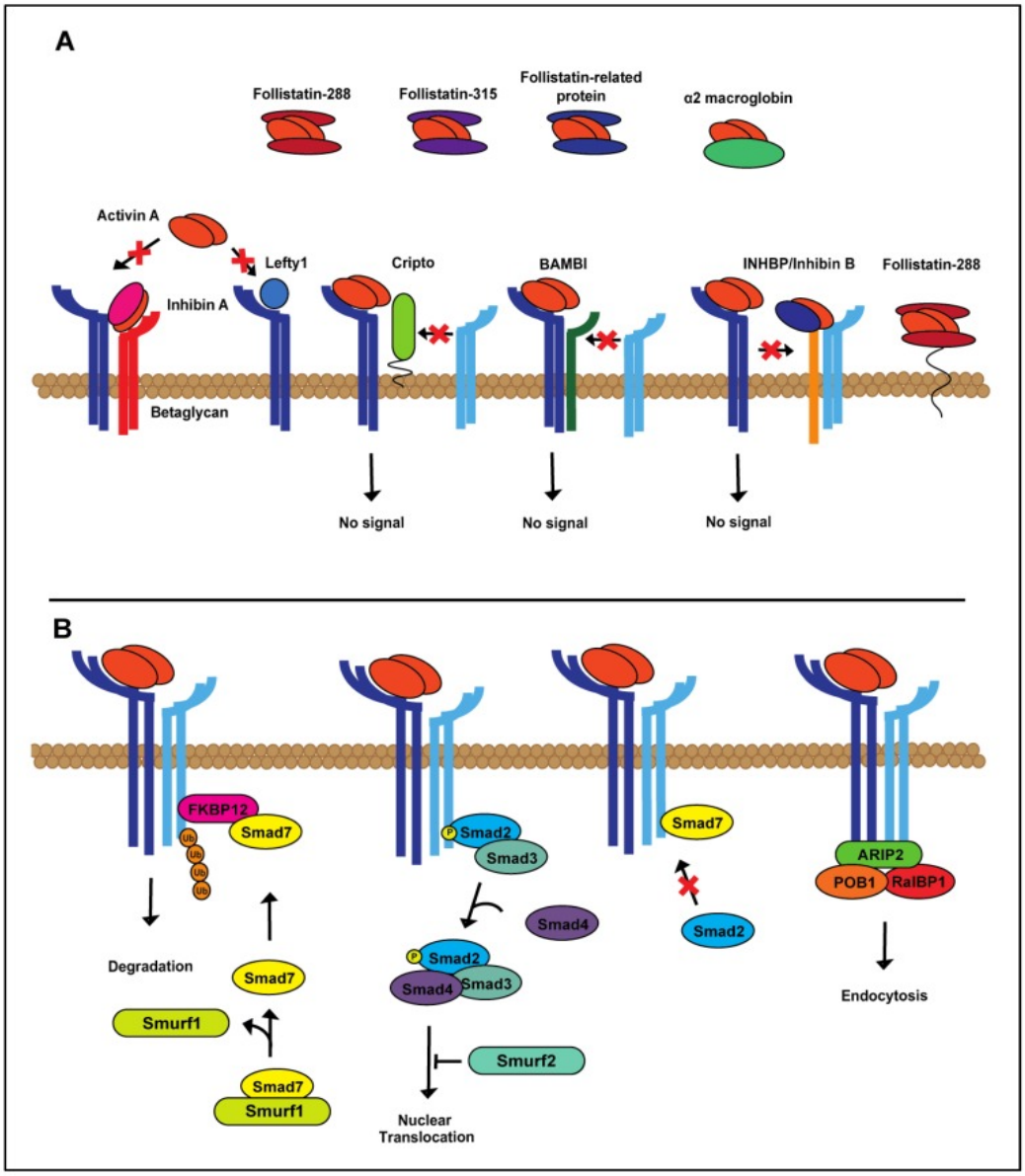

Figure 1-2. Extra- and intracellular regulation of Activin A signaling. (A) Activin A is tightly regulated at the extracellular level by both extracellular matrix (follistatin-288, follistatin-315, follistatin-related protein, α2-macroglobin, left-right determination factor 1 (Lefty1), inhibin A) and membrane-bound proteins (betaglycan, Cripto, BAMBI, INHBP/inhibin B, follistatin-288). (B) In the cytoplasm, canonical Activin A signaling is controlled during each step of the Smad cascade.

ligand, inhibin A. Inhibin A is a heterodimer composed of inhibin α and β_A subunits (29,30,56,57). Inhibin A, bound to the transmembrane receptor betaglycan (TBRIII), dimerizes with ActRII/IIB, preventing Activin A binding (31-35,58,59). TGFβ signaling demonstrates similar levels of regulation, indicating the importance of maintaining the signaling balance of this superfamily (2,4,6,41-43,60,61). Additional mechanisms of Activin A regulation on the extra- and intracellular levels are shown in Figure 1-2.

Hijacked developmental processes and their contributions in tumorigenesis

Early development and stem cell biology

Embryonic development requires particular populations of cells to undergo EMT, migration and complete implantation and gastrulation (44-47,56,62). Activin A plays a significant part in this process. Initially described as XTC-MIF, Activin A was found to be a potent morphogen and inducer of the mesodermal patterning (11,13,15,26,48,49,63,64). With increasing concentrations, Activin A can induce mesodermal cell differentiation, inducing epidermal cell fate at the lowest concentrations, as well as Spemann organizer patterning at the highest concentrations (9,26-28,45,50,51,61,65,66). Spatial patterning occurs via a diffusible Activin A concentration gradient in the extracellular matrix (ECM) (29,67). Interestingly, TGFβ plays a minor role in these developmental processes. Similar to Activin A, TGFβ diffuses along a similar gradient; however, it does not induce the same spectrum of cell fates as Activin A does and requires a much higher concentration of TGFβ ligand (29,51,54,68).

In human development, Activin A is necessary to maintain pluripotency and the subsequent differentiation of human pluripotent stem cells (hPSC) (50,69). Prolonged treatment of hPSCs with Activin A induces definitive endoderm differentiation (52,70). During

early hPSC differentiation, Activin/Nodal signaling is critical to induce epithelial-mesodermal switching, indicated by the loss of the epithelial marker, CD326 (epithelial cell adhesion molecule, EPCAM), and upregulation of mesodermal marker CD56 (neural cell adhesion molecule 1, NCAM1) (55,59,71). Activin A, in concert with Nodal, signals through Smad2/3 to induce Nanog expression, which is necessary to maintain the expression of genes involved in pluripotency (1,3,7,37). Maintaining pluripotency in cells is necessary not only to achieve proper development, but also to initiate and sustain tumorigenesis. Activin A has been shown to be necessary for the maintenance of self-renewal in human embryonic stem cells (hESC) through the induction of Oct4, Nanog, Nodal and wingless-type MMTV integration site family member 3 (Wnt3) (36) and, more importantly, the induction of basic fibroblast growth factor 2 (FGF-2) and suppression of BMP7 (72). Suppression of the downstream target inhibitor of DNA binding 2 (ID2) by Activin A and TGFβ is central in the induction of EMT, which is antagonized by BMPs (73). Of interest, EMT is associated with the acquisition of malignant traits and stem cell markers, therefore linking TGFβ signaling to the regulation of cancer stem cells (74).

EMT vs. Collective Migration

Different members of the TGFβ superfamily (TGFβ1, TGFβ2, TGFβ3, Activin A or BMP7) have been analyzed for their potential to induce EMT in epithelial cells of different origins. While TGFβ1, TGFβ2 and TGFβ3 induce characteristic features of EMT in the mammary and lung cells along with the downregulation and delocalization of E-cadherin, Activin A does not induce EMT in mammary cells or keratinocytes, but causes the scattering and spindle-like morphology of lung cells (75). As EMT is widely recognized to lead to increased cell invasion, loss of E-

cadherin is a hallmark of EMT (76). TGFβ participates in the EMT process through the regulation of transcription factors, such as Snail family zinc finger 1 (Snail), zinc finger E-box binding homeobox 1 (ZEB1) and Twist family BHLH transcription factor 1 (Twist), which suppress the E-cadherin expression. E-cadherin repressors function as EMT inducers on multiple levels, but when cells at the invasive front lose E-cadherin expression, single cell migration occurs (77). Yet, cell migration in a cohesive group is a hallmark of the tissue remodeling events that underlie embryonic morphogenesis, wound repair and cancer invasion (78). The mode of sheet migration relies on the cooperative guidance of leader and follower cells throughout the collective group. TGFβ has been shown to stimulate collective migration primarily through extracellular-regulated kinase 1/2 (ERK1/2) activation (79). On the other hand, using a three-dimensional organotypic culture system, we have described that inhibition of TGFβ signaling increases collection into the underlying extracellular matrix in a fibroblast- and MMP-dependent manner (80). Additional research has demonstrated that tumor cell knockout of TGFβ signaling, through deletion of the type II receptor, drives fibroblast-stimulated collective migration and metastasis (81).

Wound Healing and Regeneration

Embryogenesis and wound healing enlist similar processes, such as the programmed death of unneeded or damaged cells and cell migration. Wound healing is an elaborate, tightly regulated process. Briefly, following tissue injury, growth factors and cytokines are released at the wound site. Injured vessels begin to clot due to the deposition of ECM proteins, such as fibronectin and collagen, and form granulation tissue. Over the course of the next several days to weeks, immune cells and fibroblasts infiltrate the granulation tissue, ridding it of debris and

rebuilding the matrix (10,12,14,56). Throughout the process, all cell types require the ability to move, replace and reform tissue. In zebrafish, Activin A is required for tissue regeneration following injury, while inhibition of signaling completely blocks regeneration (58,82-84). In a mouse model of wound repair, increased *Inhba*, which encodes the mouse β_A subunit, was observed in wound granulation tissue within one day of injury and was sustained for seven days (2,4,6,9,61,85). As follistatin levels increased concurrently with inhibin β_A levels, it has been suggested that the availability of the Activin A ligand, not receptor occupation, regulates the wound response (9,26,28,56,85). Activin A levels become quickly elevated in wounded tissues, likely due to the early inflammatory response (11,13,15,26,63,64,86). However, as demonstrated in embryogenesis, Activin A operates in a concentration-dependent manner. When Activin A is overexpressed in the skin, wounds heal more quickly, however this is associated with substantial fibrosis (27,61,66,87-89).

TGFβ has been highly characterized to promote a reactive stroma (65,89,90). Similarly to Activin A, TGFβ can support all aspects of wound granulation tissue, such as attracting macrophages and fibroblasts to the injury site, initiate wound angiogenesis and stimulate ECM deposition and inflammation (50,67,91). During wound healing, Activin A and TGFβ function in similar roles.

Contributions to tumorigenesis

Harold Dvorak elegantly described cancer as being “the wound that does not heal”. Activin A and TGFβ are excellent examples of this phenomenon, as they both show similar functions in development and wound healing to that observed in cancer initiation and progression. Interestingly, both ligands demonstrate cell type and context-dependent roles within the tumor

microenvironment, illustrated below (Figure 1-3).

Epithelial tumors

As the role of Activin A has been explored in a variety of epithelial tumors, differences in action have emerged between cancers. It has been shown that Activin A can exert a primarily protective function (51,52,54,68,92). Experiments utilizing patient-derived prostate cancer cells and non-invasive LNCaP cells have demonstrated that treatment with Activin A results in cell

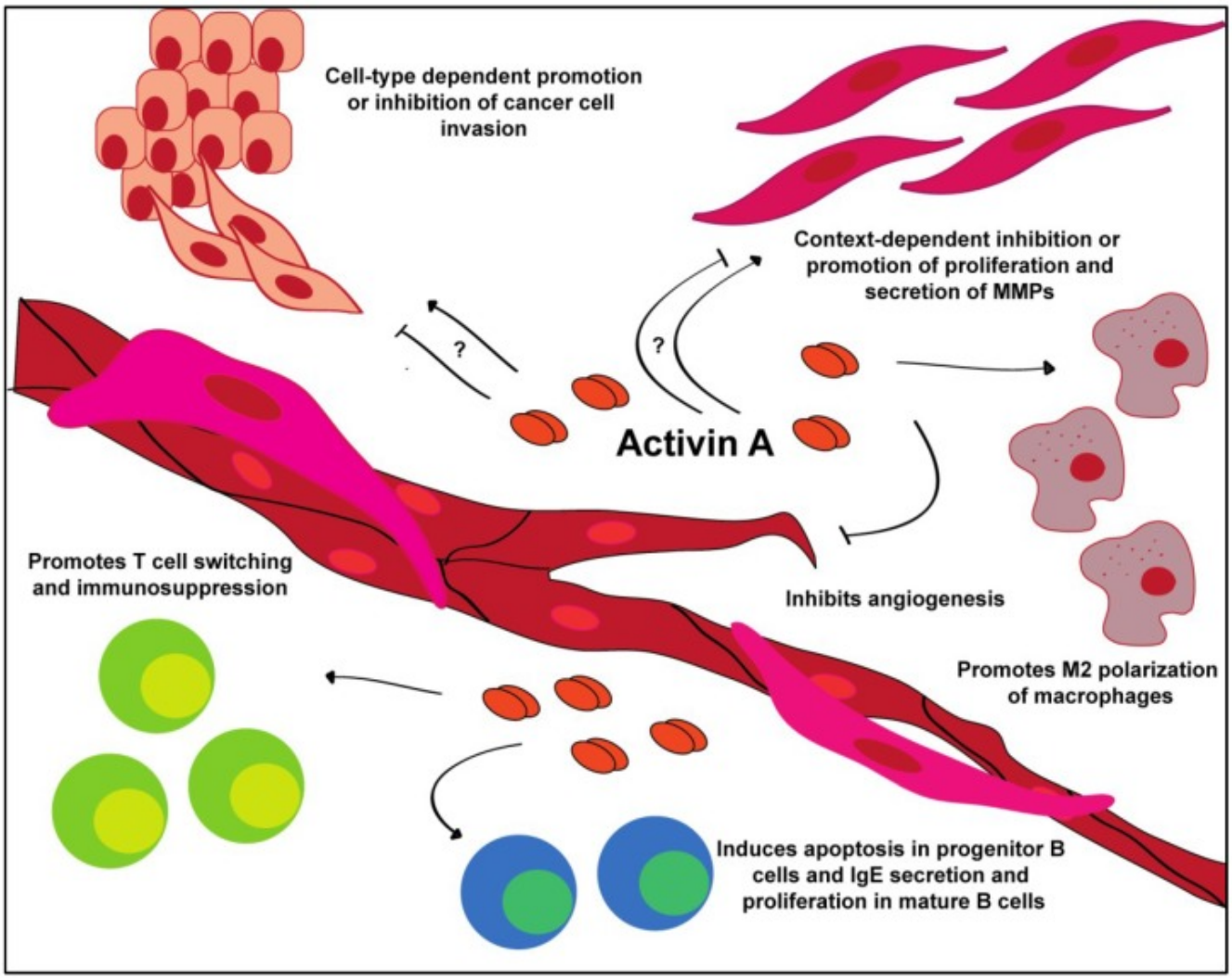

Figure 1-3. Tumor microenvironmental interactions of Activin A. Activin A promotes a variety of behaviors in a cell-type and context-dependent manner.

cycle arrest (45,93-95). Interestingly, LNCaP cells showed no response when treated with the exogenous TGFβ ligand (96,97). In contrast, Activin A treatment of the more aggressive prostate cancer cell line PC3 resulted in an increase in proliferation (69,98). Recent evidence has implicated endoglin, a TGFβ type III receptor and co-receptor, in cancer cell invasion in prostate cancer cell lines via Activin A signaling, though endoglin has primarily been shown to propagate the signal by forming a complex with TGFβ and its receptors (99). Co-expression of endoglin and ActRIIA, when expressed on PC3 or DU-145 cells, has shown suppressed cancer cell invasion, while co-expression of endoglin with ActRIIB, BMP and TGFβ does not exhibit this effect. This functional effect is likely mediated through non-canonical Smad signaling; however, the mechanism of action needs to be further explored (100). Additionally, TGFβ has an oncogenic effect on PC3 cells, as well as the breast cancer cell line, MDA-MB-231. When acting via the non-canonical MAPK/TRAF6 pathway, TGFβ induces a pro-migratory, invasive phenotype (70,101). An *in vitro* analysis of 15 breast cancer cell lines detected Activin A expression in only four cell lines (59,71,102). Functionally, when treated with Activin A, T47D cells showed the induction of cyclin-dependent kinase inhibitors p21 and p27 and the cell cycle control protein p15INK4B, as well as the downregulation of cyclin A, resulting in increased apoptosis and cell cycle arrest. Similarly, MCF7 cells, which have no detectable endogenous Activin A, are highly sensitive to the growth inhibitory effects of Activin A (1,2,5,7). In early tumorigenesis, TGFβ has been shown to have a similar effect. In an overlapping pathway to Activin A signaling, TGFβ induces cell cycle arrest through the induction of the cyclin-dependent kinase inhibitors p15INK4B, p16INK4A, p21 and p27 (8,10,12,14). However, in some cancers, tumor cells lose their ability to respond to the growth inhibitory effects of both Activin A and TGFβ. This occurs primarily through mutation or

downregulation of the receptor; however this is not always the case (2,4,6,82-84,103).

In contrast to its characteristic growth inhibitory effects, Activin A expression has also been associated with inducing an invasive phenotype in certain cancers (Figure 1-3). In lung adenocarcinoma and oral squamous cell carcinomas, for example, Activin A overexpression is correlated with positive lymph node status and poor patient prognosis (9,11,13,15,85,104,105). In head and neck squamous cell carcinoma, increased Activin A has been hypothesized to be an independent prognostic marker of survival (9,26-28,85,106). *In vitro* treatment of the lung cancer cell lines, H460 and SKLU1, with recombinant Activin A showed increased proliferation (26,29,30,63,86,107-109). Additionally, treatment with recombinant Activin A increased invasion in the ovarian cancer cell lines SKOV-3 and OCC1 without impacting proliferation (110).

MMP-7, a matrix metalloproteinase capable of degrading many components of the ECM and activating additional MMPs responsible for increased cell invasion, is upregulated in the presence of Activin A (31-35,87-89,109). This occurs through c-Jun/Smad activity inducing MMP-7 transcription via the AP-1 promoter region (41-43,89,90,111). Additionally, *in vitro* and clinical evidence suggest that Activin A may drive cell invasion by upregulating N-cadherin, a marker of mesenchymal cells and invasiveness (44-47,50,91,112). N-cadherin expression is positively correlated with Activin A, regardless of E-cadherin expression (48,49,52,92,113-115).

In a similar mechanism to Activin A, TGFβ has also been shown to promote cancer cell progression. TGFβ is a potent inducer of EMT through its canonical Smad signaling pathway, as demonstrated in various cancer cell types. TGFβ prompts the expression of Snail family zinc finger 2 (Slug), Snail and Twist, which act to repress E-cadherin expression (8,51-

54,116,117). EMT can also be induced through TGFβ non-canonical signaling pathways. It has been recently demonstrated that TGFβ can act through TRAF6 to promote receptor cleavage of ALK5/TβRI, which allows for the cleaved intracellular domain to translocate to the nucleus and, in association with p300, drive transcription of various invasion-promoting genes (8,51-54,116,117). Increased expression of TGFβ has been noted in various cancers, such as lung, breast and gastric cancers, and has been associated with poor patient prognosis (51,54,55,96,118). Additionally, TGFβ can stimulate and alter MMP expression from epithelial cells. Several groups have shown that TGFβ can negatively regulate MMP-1 and MMP-7 through canonical Smad signaling, while stimulating the production of MMP-2 and MMP-9 through non-canonical p38 and NFκB signaling pathways (1,37,49,98,119). MMP production and activation are necessary for degrading ECM components, allowing for cell migration and further invasion into the stroma.

When overexpressed in the tumor, Activin A confers differential effects. Some cancers, such as lung and head and neck squamous cell carcinoma, develop insensitivity to the growth inhibitory effects of Activin A, one of the hallmarks of cancer (56,57,101,120). During cancer progression, cells in the tumor microenvironment, such as T-helper 1 (Th-1) cells and fibroblasts, increase their production of Activin A in an attempt to inhibit the growth of the tumor; however, the cancer cells adapt and evade these signals. Tumor cells that show resistance to Activin A may do so by downregulating ALK4, which is responsible for signal transduction, or by upregulating follistatin or inhibin A production; however, these areas need to be further explored (58,59,102,121).

Immune Cells

Activin A plays a key role in the maturation and activation of the innate and adaptive immune systems (Figure 1-3). In the normal immune response, Activin A is on the forefront of fighting infection (1,2,4-6,60,61,122). In the humoral immune response, Activin A is secreted by and plays a significant role in the function of adaptive immune cells. In the T-cell population, Activin A is secreted specifically by activated CD4+CD25- (CD25: interleukin 2 receptor α) T-helper 2 (Th-2) cells (8,56,62,123,124). However, Activin A also contributes to the switching of CD4+CD25-Foxp3- (Foxp3: forkhead box P3) cells to CD4+CD25+Foxp3+ T-regulatory (Treg) cells, which correlates with immunosuppression in patients with B-cell acute lymphoblastic leukemia (11,13,15,26,63,64,103,125). Treg cells downregulate the actions of Th-1, Th-2 and T-helper 17 (Th-17) cells, limiting their ability to recognize and potentially destroy cancer cells. Similarly, TGFβ induces a similar Foxp3+ Treg cell phenotype (9,27,61,67,105,126,127). Together, T-cells induced by either Activin A or TGFβ promote a pro-tumor microenvironment.

Activin A induces stage-dependent effects on B-cells (9,26,28,65,106,127). This has been demonstrated in a variety of studies, where exogenous treatment of B-cells derived from marrow stem cells, cultured cell lines and mature B-cells can induce apoptosis in the former and proliferation and antibody secretion in the latter (29,65,67,107-109). Additionally, Activin A is secreted by activated B-cells which, in turn, stimulates the production of IgE antibodies (29,51,54,68,109,128,129). TGFβ performs a similar function as Activin A and inhibits the proliferation of progenitor B-cells; however, it also has the ability to induce growth arrest during B-cell maturation (41,45,95,111). Conversely, TGFβ can drive B-cell differentiation and stimulate the production of IgE and, in some cells, IgG (44,46,47,51,53,54,112,130,131).

The effect of Activin A in the immune system has been most heavily studied in

macrophages, though Activin A has also been shown to affect mast cells, natural killer cells and dendritic cells (48,97,113-115). As discussed above, Activin A is secreted by Th-2-helper cells, which also secrete high levels of interleukin-4 (IL-4) and interleukin-13 (IL-13). IL-4 and IL-13 promote the alternative activation pathway of macrophages, M2, that is commonly associated with wound healing and cancer (8,50,58,69,103). Interestingly, *in vitro* Activin A stimulation promoted the M2 macrophage phenotype, suggesting its functional similarities to IL-4 and IL-13 in tumor promotion (8,52,70,117). TGFβ also polarizes macrophages to the alternative M2 phenotype, promoting an immunosuppressive microenvironment (55,59,71,118). These M2 macrophages secrete cytokines and MMPs that promote a favorable tumor microenvironment (132).

Condeelis and Pollard, in their review of the multifaceted nature of macrophages, succinctly stated that “tumors recruit macrophages and create a microenvironment that causes macrophages to suppress immune functions and, instead, adapt trophic roles found during development and repair” (1,3,7,37,119). This involves creating a favorable environment for tumor cell invasion and recruitment of fibroblasts and endothelial cells to the microenvironment. For example, secretion of interleukin-2 (IL-2) and interferon-α (IFN-α) from T-cells drive the release of basic fibroblast growth factor, leading to the subsequent induction of endothelial cell and fibroblast migration (36,120). Activin A and TGFβ play important roles in this process, reasserting developmental function in the incorrect context and promote pathogenesis.

Fibroblasts

Fibroblasts in the tumor microenvironment contribute to the creation of a reactive stroma or the

transformed state of the stroma in response to disrupted homeostasis, wounding or cancer initiation (72,121). In normal physiology, fibroblasts typically express Activin A at negligible levels (73,123). Activin A stimulates proliferation of mouse 3T3 fibroblasts, airway smooth muscle cells and lung fibroblasts (74,123,124). Interestingly, we have shown that overexpression of Activin A by normal esophageal fibroblasts in a pre-neoplastic microenvironment inhibits proliferation in both an autocrine and paracrine manner; however, it switches to a tumor promoter in the presence of malignant cells (133). A similar phenotype has been noted in the context of TGFβ signaling (75,126).

Tumor-associated myofibroblasts (TAMs) of oral squamous cell carcinoma, which express markers of activation (e.g., α-smooth muscle actin (αSMA), platelet-derived growth factor-α (PDGFα), fibroblast activating protein (FAP)), secrete increased levels of Activin A (76,127,128). This was associated with increased secretion of collagen I, MMP-1, MMP-2, MMP-9 and MMP-13, as well as increased proliferation and *in vivo* tumor volume (77,128). In comparison, the development of a reactive stroma has been correlated with increased secretion of TGFβ by pre-neoplastic cells (65,78). However, it has been consistently shown that fibroblasts that lose responsiveness to TGFβ promote collective cell invasion (79,129,130). These results suggest that, in contrast to Activin A, TGFβ signaling in the tumor stroma is necessary to maintain an intact microenvironment and prevent tumor cell invasion, though it has been suggested that TGFβ may act through the reactive stroma, not the epithelial cell compartment, to promote tumor angiogenesis (2,4,6,80,134). This is a significant point where the functional consequences of TGFβ and Activin A diverge.

TAMs secrete proinflammatory cytokines and proteases to drive EMT and proliferation and migration of epithelial and endothelial cells (11,13,15,81,135,136). These results suggest

that TAMs can prime the tumor microenvironment for epithelial cancer cell invasion by rearranging the ECM. Additionally, TAMs have been associated with the recruitment of immunosuppressive cells to the tumor front (10,12,14,27,56,98). The localization of myofibroblasts to the tumor has been indicative of poor patient prognosis (30,58,82-84,137,138). The recruitment and activation of fibroblasts at the tumor front results in an aggressive and invasive phenotype, as tumor cells use fibroblasts to alter the ECM.

Endothelial cells

In contrast to its tumor promoting function in immune cells, Activin A has been consistently shown to operate as a potent anti-angiogenic factor (Figure 1-3). Treatment of endothelial cells demonstrated decreased tube formation and inhibition of proliferation via induction of p21 and decreased expression of cyclin D1 and Rb (2,4,6,9,32-35,61,85,139,140). This effect can be overcome by silencing of p21. Additionally, fibroblast-derived overexpression of Activin A downregulates vascular endothelial growth factor (VEGF) expression, one of the key components of tumor angiogenesis (141). Previous research has shown that most endothelial cells express ActRII/IIB and are therefore able to respond to Activin A ligand binding (9,26,28,42,43,56,85,140). Activin A treatment of endothelial cells results in a dose-dependent inhibition of proliferation. Experiments utilizing chick chorioallantoic membrane demonstrated a complete loss of capillary development and fibrosis when treated with Activin A (11,13,15,26,45,63,64,86,142). In our experiments, conditioned media from normal esophageal fibroblasts that overexpress Activin A completely inhibits the tube formation of endothelial cells (133). *In vivo*, breast cancer cells expressing Activin A develop larger though less vascularized, tumors compared to cells expressing follistatin, which form smaller, better

vascularized tumors (27,49,61,66,87-89,143).

Due to its potent anti-angiogenic nature, cancer cells have developed mechanisms to counteract Activin A activity. Activin A expression in neuroblastoma results in elevated cyclin-dependent kinase inhibitors and decreased vascular endothelial growth factor receptor 2 (VEGFR2) (45,51,65,89,90,144). Therefore, the oncogene N-Myc, which is consistently overexpressed in neuroblastoma, stifles this effect by directly inhibiting transcription of the inhibin β_A subunit and, subsequently, Activin A homodimer formation (27,50,51,54,67,91,142). Additionally, Activin A may be negatively regulated by interleukin-32 (IL-32), which promotes proliferation and endothelial cell function during tumor promotion. This effect is overcome with the silencing of IL-32, which results in the upregulation of Activin A (51,52,54,68,92,145).

TGFβ has been consistently shown to both induce and inhibit angiogenesis. In contrast to Activin A's function, TGFβ directly stimulates the pro-angiogenic protein, vascular endothelial growth factor A (VEGF-A), via Smad3, while the angiogenesis inhibitor, thrombospondin-1 (THBS1), is induced via phosphorylated Smad2 (45,49,93-95,145,146). These separate signaling pathways suggest differential effects of TGFβ signaling in cancer development and metastasis. Interestingly, TGFβ-induced angiogenesis can be blocked through treatment with TGFβ inhibitors, latent activating protein (LAP) and BMP and Activin membrane-bound inhibitor (BAMBI) (57,96,97,134,147). It may be suggested that TGFβ promotes endothelial cell migration and tube formation via a VEGF-dependent pathway, as it is well established that VEGF is a potent driver of tip cell migration (59,69,98,148). However, recent research has shown that TGFβ has the ability to bind to ALK1 and ALK5 on the endothelium, which may then dimerize with endoglin to activate Smad1/5/8, the typical BMP signaling pathway, and inhibit endothelial cell proliferation; however, these results remain

controversial (149,150).

Conclusions and future directions

As discussed above, Activin A plays pleiotropic roles in basic physiology and pathogenesis. Normal Activin A function induces embryonic cell fate, wound healing and inhibits proliferation. During tumorigenesis, Activin A acts as a suppressor of cancer angiogenesis, as a promoter of tumor-associated macrophages and T-cells and exerts mixed effects on epithelial tumor cells, further exemplifying the context- and cell type-dependent effects of Activin A signaling. Based on the evidence presented, the overall functional consequences of Activin A signaling alone are not sufficient to either suppress or drive cancer progression and, therefore, must work in collaboration with other pathways to dictate a particular phenotype. This may include working in cooperation with other TGFβ ligands, such as BMP4, or synergistically with other pathways, such as MAPK/ERK (37,59,62,71,102,151). Further investigation into the mechanism of Activin A signaling and the intertwining of different pathways to promote cancer progression is needed to unravel the complex signaling processes.

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CHAPTER II

ACTIVIN RECEPTOR-LIKE KINASES: A DIVERSE FAMILY PLAYING AN IMPORTANT ROLE IN CANCER

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Abstract

The role and function of the members of the TGFβ superfamily has been a substantial area of research focus for the last several decades. During that time, it has become apparent that aberrations in TGFβ family signaling, whether through the BMP, Activin, or TGFβ arms of the pathway, can result in tumorigenesis or contribute to its progression. Downstream signaling regulates cellular growth under normal physiological conditions yet induces diverse processes during carcinogenesis, ranging from epithelial-to-mesenchymal transition to cell migration and invasion to angiogenesis. Due to these observations, the question has been raised how to utilize and target components of these signaling pathways in cancer therapy. Given that these cascades include both ligands and receptors, there are multiple levels at which to interfere. Activin receptor-like kinases (ALKs) are a group of seven type I receptors responsible for TGFβ family signal transduction and are utilized by many ligands within the superfamily. The challenge lies in specifically targeting the often-overlapping functional effects of BMP, Activin, or TGFβ signaling during cancer progression. This review focuses on the characteristic

function of the individual receptors within each subfamily and their recognized roles in cancer. We next explore the clinical utility of therapeutically targeting ALKs as some have shown partial responses in Phase I clinical trials but disappointing outcomes when used in Phase II studies. Finally, we discuss the challenges and future directions of this body of work.

Introduction

The TGFβ superfamily has long been of interest in elucidating the mechanisms of normal physiological development and the development of cancer. This family portrays a seemingly simplistic mechanism of action: the ligand binds to a type II receptor dimer, which then forms a tetramer with a type I receptor dimer and transduces a signal through Smad2/3 or Smad1/5/8, ultimately driving nuclear transcription. However, there are numerous family members and several layers of regulation, which complicate the effects following signaling initiation.

The TGFβ superfamily is made up of greater than 30 ligands, which are subdivided into further groups according to structural and mechanistic similarities: transforming growth factor β ligands (TGFβ), bone morphogenic proteins (BMPs), Activins, growth and differentiation factors (GDFs), and anti-Müllerian hormones (AMH) (reviewed in (152,153)). In contrast to the large family of signaling ligands, there are a small number of receptors to transduce these signals: five type II receptors and seven type I receptors (reviewed in (154)). As there are substantially more ligands than available receptors, there is overlap in the combination of ligands and receptors. Though the focus of this review is on the type I receptors that transduce these signals, it is important to recognize the magnitude of inputs coming into these receptors, resulting in a diverse set of biological outputs, including cell growth suppression, epithelial to mesenchymal transition, and migration and invasion.

Type I receptors

The type I receptors, known collectively as Activin receptor-like kinases (ALKs), are more similar to each other than to the type II receptors, hence their separate classification. The two classes share only approximately 40% amino acid sequence similarity (155). In humans, seven ALKs have been identified. This class of kinases is approximately 40-60% homologous and share structural elements including a cysteine-rich extracellular domain with glycosylation site, a transmembrane domain, and a cytoplasmic tail with an active serine/threonine kinase domain (156-159). Due to their similarities, or, potentially, their differences, the type I receptors form heterotetrameric complexes (160,161). This results in differential downstream signaling and translational control through pairing with various type II receptors (161). As there are substantial differences in the biological and functional effects of the type I receptors, the focus of this review will describe their primary function as well as their involvement in cancer and therapeutic potential. However, though this review will focus on ALKs, it is important to note the TGFβ type II receptors, as they play a necessary part in this signaling cascade. The tumor suppressive function of the TGFβ type II receptors have been extensively documented. Several studies have illustrated the presence of TGFβ type II receptor mutations and inactivations. Of particular note, approximately 90% of microsatellite (MS) unstable and 15% of MS stable colorectal cancers have *TGFBR2* inactivating mutations (162). *TGFBR2* inactivation is more frequently observed in MS unstable cancer types, such as gastric cancer and glioma, than in MS stable cancer (163). Interestingly, this does not appear to be a phenomenon that occurs among ALKs.

In contrast to the previously stated MS unstable colorectal cancer observations, individuals suffering from hereditary nonpolyposis colorectal cancer, which frequently have

TGFBR2 inactivating mutations, often have overall better outcomes than those with sporadic colorectal cancer, suggesting a protective effect of *TGFBR2* inactivation in these cases (163,164). For a more complete review of the literature, please see (163,165). The potential duality of function of the TGFβ family receptors is not unique and is a characteristic that translates to ALKs.

As there are differences in the biological and functional effects of the ALKs, the focus of this review will describe their primary tasks as well as the involvement of ALKs in cancer and therapeutic potential.

ALK1

Function

ALK1 (ACVL1) has been well studied for its role in vasculogenesis. ALK1 primarily acts as a BMP receptor due to the high binding affinity of BMP9 and BMP10, which leads to type II receptor/ALK1 complex formation. Upon activation, ALK1 signals via Smad1/5/9 most commonly in endothelial cells, contributing to both angiogenesis and lymphatic vessel formation (166-168). Interestingly, TGFβ has also been found to induce Smad1/5/9 signaling in endothelial cells, however the co-receptor endoglin and ALK5 are required for full activation (169). During wound healing, ALK1 expression increases to induce blood vessel branching and, upon wound closure its expression is downregulated (170). Inhibition of endothelial ALK1 signaling through the use of an ALK1 neutralizing antibody substantially reduces vasculogenesis and angiogenesis, even when growth factors, such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), are present (171).

Interestingly, some studies have indicated that ALK1/Smad1/5/9 works synergistically

with the Notch pathway to regulate angiogenesis (172). Rostama and colleagues found that Delta-like ligand 4 (Dll4)/Notch with BMP9/ALK1 activation induces cell quiescence through p21 and thrombospondin-1 (THBS-1), as well as induces the expression of Hey genes in lung endothelial cells. Additionally, upon loss of Dll4, ALK1/Smad1/5/9 becomes upregulated, therefore compensating for the loss of Notch signaling (172). Similarly, treatment of primary human endothelial cells with BMP9 induces Hey1 and Hey2 genes through cooperation with Notch, however treatment with soluble ALK1 inhibited expression, demonstrating the close relationship of these two pathways (173).

Loss of function of ALK1 is a primary cause of autosomal dominant vascular dysplasia syndrome, as known as hereditary hemorrhagic telangiectasia type 2 (HHT2) (171). With an incidence of 1 in 8000, approximately 80-90% of HHT2 cases have mutations in ALK1 or endoglin (TBRIII), a TGFβ family co-receptor (166). Mutation of ALK1 in HHT2 results in a haploinsufficency, where the affected ALK1 allele can then induce mRNA synthesis or degradation of the non-functional protein (166).

Role in cancer and therapeutic potential

As ALK1 has a well-established role in vasculogenesis, as described above, investigating the potential contribution of ALK1 in cancer seems like a logical step. Alk1 global knockout mice are phenotypically similar to BMP9 knockout mice, presenting with enlarged lymphatic vessels and development of cancer (168). ALK1 expression is induced in the vasculature of breast tumors (170). Several strategies have been explored to block ALK1 signaling in endothelial cells, thereby downregulating or inhibiting tumor angiogenesis. *In vivo*, the extracellular domain of ALK1, coupled to a mouse-derived Fc region (ALK1-Fc), has been used as a ligand trap for BMP9/10, thus blocking their binding to endothelial ALK1 (170). Similarly, dalantercept (ACE-

Table 2-1. Comprehensive list of the inhibitors discussed in this review. (Adapted from Loomans & Andl, 2016)

Receptor Target	Inhibitor	Use
ALK1	ALK1-Fc	Laboratory
	PF-03446962	Phase I
	Dalantercept/ACE-041	Phase I/II
ALK2	DMH1	Laboratory
	K02288	Laboratory
	LDN-212854	Approved – FOP treatment
ALK3	LDN-193189	Laboratory
	DMH2	Laboratory
	VU0465350	Laboratory
ALK4	SB-431542	Laboratory
	SB-505124	Laboratory/Phase I
ALK5	SB-431542	Laboratory
	EW-7197	Laboratory
	SB-505124	Laboratory/Phase I
	LY-2157299	Phase I
	GW6604	Phase I
ALK7	SB-431542	Laboratory
	SB-505124	Laboratory/Phase I

041) is a soluble form of ALK1 that binds to BMP9/10, therefore preventing the activation of endogenous ALK1 and inhibiting signaling complex formation (174,175).

Dalantercept/ACE-041 showed potential in a Phase I dose-escalation study, where 14 of 29 patients had partial response or stable disease (175). However, when it was used in Phase II for recurrent or persistent endometrial carcinoma, it was deemed ineffective as a single-agent therapeutic (176). Combination therapy of dalantercept with the vascular endothelial growth factor receptor (VEGFR) inhibitor sunitinib has shown some promise in metastatic renal cell carcinoma *in vivo*. When combined with sunitinib, dalantercept induced tumor necrosis, stifled cellular growth and revascularization, and downregulated the expression of pro-angiogenic genes, as well as endothelial cell-specific Notch pathway genes (177).

An additional approach to blocking ALK1 signaling has been through the use of a monoclonal antibody specifically targeting ALK1. PF-03446962 prevents BMP and TGFβ ligands from binding to the ALK1 extracellular domain (167). It was tested in a Phase I study for advanced solid tumors and has since been approved for use in colorectal cancer, mesothelioma, and endometrial cancer (167,174). Most recently, PF-03446962 was used in a Phase I

study for hepatocellular carcinoma. While the majority of patients had adverse effects related to the treatment, 50% of those treated had stable disease, however no complete or partial responses were observed (178). Collectively, these studies have indicated that ALK1 may be a feasible therapeutic target for advanced solid tumors. For a complete list of ALK1 inhibitors and additional inhibitors discussed in this review, please refer to Table 2-1.

ALK2

Function

ALK2 (ACVR1) is a bone fide BMP receptor. Various TGFβ, Activin, and BMP ligands, such as BMP9 and Activin B, have been found to induce ALK2 signaling (179,180). In Leydig cells, which are found in the testes, ALK2 can inhibit signaling by blocking Activin A access to the type II receptor by forming a type II/type I receptor complex in the absence of ligand (181). When it acts in this manner, ALK2 has an inhibitory function. Interestingly, induction of signaling through non-TGFβ family ligands has been noted. For example, Tsai and colleagues found that stress-induced phosphoprotein 1 (STIP1) can bind directly to ALK2, independent of a type II receptor, to induce downstream Smad signaling (179).

Primarily studied for its role in osteogenesis and chondrogenesis, ALK2 is required for chondrocyte proliferation and differentiation (182). This role has been illustrated using an Alk2 knockout mouse model, in which Alk2 was conditionally deleted in cartilage. These mice show defects in bone formation, as observed by shortened cranial bases and hypoplastic cervical vertebrae. Adult Alk2 conditional knockout mice develop progressive kyphosis, or convex curvature of the spine (182).

The importance of ALK2 in osteogenesis is fully grasped in the development of

fibrodysplasia ossicans progressive (FOP). FOP is a sporadic, rare disease (incidence of 1 in 2 million) that is characterized by progressive ossification of muscles, tendons, ligaments, and connective tissues (183,184). The progression of FOP results in chronic pain and growth impediments, often leading to difficulty breathing and, ultimately, death (184). Gain-of-function mutations in ALK2 have been identified as the cause of FOP. Approximately 50% of the identified ALK2 mutations occur in the GS activating domain, a serine/threonine rich sequence near the kinase domain of these type I receptors (184). Of the GS mutations, the R206H mutation comprises approximately 90% (185). Though many of the ALK2 mutations occur in the same region, genotype-phenotype correlations seem to exist, as the clinical presentation of FOP varies depending on the ALK2 mutation (184). Interestingly, the type II receptor is required for the gain-of-function effect (186). Therefore, it stands to reason that not only may the location of the ALK2 mutation dictate the severity of FOP, but also the expression level of the BMP type II receptor may contribute to its phenotype.

Role in cancer and therapeutic potential

Few examples of alterations in ALK2 signaling are have been found in the context of cancer. One of the best-known examples occurs in diffuse intrinsic pontine glioma (DIPG), a rare type of childhood tumor. Buczkowicz and colleagues found that approximately 20% of DIPG tumor samples had mutations similar to those found in FOP, indicating ALK2 gain-of-function (187). Hyperactivation of ALK2 signaling has also been noted in ovarian cancer. ALK2 activation, via autocrine BMP9 signaling, induces transcription of ID1 and ID3, thus increasing proliferation (179,188). Though BMP9 can also signal through ALK1, as discussed above, Herrera and colleagues found that this proliferative phenotype was specific to ALK2 signaling in

immortalized ovarian surface epithelial cells and ovarian cancer lines (188).

In contrast, maintaining ALK2 signaling has been indicated as a regulator of tumor suppression. Olsen and colleagues found that treatment of primary multiple myeloma samples with BMP9 resulted in signaling through ALK2 and the induction of apoptosis (189). In melanoma, treatment with BMP7 upregulates ALK2, inducing mesenchymal-to-epithelial transition via downregulation of Twist, leading to an overall reduction of invasion (190).

Specifically targeting ALK2 in therapeutics has proven challenging. DMH1 is a dorsomorphin analog and selective inhibitor of ALK2 (191). It reduces the ability of ALK2 to phosphorylate Smad1/5/9 without affecting other kinases such as ALK5, AMPK, or VEGFR (191-193). DMH1, however, has not been reported for use yet in the clinic. An additional dorsomorphin analog, LDN-212854, has been used as a means to treat or prevent FOP, yet this inhibitor also non-specifically inhibits ALK1 and ALK3 (194). Thus far, the most effective ALK2 inhibition strategy has been through the use of K02288, a 2-aminopyridine compound with high affinity for both ALK1 and ALK2. This inhibitor binds to a conserved binding pocket in ALK1 and ALK2, which results in reduced angiogenesis and vessel sprout *in vitro* (195). Targeting ALK2, along with the non-specific inhibition of the other ALK kinases ALK2 and ALK3, has been shown as an effective strategy *in vitro*, one that may show efficacy upon further clinical testing.

ALK3

Function

Though some functions of ALK3 (BMPR1A) suggest similarity to ALK2, this protein has substantial sequence similarity with a different ALK family member, ALK6 (196). Currently, it is known that many BMPs (BMP2, 4, 5, 6, 7, 8, 14, 15), GDF6, GDF7, and AMH can bind to

ALK3 with some affinity to initiate differential downstream action (reviewed in (197)). Global knockout of Alk3 is embryonic lethal (198). ALK3 is expressed in cells of the osteo lineage and bone marrow and is necessary for post-natal bone formation; as such, it is suggested that the main function of ALK3 is osteogenesis (183). Mice with conditional knockout of Alk3 in cartilage lack growth of the long bones, however this tissue gets replaced with bone-like tissue, supporting a role for ALK3 in cell fate and osteogenesis (199). Conditional deletion of Alk3 from osteoblasts produces a similar phenotype. These mice have increased bone formation in the trabecular bone, vertebrae, tail, and ribs, coupled with a reduction in osteoclastogenesis (200).

Unlike ALK2, ALK3 has additional critical roles in development. Deletion of alk3 in *Xenopus* results in dorsalized embryos and defects in the eye (201). Myocardial- and neural crest-specific knockouts of ALK3 show drastic developmental defects (reviewed in (202)). Epicardial-specific deletion of Alk3 in mice results in atypical developmental of the atrioventricular sulcus and annulus fibrosis within the cardia (203). Additionally, when Alk3 is deleted early (at weaning or early adulthood) from the foregut, mice have improper gastric patterning, as well as a reduced number of parietal cells and increased number of endocrine cells (204).

Affecting another area of the gastrointestinal tract, the loss of or mutations in ALK3/BMPR1A has been associated with the development of juvenile polyposis syndrome (JPS), a hereditary condition that is characterized by the presence of hamartomatous polyps and associated with an increased risk for colorectal cancer. Germ line mutations in ALK3 are found in approximately 20% of JPS cases, while 45% of cases have mutations in ALK3 and/or the co-Smad, Smad4, therefore contributing to loss of ALK3 signaling (205,206). Additionally,

case reports of JPS have reported ALK3 mutations occurring independently of Smad4 mutations (207,208).

Role in cancer and therapeutic potential

Loss of ALK3 is best known to contribute to increased risk for colorectal cancer (CRC). As described above, individuals with JPS, which is associated with loss of ALK3 signaling, have an increased risk of cancer development, particularly CRC. However, it has also been noted that loss of ALK3 has also been associated increased risk of esophageal carcinoma, adrenal hamartoma, and Wilm's tumors (207,209). Without JPS as a predisposition, mutations in ALK3, along with several other mismatch repair genes including Smad4, account for less than 5% of CRC (210). Chang and colleagues found similar results. In a cohort of 103 patients, one patient was found to have a *de novo* mutation in ALK3 (211). Similarly to CRC, individuals with loss of ALK3, but unaffected Smad4 expression in pancreatic ductal adenocarcinoma, had significantly poorer survival compared to those who are ALK3-positive (212).

In contrast to loss of signaling contributing to CRC, abrogation of ALK3 in other cancer contexts appears to promote aggressiveness. Pickup and colleagues found that conditional knockout of ALK3 in breast cancer cell lines results in delayed tumor onset *in vivo*, however these cells acquire more mesenchymal markers. Analysis of patient data from The Cancer Genome Atlas indicated that individuals with high ALK3 expression had overall poorer survival, regardless of subtype (213). Additionally, BMP2-induced ALK3 signaling in liposarcoma has been associated with increased extracellular matrix remodeling, disease progression, and, therefore, poorer patient outcome (214).

As the role of ALK3 in cancer is a double-edged sword, the feasibility of targeting this

receptor *in vivo* remains uncertain. Currently, the only reported ALK3 inhibitors in use are LDN-193189, DMH2, and VU0465350. These inhibitors have been used to treat liver disease by enhancing liver regeneration (215).

ALK4

Function

ALK4 (ACVR1B) is a versatile receptor that has a critical role in development. In *Xenopus*, ALK4 activates both sides of the developmental pathway: the TGFβ-driven left side with the ligands Xnrl and derriere and the BMP-driven right side with the ligand Vgl (216). This activation modulates mesoderm induction and dorsoanterior/ventroposterior development during primary axis formation (216). Constitutive activation of ALK4 induces *Xenopus* mesodermal and dorsoanterior markers, similarly to Activin expression models (217).

In mouse models, global knockout of Alk4 (Acvr1b) is embryonic lethal due to developmental impairment of the epiblast and extraembryonic ectoderm, leading to improper gastrulation (218). Activation of ALK4 can occur through a multitude of ligands, such as Activins, GDFs, and Nodal (219). Conditional knockouts of Alk4 in various adult tissues have been generated to analyze the impact of Alk4 systemically. Activin A signaling, mediated via ALK4, has a substantial role in reproduction. Trophoblast invasion is regulated through a canonical ALK4-mediated pathway, where upregulated Snail induces MMP-2 expression; knockdown of ALK4 attenuated this effect (220).

Conditional knockout of uterine Alk4 results in subfertility due to defects in placental development (221). Signaling through ALK4/ALK7 in the male reproductive tract is required for germ cell development and Sertoli cell proliferation (222). This may be initiated by Nodal or

GDFs, along with Cripto, as these mechanisms can trigger downstream phosphorylation of Smad2 (223,224).

Additional conditional mouse models have been generated to examine the tissue specific effects of ALK4. Squamous cell deletion of Alk4 leads to substantial hair loss, increased epidermal thickness, and growth stunting in approximately 25% of Alk4-null mice. Interestingly, this appears to have a dose-dependent effect, as those with the highest Cre-driven Alk4 expression had the most severe phenotype. Molecularly, Alk4-deleted tissues had increased expression of the transcription factor Lef1, which regulated hair-specific expression of keratin, and increased proliferation (225). A subsequent conditional model of Alk4 in adult tissues has also been developed, with Alk4 loss observed in skin, liver, spleen, pancreas, and kidney (226).

Role in cancer and therapeutic potential

The best-characterized alterations of ALK4 expression have been noted in pituitary and pancreatic cancers. Alternatively spliced forms of ALK4 have been identified in somatotroph, corticotroph, and nonfunctioning pituitary adenomas, which are generally not found in normal tissue (227). These splice variants of ALK4 are truncated, lacking the kinase domain and therefore cannot propagate anti-proliferative signals (227,228). Restoration of full-length ALK4 reverses these effects (228). Conversely, rather than alternative splicing, pancreatic cancers frequently show ALK4 deletions. Su and colleagues identified loss of heterozygosity of ALK4 in 34% of cancer xenografts and 45% of pancreatic cancer cell lines, supporting the hypothesis that ALK4 acts as a tumor suppressor in this cancer type (229). In ALK4-positive pancreatic cancer cell lines, invasion could be inhibited through treatment with SB-431542, a chemical

inhibitor of ALK4/5/7 (230).

Though the role of ALK4 has not been comprehensively explored in most cancer contexts, several studies have suggested that ALK4 expression can have either oncogenic or tumor suppressive influences. Both prostate cancer cell lines and testicular carcinomas have been shown to retain variable levels of ALK4 expression (231,232). Landis and colleagues found that, in ErbB2/Her2/Neu positive tumors, TGFβ signaling through ALK5 was frequently lost, however ALK4-mediated Activin A signaling remained active along the invasive front of the tumor (233). Similarly, B16 melanoma cells, upon treatment with Activin A, showed dose-dependent regulation of CDH1 and HMGA2 expression. However, in metastatic cultured B16 cell lines, ALK4 expression was substantially reduced (234).

Targeting ALK4 in therapeutics appears to be, generally, an unfeasible target, as most noted alterations in ALK4 signaling are deletions or inactivations. In some cancers, such as prostate, testicular, or Her2-positive breast cancers, as discussed above, targeting ALK4 may have potential. However, the existing inhibitors not only target ALK4, but the additional type I receptors ALK5 and/or ALK7. SB-505124 and SB-431542 potently inhibit ALK4/5/7, as shown by decreased phosphorylation of Smad2 and Smad1/5/9 (235,236). These two inhibitors are not currently used clinically. Additional inhibitors, such as LY-362947, LY-2157299, and SD-208, among others, have also been developed (reviewed in (237)).

ALK5

Function

Similar to the previously discussed receptors, ALK5 (TGFBR1) plays critical roles in development and reproduction. ALK5 has been best characterized as the primary receptor for the

TGFβ ligands (TGFβ1, TGFβ2, TGFβ3), however GDF8 and GDF9 have been additionally reported to signal through this receptor (238,239). GDF8 can signal via ALK5 to activate Smad3, ERK1/2, and steroidogenic acute regulatory protein (StAR) in granulosa cells. This effect can be blocked by inhibition of ALK5 (238). GDF9 signals through BMPRII and ALK5 recruiting Smad2 and Smad3 to activate adrenocortical and Sertoli cells (239); both are required for folliculogenesis (238,240).

ALK5 is required for proper embryonic development; Alk5 knockout mice are embryonic lethal (241). Even Alk5 mutant mice with a D266A knock-in mutation in the L45 loop, therefore not allowing for Alk5 to phosphorylate Smad2, only survive until E10.5 due to defects in vasculature formation (242). Targeted deletion of Alk5 in the neural stem compartment is embryonic lethal at E15 due to failure of upper lip palate closure (243). Because of its embryonic lethality, investigators have utilized conditional knockouts of Alk5 to examine its role in the development of various tissues. Deletion of Alk5 in the endocardium, using Tie2-Cre *in vitro* and *in vivo*, demonstrated that this signaling pathway is necessary for not only epithelial-to-mesenchymal transition of the cardiac cells, but also differentiation and maintenance of tight junctions, as observed through downregulation of N-cadherin and VE-cadherin (244). Additional vascular defects were noted in mice with Alk5 knockout in skin lymphatic endothelial cells. These vascular networks lacked organization and complexity and were hyperproliferative (245).

Previously, we discussed that ALK5 is necessary for reproductive function. Mice with conditional knockout of Alk5 in the uterus are sterile. Deletion of Alk5 disrupts development of the oviductal diverticula and myometrium. Additionally, these mice have hyperproliferative uteruses and irregular glands (246). This phenotype is exemplified during implantation, as trophoblasts lack organization, the uterine natural killer cell population is reduced, and arterial

remodeling diminished (221). These results suggest that ALK5 is necessary to not only transduce TGFβ family signaling, but that it is also needed to mediate part of the uterine immune response. Though loss of Alk5 expression in the uterus results in defective processes, constitutive expression of the receptor is additionally problematic. Conditional gain-of-function of uterine Alk5 resulted in increased myometrium thickness, causing hypermuscularized uteri. In the endometrium, constitutive activity of Alk5 promoted fibroblast differentiation and a smooth muscle gene signature. Interestingly, deletion of Alk5 in the uterus had substantial epithelial effects, while constitutive activation heavily altered the uterine microenvironment (247).

Role in cancer and therapeutic potential

Though research suggests that alterations of ALK5 promote cancer progression, ALK5 alone appears not to be sufficient for this process and needs to cooperate with an oncogenic driver. Examples of this have been explored in breast, colorectal, head and neck squamous (HNSCC), and pancreatic cancers. Landis and colleagues found that phosphorylation of Smad2 was substantially downregulated in a mammary cancer model of ErbB2/Her2/Neu amplification, a result of Alk5 loss (233). APC is mutated in approximately 70% of sporadic CRC and is often described as the “first hit” (248). The commonly used APC^{min} mouse model, combined with loss of heterozygosity of Alk5, develop approximately three times more tumors than APC^{min}/Alk5 wild-type mice, indicating that diminished Alk5 signaling can be a second-hit accelerating tumor formation in CRC. Alk5 wild-type and knockout mice alone did not develop CRC tumors (249). Alterations in ALK5 in CRC frequently occurs through the deletion of three alanines located in a nine alanine repeat, termed TGFBR1*6A (250). Homozygous variants of

TGFBR1*6A has been associated with increased risk of CRC, though a more substantial rate of this deletion has been observed in CRC metastases, compared to primary tumors (249,250).

In HNSCC, PI3K pathway mutations occur at a frequency of approximately 30% (251). Of the PI3K pathway mutations, loss of PTEN expression is common (252). Additionally, HNSCC patient samples and cell lines have marked reduction in ALK5 protein expression. A combination of these two models, knockout of Pten and Alk5, in HNSCC promoted the expansion of the cancer stem cell niche, reduced cellular senescence, and increased cancer-associated inflammation (253). This association of ALK5 has also been strengthened in a mouse model of pancreatic cancer. Homozygous loss of Alk5, with mutant Kras, led to the development of pre-cancerous lesions at 100% frequency. While only 50% of Alk5 heterozygotes showed pre-cancerous lesions, those that were developed were larger than those in knockout mice (254).

Though loss of ALK5 often confers a tumor cell advantage, ALK5 activation has been noted in osteosarcoma and sex-cord stromal tumors, indicating a dual role for this signaling receptor. Treatment of the osteosarcoma cell line MG63 with TGFβ, which acts primarily through ALK5, induced cellular proliferation via the Smad2/3/4 axis. This effect could be inhibited through treatment with SB-431542 (255). Additionally, constitutively active Alk5 in ovarian granulosa cells, described previously, promoted the development of sex-cord stromal tumors within two months. These tumors also had elevated expression of the Hedgehog proteins Gli1 and Gli2 (256).

As described above, ALK5 deletion or mutation in tumors appears to be the common form of pathway alteration. That being said, efforts have been directed at inhibiting the ALK5

cascade and, surprisingly, have had some success. Several of these inhibitors such as LY-2157299, SB-505124, and GW6604 have been tested in Phase I and Phase II clinical trials (reviewed in (257)). These inhibitors target the ATP binding pocket of the kinase, removing its signal transduction ability. A limitation of several ALK5 inhibitors is their lack of selectivity between ALKs; for example, SB-431542 inhibits not just ALK5, but also ALK4 and ALK7 (236).

However, these off-target effects of inhibitors can be utilized. Dasatinib, a commonly used Src inhibitor, has also been used *in vitro* to inhibit ALK5 in pancreatic ductal adenocarcinoma; it has been shown to inhibit Smad2 phosphorylation and cell invasion, similarly to that observed following treatment with the ALK4/5/7 inhibitor SB-431542 (258). Efforts have been directed at developing ALK5-specific inhibitors. EW-7197 has been used as an *in vitro* treatment for melanoma. Use of EW-7197 enhanced the number of tumor-infiltrating lymphocytes, particularly CD8+ cytotoxic T-cells (257,259), thereby stimulating the immune response.

ALK6

Function

ALK6 (BMPRIB) acts primarily as a BMP receptor, with preferential binding to BMP2, BMP4, BMP6, BMP7, BMP15 and GDF5, however ligand binding of Müllerian-inhibiting substance (MIS) has also been observed (260,261). In *Xenopus*, the necessity of ALK6 during development has been shown, as loss of ALK6 causes defects in neural crest formation and pigmentation, resulting in an embryonic lethal phenotype (201).

In vertebrate development, expression of ALK6 is tightly controlled, and primarily found in mesenchymal pre-cartilage, chondrocytes and osteoblasts. During osteoblast differentiation,

ALK6 expression is upregulated, indicating a role for this protein in bone formation (183). This is further indicated in the association of ALK6 mutations in the development of brachydactyly type A1 and type A2. Brachydactyly is an autosomal domain disorder affecting the digits. Type A1 is an inherited disorder characterized by malformation of the middle 2-5 fingers, while type A2 is autosomal and characterized by shortening of the index fingers and, sometimes, the first and second toes (262,263). Two heterozygous mutations in ALK6 have been identified in association with the development of type A1, which work to halt kinase function (263). Similarly, linkage analysis of two families identified mutations falling within the GS and kinase domains. Interestingly, only the GS domain mutation rendered the protein kinase-dead, while the kinase domain mutations appeared to have no effect on activity, indicating that these mutations differentially effect ALK6 function (262). Additional evidence implicating ALK6 in bone formation is the development of the skeletal disorders Grebe dysplasia and acromesomelic du Pan dysplasia (261,264). Various mutations affecting the activity of the ALK6 kinase domain have been associated with these disorders (261,265). ALK6 expression is highest in the brain, lung, and ovary in adult tissues (reviewed in (196)). In the ovaries, ALK6 is necessary for folliculogenesis. Its expression fluctuates throughout the stages, however reduced or disrupted ALK6 expression on the granulosa cell surface has been associated with reduced growth of the follicles (266). Adult sheep with a point mutation in ALK6 have impaired follicle development and an increased ovulation rate (267).

Role in cancer and therapeutic potential

As is a recurrent theme in the TGFβ family, the role of ALK6 in cancer appears to be both tumor promoting and suppressive. Examples of the oncogenic properties of ALK6 have been explored in chronic myeloid leukemia (CML), epithelial ovarian cancer, luminal breast cancer,

and CRC. Upregulation or overexpression of ALK6 has been identified on CML cells, compared to healthy donors. It is suggested that microenvironmental influence of BMP2 and BMP4 contribute to the noted change in ALK6 expression (268). A similar observation has been made in epithelial ovarian cancers, where patient samples with ALK6 expression had a worse prognosis compared to patients without ALK6 expression (260). In the investigation of ALK6-ligand interactions in cancer, BMP2 binding and signaling through ALK6, in conjunction with IL-6, has been shown to promote the development of luminal breast cancer through the upregulation of Smad5 and GATA3, as well as downregulation of FOXC1 (269). Using MCF10A cells, Chapellier and colleagues demonstrated increased colony formation in soft agar and greater ability to form tumors *in vivo* (270). Functionally, *in vitro* knockdown of ALK6 in SW480 CRC cells, which are Smad4 positive, decreased invasion (271).

Though seemingly oncogenic, ALK6 appears to act as a tumor suppressor in gliomas and glioblastomas. Expression of ALK6 is downregulated in various malignant gliomas, including astrocytomas and glioblastomas, compared to normal astrocytes, as measured by mRNA expression and reduced phosphorylation of Smad1/5/9. Re-expression of ALK6 decreased the ability of these cells to have anchorage-independent growth (272). Additionally, treatment of glioblastoma samples with BMP7 decreased proliferation and sphere number (273). These studies indicate that when the ALK6 pathway is active in gliomas and glioblastomas, it acts as a suppressor. Interestingly, the function of this pathway is likely dependent upon the activating ligand, as stimulation with BMP2 and BMP7 appears to have differential effects. That being said, despite having limited therapeutic options in development or available, treatment with BMP ligands themselves shows some potential *in vitro* (274-276).

ALK7

Function

Little is known regarding ALK7 (ACVR1C). Until recent years, the type II receptor and ligands interacting with ALK7 were unknown (277). Several ligands have now been identified, including GDF3, Activin B, and Activin AB (278-280). ALK7 shows high sequence similarity to ALK4 and ALK5, however, the structure of the extracellular domain diverges from the other type I receptors (159,277). Similarly to other ALKs, ALK7 substantially impacts development. In *Xenopus*, active ALK7 is associated with the induction of the mesoendodermal markers (281). In post-natal development and adulthood, ALK7 is primarily expressed in the central nervous system, where the signaling pathway is suggested to be involved in neuronal proliferation and differentiation, as well as the pancreas and colon (157,277). Alk7 knockout mice have been shown to metabolic issues, including reduced insulin sensitivity, impaired glucose tolerance, and enlargement of pancreatic islands (279).

Role in cancer and therapeutic potential

Loss of ALK7 in cancer has been, thus far, consistently associated with poor outcomes. In gallbladder cancer, expression of ALK7 has been associated with better survival compared to patients with ALK7-negative squamous cell, adenosquamous, and adenocarcinomas of the gallbladder (282). A similar pattern was found in breast cancer, where ALK7 expression is lost with increased cancer grade and stage (283). *In vitro* utilization of a triple negative breast cancer cell line showed that re-expression of ALK7, along with Activin B treatment, can restore the functional effects of this pathway and inhibit proliferation. A more molecular examination of ALK7 in ovarian cancer has indicated that ALK7 signaling, via the Smad2/3 axis, can

upregulate and downregulate cyclin G2 and Skp1 and Skp2, respectively, whereby inhibiting cell cycle and acting as a tumor suppressor (284). ALK7 regulation in cancer may be a result of post-transcriptional regulation. Ye and colleagues found that loss of ALK7 in ovarian cancer patient samples was associated with high expression of mir376c. Additionally, high levels of mir376c were found in patients who exhibited chemoresistance. *In vitro* overexpression of ALK7 could partially overcome cisplatin-induced cell death (285).

As previously discussed, specific inhibition of ALK7 has proven no small feat. Of the current inhibitors, SB-431542 and SB-505124 are the most highly utilized in the laboratory, with SB-505124 being used in Phase I clinical trials (234,235). However, as these are not specific inhibitors (also target ALK4 and ALK5), there is no sure way to know if targeting ALK7 is yet beneficial, or if targeting the combination of receptors is more efficacious.

Conclusion

As presented here, signaling of the TGFβ signaling family is mediated by the formation of heteromeric complexes of type I and type II receptors. Downstream signaling occurs upon ligand binding and affects various cellular processes in normal cells with implications for tumorigenesis through the regulation of apoptosis, migration and invasion, angiogenesis and the immune response. The promise of using ALKs as therapeutic targets has been successful in Phase I clinical trials, yet the challenge lies in restoring homeostasis in the face of multiple overlapping downstream signaling cascades and the potential of off-target effects resulting in serious side effects. Additional difficulties are met by the similarity of these receptors and the aim to specifically inhibit one type of receptor. Aside from the receptor inhibition as described in this review, other pre-clinical tests aimed to neutralize the ligands. A neutralizing antibodies against

TGFβ, 1D11, demonstrated successful suppression of metastasis in a mouse breast cancer model (286). Clinical trials testing a number of approaches to inhibit TGFβ signaling are recruiting or ongoing (clinicaltrials.gov). Ligand traps consisting of extracellular domains of human ACVR (mostly type 2) have been developed and are valuable in Activin-induced muscle wasting, cachexia, one of the main complications of cancer.

Taken together, although most cancers show alterations of the TGFβ pathway, use of inhibitors has shown some encouraging early results, yet many hurdles must be overcome before they could be considered for first-line treatments.

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CHAPTER III

ACTIVIN A BALANCE REGULATES EPITHELIAL INVASIVENESS AND TUMORIGENESIS

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Preface

The Activin A signaling pathway has been an area of continuous research over the last several decades. Since the isolation of Activin A and its characterization in regards to folliculogenesis and embryonic development, the role of Activin A in post-natal tissues has been widely disputed. Due to its similarities to the TGFβ pathway, there have been substantial arguments suggesting that Activin A may act in a comparable manner to the dual role of TGFβ (also known as the 'TGFβ switch') in the context of normal physiology and, subsequently, disease. In this work, we examined this relationship in the context of esophageal homeostasis. Working in close collaboration with Dr. Gregoire Le Bras, a post-doctoral fellow in the lab, we investigated and determined that Activin A works to regulate cell invasion through the maintenance of epithelial homeostasis along the spectrum of normal (E-cadherin expressing) to dysplastic (E-cadherin and TGFβ receptor type II dominant negative) esophageal squamous cells. Maintaining the delicate balance of Activin A expression is critical for the preservation of

the esophageal epithelium and stromal microenvironment. The work presented in this chapter laid the groundwork for work described in the succeeding chapters.

Abstract

Activin A is a member of the TGFβ superfamily. Activin A and TGFβ have multiple common downstream targets and have been described to merge in their intracellular signaling cascades and function. We have previously demonstrated that coordinated loss of E-cadherin and TGFβ receptor II results in epithelial cell invasion. When grown in three-dimensional organotypic reconstruct cultures, esophageal keratinocytes expressing dominant-negative mutants of E-cadherin and TGFβ receptor II showed activated Smad2 in the absence of functional TGFβ receptor II. However, we could show increased levels of Activin A secretion and recombinant Activin A was able to induce Smad2 phosphorylation. Growth factor secretion can activate autocrine and paracrine signaling, which affects crosstalk between the epithelial compartment and the surrounding microenvironment. We show that treatment with the Activin A antagonist follistatin or with a neutralizing Activin A antibody can increase cell invasion in organotypic cultures in a fibroblast- and MMP-dependent manner. Similarly, suppression of Activin A with shRNA increases cell invasion and tumorigenesis *in vivo*. Therefore, we conclude that maintaining a delicate balance of Activin A expression is critical for homeostasis in the esophageal microenvironment.

Introduction

Activin A (Act A), like other members of the TGFβ superfamily, is a regulator of embryonic development and tumorigenesis (287-289). Act A binds to type II Activin receptors, ActRII/IIB, that have intrinsic serine/threonine kinase activity. Binding of Act A results in the recruitment of

Activin receptor type I, ALK4, and the subsequent phosphorylation and activation of Smad 2/3 (290,291). TGFβ and Activin A signaling intersect at the level of Smad2/3 resulting in common downstream effector activation.

Previous research has shown that coordinated loss of E-cadherin and TGFβ receptor II (TβRII) occurs in the majority of esophageal squamous cell cancers (292). Based upon this evidence, we have established an organotypic culture model system utilizing esophageal keratinocytes expressing dominant-negative mutants of E-cadherin and TβRII to investigate the biological consequences of their loss. Keratinocytes expressing dominant-negative forms of E-cadherin and TβRII and tumor tissues with coordinated loss of E-cadherin and TβRII retained the expression of phosphorylated Smad2, thus suggesting that Act A compensates for the loss of TGFβ signaling and induces the activation of Smad2.

Similar to TGFβ, Act A is described as having pro- and anti-tumorigenic functions. Members of the TGFβ family, including Act A, can downregulate E-cadherin, promoting cell invasion (293). Our previous study showed increased cell invasion in Boyden chamber assays in response to Act A stimulation through an E-cadherin-dependent increase of the CD44 variant form in the esophageal cancer cell line TE-11 (294). In the current study, we further demonstrate the increased secretion of Act A in invasive organotypic cultures. Act A can suppress proliferation in different tumor cells (7,295,296), mainly through the induction of p15INK4B (297), while others describe an oncogenic role for Act A (289,298-301). From these reports and similar observations made for TGFβ1, which also has been shown to have dual functions (302,303), Act A is likely to have a role in the regulation of epithelial homeostasis. Interestingly, other studies demonstrated that Act A is necessary for the maintenance of self-renewal in human embryonic stem cells through the induction of Oct4, Nanog, Nodal, Wnt3,

and FGF-2 and by suppressing BMP signaling (72). These observations illustrate Act A function as a mediator of stemness with the potential for being a cancer stem cell marker (304,305). Furthermore, suppression of ID2, a downstream target of Act A and TGFβ1, is central to the induction of epithelial-mesenchymal-transition (73), a mechanism known to induce single-cell invasion, which is antagonized by BMP. Another Act A target, ID1, regulates epidermal homeostasis (306). However, the role of ID1 in proliferation could only be demonstrated in three-dimensional cultures, not in monolayer experiments (306).

To better understand the complex role of Act A in esophageal cell invasion, we employed three-dimensional organotypic cultures to reconstitute the epithelium with squamous esophageal epithelial cells expressing wild-type full-length E-cadherin (E), dominant-negative mutant E-cadherin (EC) or dominant-negative mutant E-cadherin and TGFβ receptor II (ECdnT). We found that Act A secretion is increased in ECdnT cells. In addition, altered cell invasion of ECdnT cells *in vitro* was dependent upon fibroblasts and MMP activation. *In vivo,* we show that loss of Act A can initiate tumorigenesis using xenograft models. Overall, our data indicate that Act A concentrations contribute to homeostasis in the esophageal microenvironment and, in the absence of functional TGFβ signaling, can shift the balance towards tumor invasiveness.

Material and Methods

Cell culture and tissues

Primary esophageal epithelial cells (keratinocytes) from normal human esophagus were established as described previously (292). Fetal esophageal fibroblasts were isolated as previously described (292) and head and neck cancer-associated fibroblasts were purchased

from Asterand (Detroit, MI). Fibroblasts were grown in DMEM with 5% fetal bovine serum (FBS, Hyclone, Thermo Fisher Scientific, Waltham, MA), 100 units/mL penicillin, and 100 µg/mL streptomycin (Gibco, Invitrogen, Carlsbad, CA). Human umbilical vein endothelial cells (HUVEC) were grown in EBM-2 basal media (Lonza Biosciences, Walkersville, MD) supplemented with endothelial growth medium 2 (EGM-2) growth factors (Lonza Biosciences). A tissue microarray with 83 spotted squamous esophageal tissues, AccuMax Tissue Microarray, was purchased from ISU Abxis (distributed by Accurate Chem, Westbury, NY).

Lentivirus infection

shRNA-mediated loss of Act A in esophageal squamous cells was performed using shRNA directed towards three different target sequences. Act A shRNA and control pGIPZ plasmids were purchased from Thermo Scientific. Virus was generated using HEK293T cells. Cells were then transduced and sorted using flow cytometry prior to experimental use.

Xenograft animal experiments

The current study protocol was reviewed and approved by the Vanderbilt University Animal Care and Use Committee. Briefly, a total of 1×10^6 cells (*INHBA*-high with normal esophageal fibroblasts) were suspended in 150 µl of Matrigel (BD Bioscience, Franklin Lakes, NJ) and kept on ice prior to subcutaneous injection into the flanks of 8–10 week old female NOD/SCID mice (The Jackson Laboratory, Bar Harbor, ME). Tumor growth was monitored weekly by caliper measurements.

Organotypic Culture

Organotypic reconstructs were grown as previously described (292) with the exception that each culture was rinsed in 1xPBS and incubated with Epidermalization 3 medium lacking serum for two more days before harvesting. The following treatments were added to the organotypic cultures at the time of epithelial seeding and renewed with every media change: 5 ng/ml recombinant human TGFβ1, 10ng/ml Activin A, 100 ng/ml follistatin and 600 ng/ml neutralizing antibody against Act A (all from R&D Systems, Minneapolis, MN), or 1 μM A83-01 (Tocris, Bristol, UK) and 1 μM GM6001 (Millipore EMD, Billerica, MA). Puromycin treatment at 5 μg/ml on day 5 was applied to induce fibroblast cell death. The culture matrix was washed serially with 1xPBS, before the epithelial cells were seeded.

Spheroid formation

Spheroid assays were performed as described previously (292). In brief, cells were resuspended in 2% Matrigel and then cultured on a Matrigel layer in chamber slides (Nalgene Nunc, Naperville, IL). Cells were fixed in 4% paraformaldehyde (Fisher Scientific, Hampton, NJ) overnight at 4°C. Incubation with Alexa568–conjugated phalloidin was overnight at 4°C.

Proliferation assays

Cells were plated at 500 cells per well in a 96-well plate for proliferation assays. WST-1 reagent (Roche, Nutley, NJ) was added to each well at the time points indicated and incubated at 37°C for a minimum of 1 hour. Absorbance measurements at 450 nm were taken using a BioTek Synergy 4 plate reader (Winooski, VT). Measurements were taken in 24-hour increments.

Immunofluorescence

Organotypic culture tissue, previously fixed in formalin for 24 hours and embedded in paraffin, was sectioned at 5 μm, deparaffinized and heated in 1xTBE buffer in a pressure cooker for 12 minutes for antigen retrieval. Samples were blocked in 1xPBS with 5% Bovine Serum Albumin (1xPBS-BSA 5%, Sigma-Aldrich, Saint Louis, MO) for 1 hour prior to incubation with primaries antibody in 1xPBS-BSA 5% overnight at 4°C. Tissues were then rinsed three times in 1xPBS and incubated with secondary antibodies in 1xPBS-BSA 5% for 1 hour at room temperature. After additional rinses with 1xPBS, the sections were mounted with Vectashield mounting medium containing DAPI (Vector Laboratories, Burlingame, CA). Images were taken on a Zeiss microscope, using Axiocam and Axiovision software (Carl Zeiss Microscopy, Thornwood, NY).

Immunohistochemistry for TβRII and pSmad2 in the tissue microarrays and OTC samples was done using the Vectastain Elite kit (Vector Laboratories) following the manufacturer's protocol.

Western Blot

Western blots were performed as previously described (294). The results are representative of at least three independent experiments.

Flow cytometry

Flow cytometry was used to separate lentivirus-transduced from the non-transduced population based upon GFP+ status. Gates were set to divide high and low GFP+ expressing cells. Flow cytometry experiments were performed using a BD AriaIII flow cytometer (BD

Biosciences, Franklin Lakes, NJ) in the VUMC Flow Cytometry Shared Resource. After sorting, the cells were returned to culture for future experiments.

ELISA

Capture ELISAs for Act A and TGFβ1 were purchased from R&D Systems. The protocols were performed according to the manufacturers instructions.

Invasion assays

Matrigel-based invasion chamber assays were purchased from BD Biosciences and performed according to manufacturer's directions. Act A was added either to the top insert or the bottom chamber to assess hapto- versus chemotactic invasion during 16–24 hours of incubation.

For trans-endothelial invasion assays (xCELLigence system, Roche Diagnostics), Roche E-plates (Roche Diagnostics) were treated with 100 µl of 0.1% sterile gelatin (Sigma-Aldrich) overnight at 4°C. Plates were washed once with sterile 1xPBS before the addition of HUVEC cells (Lonza Biosciences). 25,000 HUVEC cells/100 µl were seeded on E-plates and incubated for 18 hours at 37°C. The cell index was monitored on the xCELLigence system while the monolayer was formed. Following the formation of the HUVEC monolayer, which is indicated by the plateau in the cell index, the endothelial cell growth medium EGM-2 was removed and 100 µl of RPMI containing 5% serum media was added. The cell index was monitored for 4 hours and allowed to stabilize. Then, esophageal epithelial cells pre-treated with Act A for 24 hours were added to each well at a density of 5,000 cells/100 µl. The cell index was normalized to the HUVEC monolayer and invasion was monitored over time as epithelial cells disrupt the endothelial cell layer (changes in initial slope). Rate of invasion of

the cell lines were calculated in real time according to the RTCA software version 1.2.

Zymography

Zymography was performed as previously described (294). Briefly, conditioned medium was separated by SDS-PAGE at 4°C in gel containing gelatin. The gels were then washed in Triton 2.5% (v/v) in ddH2O twice for 30 min and incubated overnight in at 37°C in a development buffer (0.05M Tris-HCl pH 8.8, 5mM CaCl2, 0.02% NaN3). Then, the gels were stained in 0.1% Coomassie Blue R-250 for an hour and destained in methanol/acetic acid/water solution (10%: 20%: 70%, v:v:v). Images were taken on Gel Doc XR system (Bio-Rad, Hercules, CA).

Antibodies and other reagents

Alexa568-conjugated Phalloidin (Invitrogen), αSMA (Sigma-Aldrich) and podoplanin (eBioscience, San Diego, CA) were used for immunofluorescence. Anti-TβRII (clone L21), PAI-1 (Santa Cruz Biotechnologies, Santa Cruz, CA), Ki67 (Vector Laboratories). and phospho-Smad2 (Cell Signal Technologies, Danvers, MA) were used for immunohistochemistry. Antibodies used to detect protein by Western blotting were E-cadherin (BD Biosciences) and vimentin (Sigma-Aldrich);

Table 3-1. Information about Oncomine/Nextbio datasets used for *INHBA* expression analysis.

Database	Dataset ID	Expression Analysis	ESCC compared to	Platform	Fold Change	p-value
Oncomine	GSE29001	mRNA	Normal esophageal basal layer	GPL571 Affymetrix Human Genome Array U133A 2.0	10.2	0.0001
Oncomine/ NextBio	GSE20347	mRNA	Normal adjacent	Affymetrix GeneChip Human HG_U133A	7.13	2.30E-05
Oncomine/ NextBio	GSE23400	mRNA	Normal adjacent	Affymetrix GeneChip Human HG_U133	6.96	1.10E-11

total Smad 2, phospho-Smad2, were purchased from Cell Signaling Technologies; α-tubulin was purchased from Abcam (Cambridge, MA).

Dataset analysis

Datasets made publicly available from Oncomine (http://www.oncomine.org/), GEO Datasets (http://www.ncbi.nlm.nih.gov/gds/; (307)), and NextBio (http://www.nextbio.com/b/nextbio.nb; (308)) were used to query clinical correlations with Act A (Table 3-1). The collected information from each dataset was analyzed and visualized in Prism version 6.00 for Mac (GraphPad software, La Jolla, California, www.graphpad.com).

Biostatical analysis

Biostatistical analysis was performed using Prism version 6.00 for Mac. *In vitro* and *in vivo* experiments were analyzed using Student's t-tests or one-way ANOVAs. Statistical significance was set at $p<0.05$. All experiments were done in triplicates with at least three biological replicates.

Results

Increased epithelial cell invasion is associated with increased Act A secretion

The R-Smads (Smad2 and Smad3) are common downstream mediators of both TGFβ and Act A signaling. We performed immunohistochemistry using antibodies against TβRII and pSmad2 to determine their expression in non-invasive and invasive organotypic cultures. Keratinocytes expressing wild-type E-cadherin (E) form a non-invasive epithelial sheet, while expression of dominant-negative E-cadherin (EC) or combined expression of dominant-negative E-cadherin and TβRII (ECdnT) led to a gradual increase of invasiveness, as shown previously (294). We

have previously shown that expression of dominant-negative E-cadherin correlates with lower TβRII levels in EC cells, potentially due to a lack of its stabilization at the cell membrane (292). Even though the signal for TβRII was lower in EC and ECdnT cells than in the normal control epithelium, represented by the E cells (Figure 3-1a), we observed a strong signal of nuclear pSmad2 in invasive ECdnT cells. Nuclear localization of pSmad2 in the absence of TβRII staining supports the notion that Smad2 can be activated and phosphorylated through a separate signaling pathway, when TβRII expression is low or disrupted. We subsequently

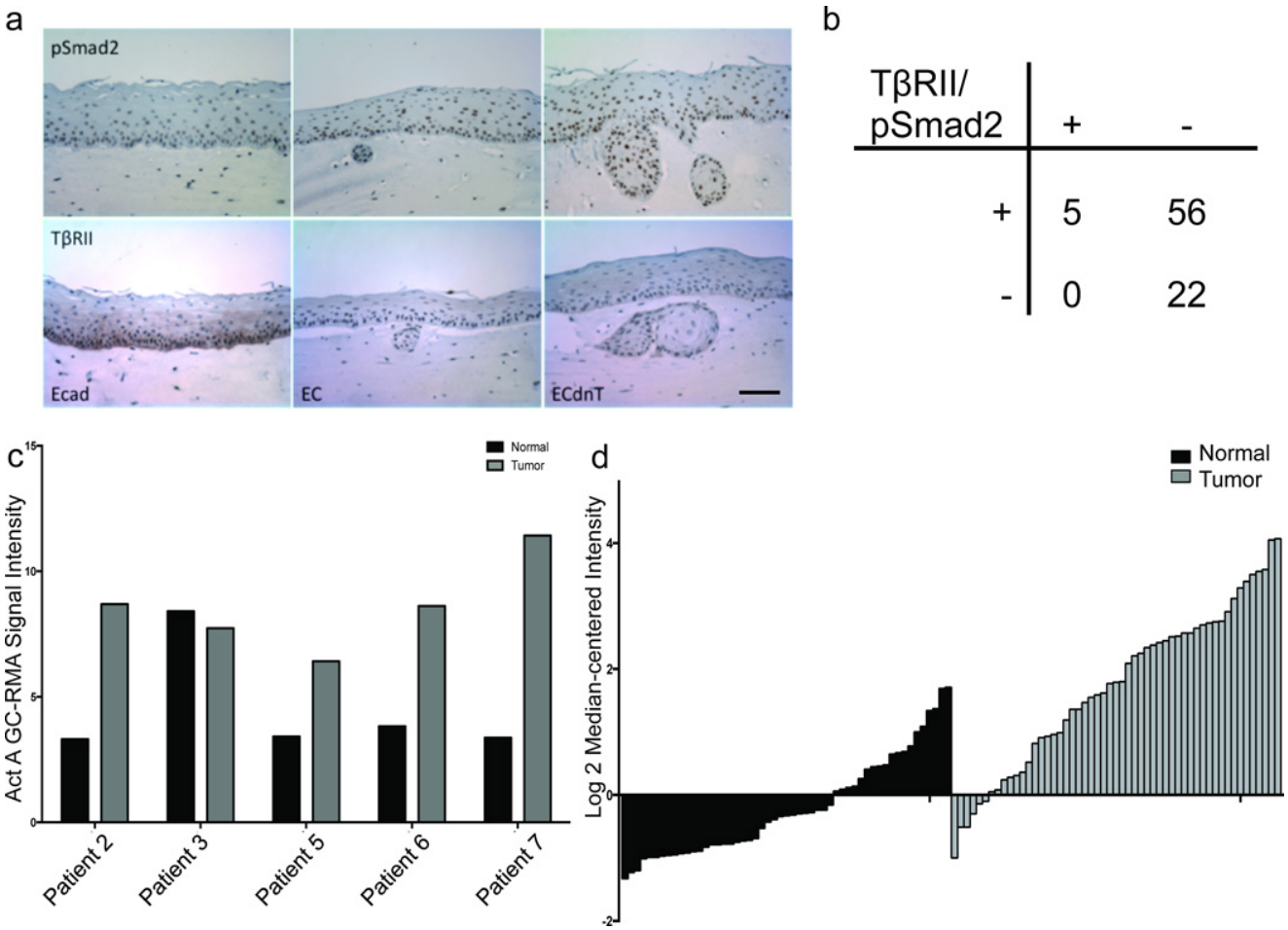

Figure 3-1. Activin A (Act A) specifically stimulates phosphorylation of Smad2 and Act A upregulation is common in esophageal squamous cell carcinoma (ESCC). (a) Immunohistochemistry staining with antibody against phosphorylated Smad2 (pSmad2) and TGFβ receptor II (TβRII) showed increased nuclear signal for pSmad2 in the invasive dominant-negative E-cadherin and TβRII (ECdnT) organotypic cultures. Scale bar, 50μm. (b) Analysis of immunohistochemistry staining for TβRII and pSmad2 in 83 ESCC cases in a tissue microarray showed no significant correlation. Fisher's exact test, two-tailed p=0.3182. (c) Five paired normal adjacent and ESCC tissues (GSE17531) were analyzed for *INHBA* mRNA expression, which identified upregulation of *INHBA* in four ESCC samples. (d) Waterfall plot of a publically available data set (GSE23400) represented upregulation of *INHBA* in the ESCC (gray bars) samples versus normal (black bars).

analyzed 83 esophageal squamous cell carcinoma tissues (ESCC) for E-cadherin (292), TβRII and pSmad2 expression. Seventy-one percent of the tumor tissues retained Smad2 activation in the absence of TβRII (Figure 3-1b). To determine the levels of Act A gene expression in ESCC, we analyzed published datasets and identified upregulation of Act A in ESCC tumor samples compared to normal tissues (Figure 3-1c,d; Table 3-1) (309,310).

As esophageal epithelial cells invade in a fibroblast-dependent manner (292,294), we

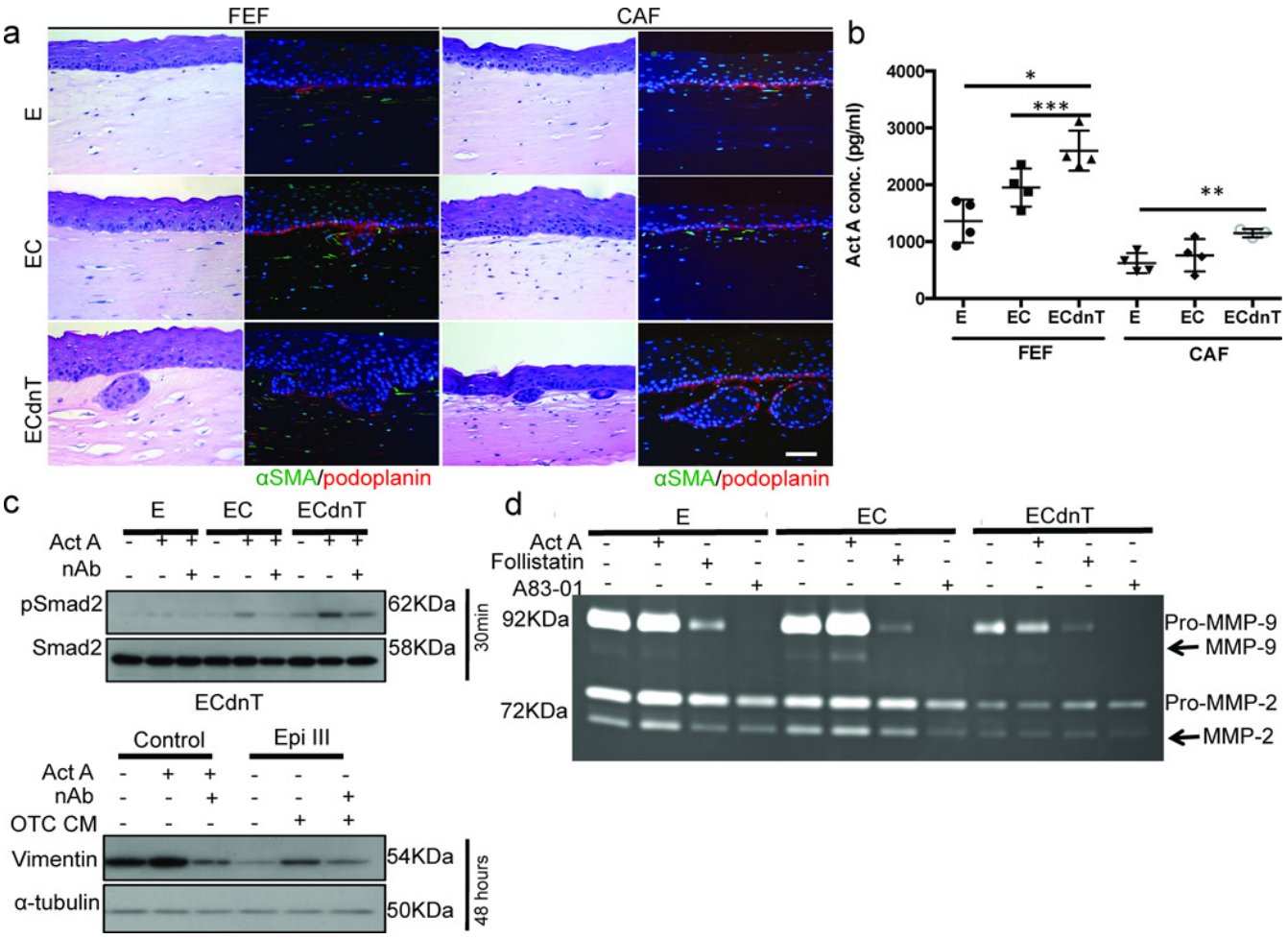

Figure 3-2. Increased epithelial cell invasion is associated with higher levels of secreted Activin A. (a) Esophageal epithelial cells expressing full-length E-cadherin (E), dominant-negative mutant E-cadherin (EC), or dominant-negative mutant E-cadherin and TGFβ receptor II (ECdnT) were grown in organotypic cultures with either fetal esophageal fibroblasts (FEF) or cancer-associated fibroblasts (CAF) embedded in the underlying matrix. Immunofluorescence staining with antibody against αSMA (green) and podoplanin (red) showed similar expression patterns in the cultures. Scale bar, 50μm. (B) Act A concentration in conditioned media from organotypic cultures is higher in invasive cultures as measured using indirect ELISA. *p=0.003, **p=0.005, ***p=0.03 (c) Stimulation of epithelial cells with Act A in monolayer plastic culture demonstrated phosphorylation of Smad. Neutralized antibody (nAb) against Act A prevented the induction of pSmad2 by Act A. Following stimulation with Act A or with conditioned media from organotypic culture increased expression of vimentin after 48h by Western blot. The increase was reversed in the presence of nAb. (d) Inhibition with the Act A antagonist, follistatin, or a pan-TGFβ inhibitor A83-01 suppressed MMP-9 secretion in E, EC, and ECdnT cells as measured by gelatin zymography. Upper bands reflect pro-MMP, lower bands activated, cleaved MMP (arrow).

employed organotypic cultures comparing the effects of the presence of embedded fetal esophageal fibroblasts (FEF) and cancer-associated fibroblasts (CAF) on cell invasion and Act A secretion (Figure 3-2a,b). E or EC cells grown in organotypic cultures with FEFs or CAFs had minimal invasive potential, while ECdnT cells showed cell invasion in either context (Figure 3-2a). Immunofluorescence staining with antibodies against the fibroblast-specific marker αSMA (green) exclusively labeled FEFs and CAFs in the matrix, while podoplanin (red) expression, a putative marker of collective invasion, stained the basal membrane of the epithelial cells (Figure 3-2a).

Since our analysis of human ESCC samples implicated Act A in the regulation of Smad2 and cell invasion (Figure 3-1), we determined the levels of Act A in the conditioned media from organotypic cultures with different levels of invasion. We performed ELISA and observed a positive association between Act A secretion levels and epithelial cell invasion (Figure 3-2b). To test for specificity of Act A-mediated phosphorylation of Smad2 in this model system, we isolated protein from keratinocytes grown in a monolayer on plastic for Western blot analysis. We compared protein lysates from conditions with Act A stimulation or neutralizing antibody (nAb) to demonstrate that phosphorylation of Smad2 could be induced by Act A with high specificity. Increased signal for pSmad2 was detected following Act A stimulation, which was reduced in the presence of the neutralizing antibody. Similarly, treating ECdnT cells with Act A or conditioned media from organotypic cultures induced vimentin, a mesenchymal cytoskeleton component and a marker of tumor cell invasion. Conversely, Act A neutralizing antibody inhibited vimentin expression (Figure 3-2c).

We speculated that Act A may alter extracellular matrix degradation, since epithelial cell invasion is strongly associated with the digestion of the extracellular matrix by matrix

metalloproteases. Therefore, we collected conditioned media from cells grown in monolayer and analyzed the secretion of the collagen-digesting matrix metalloproteases MMP-9 and MMP-2 using gelatin-zymography (Figure 3-2d). When using recombinant follistatin, the endogenous inhibitor of Act A (311), or A83-01 (an inhibitor for ALK4/5/7), MMP-9 secretion was reduced compared to untreated or Act A stimulated monolayer cultures (Figure 3-2d).

Stimulation with Act A increases spheroid size and cell invasion *in vitro*

To identify the functional consequences of Act A-mediated Smad2 signaling, we grew E, EC

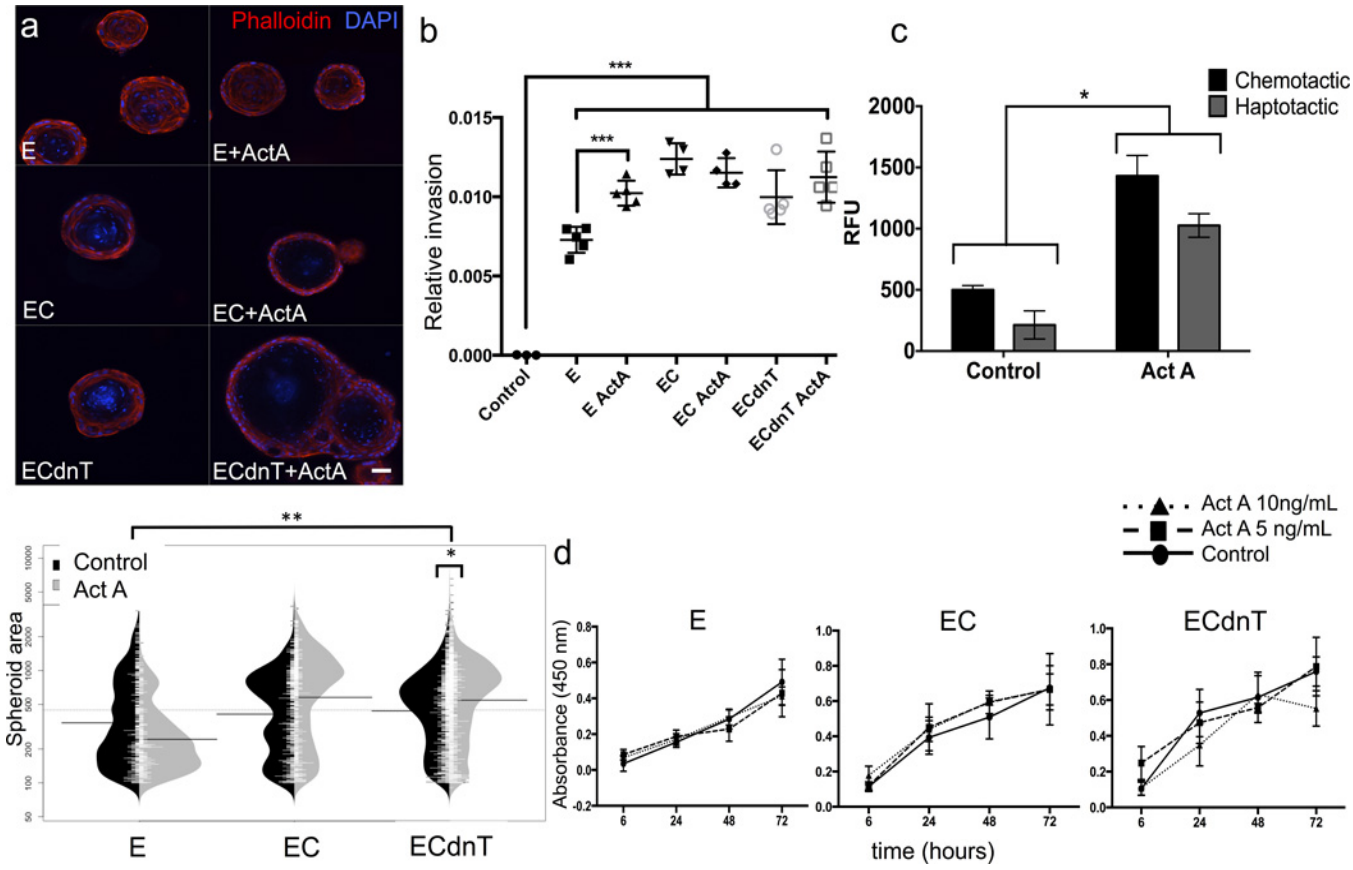

Figure 3-3. Stimulation with Activin A increases spheroid size and cell invasion *in vitro*. (a) Esophageal epithelial cells, when grown embedded in 2% Matrigel, form spheroids. Immunofluorescence with Alexa568-conjugated Phalloidin stained the actin cytoskeleton. DAPI (blue) was used as a counterstain for nuclei. Scale bar, 50µm. Stimulation with recombinant Act A increased spheroid size and number as quantified in the graph, * and ** $p<0.0001$. (b) Transendothelial cell migration was measured after stimulation of esophageal epithelial cells with Act A, *** $p<0.0001$ (c) Haptotactic (stimulation in top chamber) and chemotactic (addition to the media in the bottom chamber) invasion of ECdnT cells in response to Act A was measured in Boyden chamber assays. kSFM is media only control, * $p<0.0001$ (d) Proliferation index was determined after stimulation with two concentrations of Act A using the WST-1 assay. Cell proliferation was unchanged by stimulation with Act A.

Table 3-2. Biostatistical analysis of spheroid size for each treatment.

Condition	Mean difference (95%CI)	p-value
E Act A vs. E control	-191.4 (-310.6,-72.1)	<0.0001
EC Act A vs. EC control	265.8 (168.5,363.2)	<0.0001
ECdnT Act A vs. ECdnT control	172.0 (103.1,241.0)	<0.0001
E Act A vs. ECdnT Act A	-435.9 (-538.7,-333.0)	<0.0001
E Act A vs. EC Act A	-516.0 (-628.7,-403.3)	<0.0001
EC Act A vs. ECdnT Act A	80.2 (-2.9,163.3)	ns
E control vs. EC control	-58.8 (-163.7,46.1)	ns
EC control vs. ECdnT control	-13.6 (-99.2,72.0)	ns
E control vs. ECdnT control	-72.4 (-164.0,-19.2)	<0.0001

and ECdnT cells embedded in Matrigel to induce spheroid formation (Figure 3-3a). In the presence of Act A, ECdnT cells form more and larger spheroids compared to E and EC cells (Graph and Table 3-2). In a transendothelial invasion assay, we observed decreased impedance as a measure of cell invasion through the endothelial cell layer (Figure 3-3b). Epithelial cells pre-treated with Act A were seeded on a layer of endothelial cells (HUVEC) and a significant disruption of the HUVEC layer was observed with Act A stimulation in normal E cells. More invasive EC and ECdnT cells disrupted the HUVEC layer to a comparable degree with or without Act A stimulation.

When assessing Act A function in Boyden chamber invasion assays (Figure 3-3c), we added Act A either to the top chamber to act as a stimulant for the ECdnT cells or to the bottom to act as a chemoattractant. Act A increased haptotactic as well as chemotactic cell invasion. This observation was in line with our previous report that Act A increased cell invasion in esophageal cancer cell lines (294).

Finally, we show that the increased cell invasion is independent of cell proliferation, as WST-1 assays did not show differences in proliferation index in response to two different concentrations of Act A stimulation compared to untreated normal media (Figure 3-3d).

Imbalance of Act A induces epithelial cell invasion

Organotypic cultures allow us to measure the effects of the stromal compartment and epithelial

cell homeostasis in a physiologically relevant context, while modifying signaling activation (Figure 3-4). As described earlier, wild-type E-cadherin expressing cells grow as a normal stratified epithelium without invasion into the matrix (Figure 3-4a). When treated with Act A, epithelium formation of E cells remained unchanged (Figure 3-4a). However, the addition of follistatin induced cell invasion, while Act A neutralizing antibody did not. Similarly, EC cells,

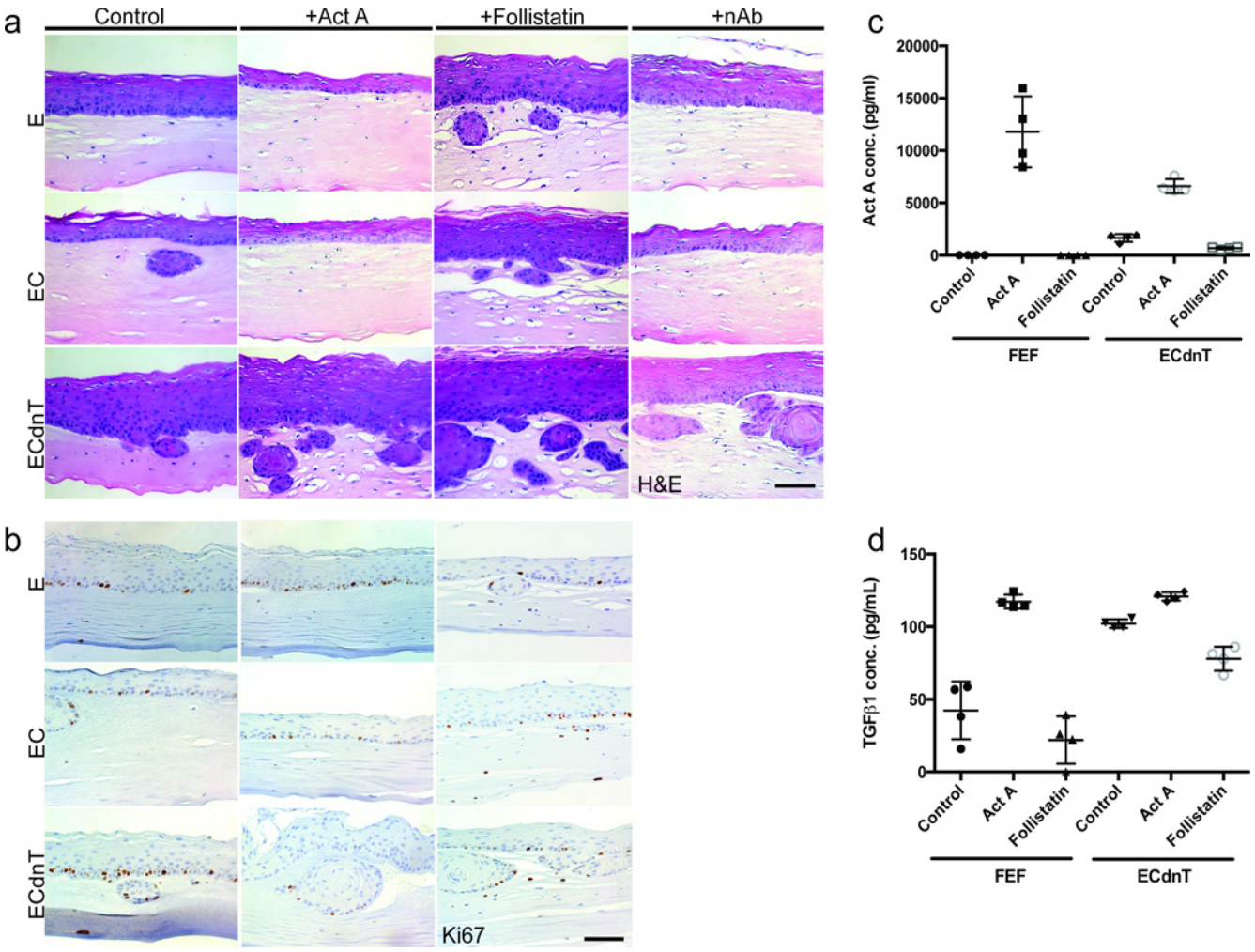

Figure 3-4. Imbalance of Activin A induces epithelial cell invasion. (a) Esophageal epithelial cells expressing wild-type full-length E-cadherin (E), dominant-negative mutant E-cadherin (EC), or dominant negative E-cadherin and TβRII (ECdnT) were grown in organotypic cultures in the presence of recombinant Act A, its antagonist follistatin or a neutralizing antibody (nAb) against Act A; H&E staining. Stimulation with Act A inhibited invasion of E and EC cells, but failed to suppress ECdnT cell invasion. Follistatin increased cell invasion in all cell types, whereas the nAb prevented invasion of E and EC cells, without an effect on ECdnT cells. (b) Immunohistochemistry staining with a Ki67 antibody showed no differences in cell proliferation. Scale bars, 50μm. (c) Indirect ELISA with antibody against Act A measured increased levels after addition of recombinant Act A in fetal esophageal fibroblasts (FEF) and ECdnT. Untreated ECdnT cells (Control) secreted higher baseline levels of Act A than FEF, which were reduced by follistatin. (d) TGFβ1 concentration was increased in response to stimulation with Act A and overall baseline secretion was higher in control ECdnT cells than fibroblasts, as measured by indirect ELISA. Follistatin inhibited TGFβ1 secretion.

which have minimal invasive potential, show slightly increased invasion when treated with follistatin. However, Act A treatment or addition of the neutralizing Act A antibody prevented EC cell invasion. This strongly supports our idea that the cells are sensitive to changes in Act A and show differential responses when exposed to varying Act A concentrations in a context-dependent manner.

ECdnT cells have the highest potential for cell invasion and could be considered dysplastic or pre-malignant ((294), unpublished data). In contrast to E and EC cells, treatment with Act A did not inhibit ECdnT cell invasion. Conditions that disrupt Act A function, such as follistatin treatment or Act A neutralizing antibody, enhanced ECdnT cell invasion. Although the degree of cell invasion varied between the cell lines and treatment, the proliferation index as analyzed by staining for Ki67-positive cells was unchanged (Figure 3-4b). Again, invasion and changes in Act A signaling were not dependent on cell proliferation. However, as disruption of Act A signaling using follistatin or the neutralizing Act A antibody had differential effects, the balance of Act A signaling appeared to be integral for the induction of cell invasion.

In organotypic cultures, the respective treatments affected stromal as well as epithelial compartments. To analyze the role of Act A in autocrine and paracrine signaling, we determined Act A secretion levels in conditioned media from fibroblast and epithelial monolayer cultures stimulated with Act A or treated with the inhibitor follistatin (Figure 3-4c). FEFs did not secrete Act A though ELISA could detect the exogenous addition of Act A. Follistatin treatment had no effect on fibroblasts. Act A levels in ECdnT indicate endogenous expression and secretion, which was reduced in the presence of follistatin. Treatment with recombinant Act A elevated the levels measured by ELISA. Interestingly, TGFβ1 levels were influenced by Act A treatment in a similar pattern. Endogenous TGFβ1 secretion was at a

detectable level in fibroblasts, albeit higher in ECdnT. Follistatin reduced TGFβ1 secretion in both cell types. This may indicate a feedback mechanism of Act A and TGFβ signaling.

Induction of cell invasion by Act A is fibroblast- and MMP-dependent

Epithelial cells grown in a monolayer on plastic have different gene expression patterns and behaviors compared to cells grown in a physiological context, such as three-dimensional organotypic cultures. We have previously shown that fibroblast-secreted factors are necessary

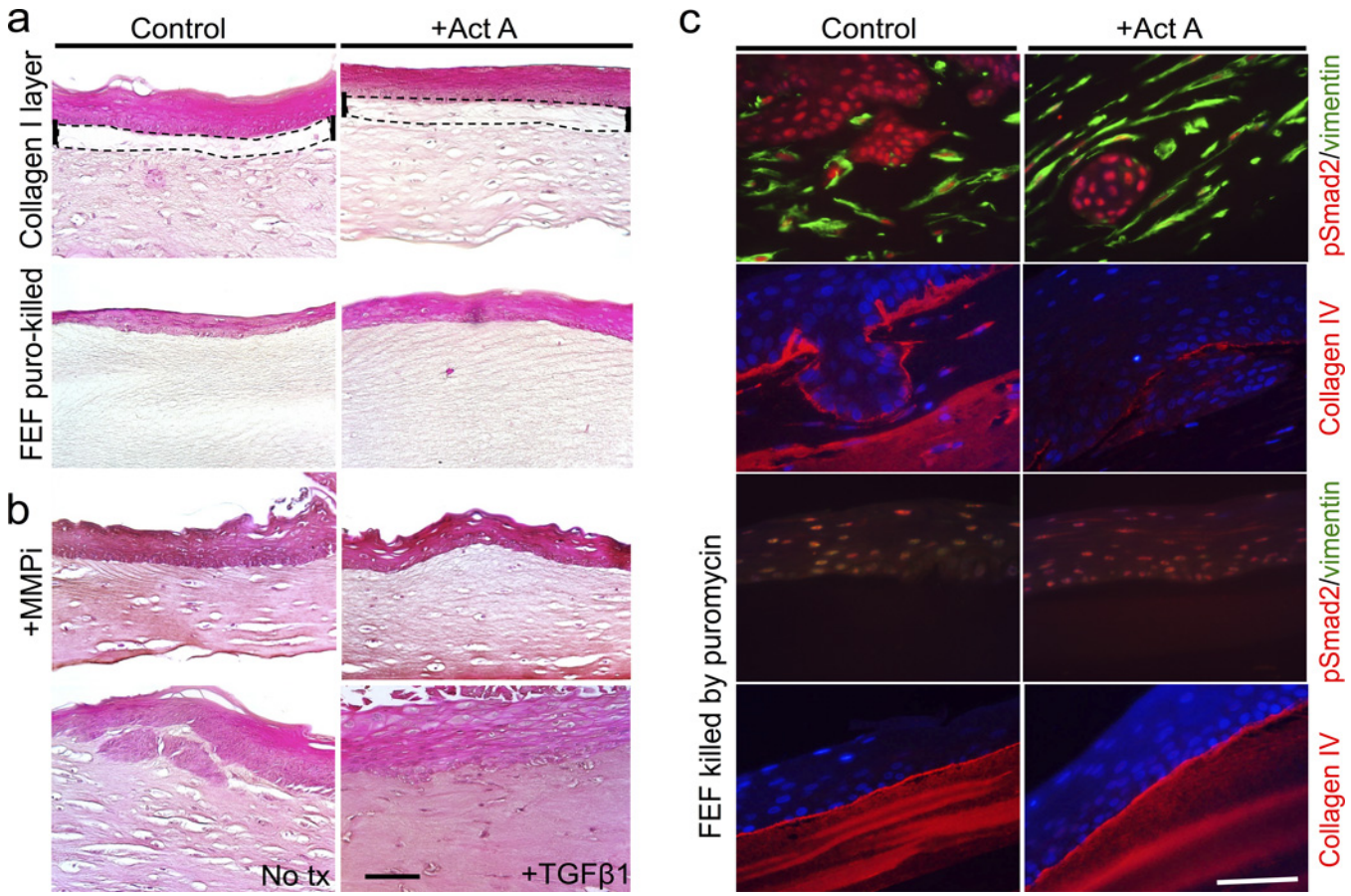

Figure 3-5. Regulation of cell invasion by Activin A is fibroblast- and MMP-dependent. (a) Separating the cellular matrix and epithelium of the organotypic cultures growing ECdnT cells through a collagen I layer, dashed lines, prevented cell invasion in the absence (control) and presence of Act A (+Act A). When the cellular matrix of the organotypic culture was treated with puromycin to kill the embedded fibroblasts before the ECdnT cells were seeded, epithelial formation occurred but invasion was inhibited with and without Act A stimulation. (b) Treatment of ECdnT organotypic cultures with a pan-MMP inhibitor, GM6001, suppressed cell invasion, which was not restored in the presence of Act A. Untreated (No tx) control ECdnT cells in organotypic culture invaded into the underlying matrix. TGFβ1 treatment inhibited epithelial cell invasion. Scale bars, 50μm. (c) Immunohistochemistry showed nuclear localization of phosphorylated Smad (pSmad2, red) in control and Act A-stimulated conditions. Collagen IV (red) was disrupted in invasive cultures after Act A treatment. Loss of the fetal esophageal fibroblasts (FEF), labeled green with antibody against vimentin (no staining in lower panels), had no effect on the nuclear localization of pSmad2. The collagen IV layer was not disrupted in noninvasive cultures in the absence of FEFs.

to induce cell invasion of ECdnT cells (294), yet we observed that Act A could initiate ECdnT invasion in Boyden chamber assays in the absence of fibroblasts or fibroblast-conditioned media (Figure 3-3b,c). We aimed to create an environment to address epithelial-fibroblast crosstalk and its effects on cell invasion. First, we overlaid the cellular matrix with a collagen I layer to prevent direct physical interaction of the epithelial cells with the embedded fibroblasts and potentially minimizing access to fibroblast-secreted factors (Figure 3-5a). ECdnT cells did not invade into the stroma in the presence of a collagen I layer, regardless of Act A stimulation. As the collagen I layer could alter the pliability and stiffness of the matrix affecting cell invasion, we next performed the experiment without the collagen I layer, however treated the stromal layer with the antibiotic puromycin to kill the embedded fibroblasts. Fibroblasts were allowed to crosslink and contract the collagen I/Matrigel matrix during the initial 5 days of culture then fibroblasts were killed by puromycin-treatment. ECdnT cells in the absence of fibroblasts did not invade into the extracellular matrix in control or Act A stimulated cultures (Figure 3-5a). This supports our previous observation that fibroblast paracrine signaling is important for epithelial cell invasion.

As MMP-9 secretion was induced by Act A treatment and correlated with increased cell invasion of ECdnT cells in organotypic cultures, we set out to determine if MMPs are necessary for Act A-induced ECdnT cell invasion. Using a pan-MMP activity inhibitor GM6001, we could abolish ECdnT cell invasion which could not be restored by Act A addition (Figure 3-5b). This finding indicates that blocking MMP activity downstream of Act A disrupts the signaling cascade necessary for ECdnT cell invasion. As a control, we treated ECdnT cells with TGFβ1, which inhibited ECdnT cell invasion (Figure 3-5b). These data show that Act A and TGFβ1 have independent functions in this cell system, but also highlight the complexity of

their balance. Immunofluorescence staining with antibodies against pSmad2 showed a strong nuclear signal in untreated control and Act A stimulated-organotypic cultures (Figure 3-5c). Collagen IV (red) was deposited at the basement membrane, but was disrupted after Act A treatment. The collagen IV layer was not disturbed in non-invasive conditions without fibroblasts regardless of Act A stimulation. Absence of fibroblasts (lack of vimentin-positive cells, green), while inhibiting cell invasion, only marginally diminished pSmad2-positive signal in the epithelial layer.

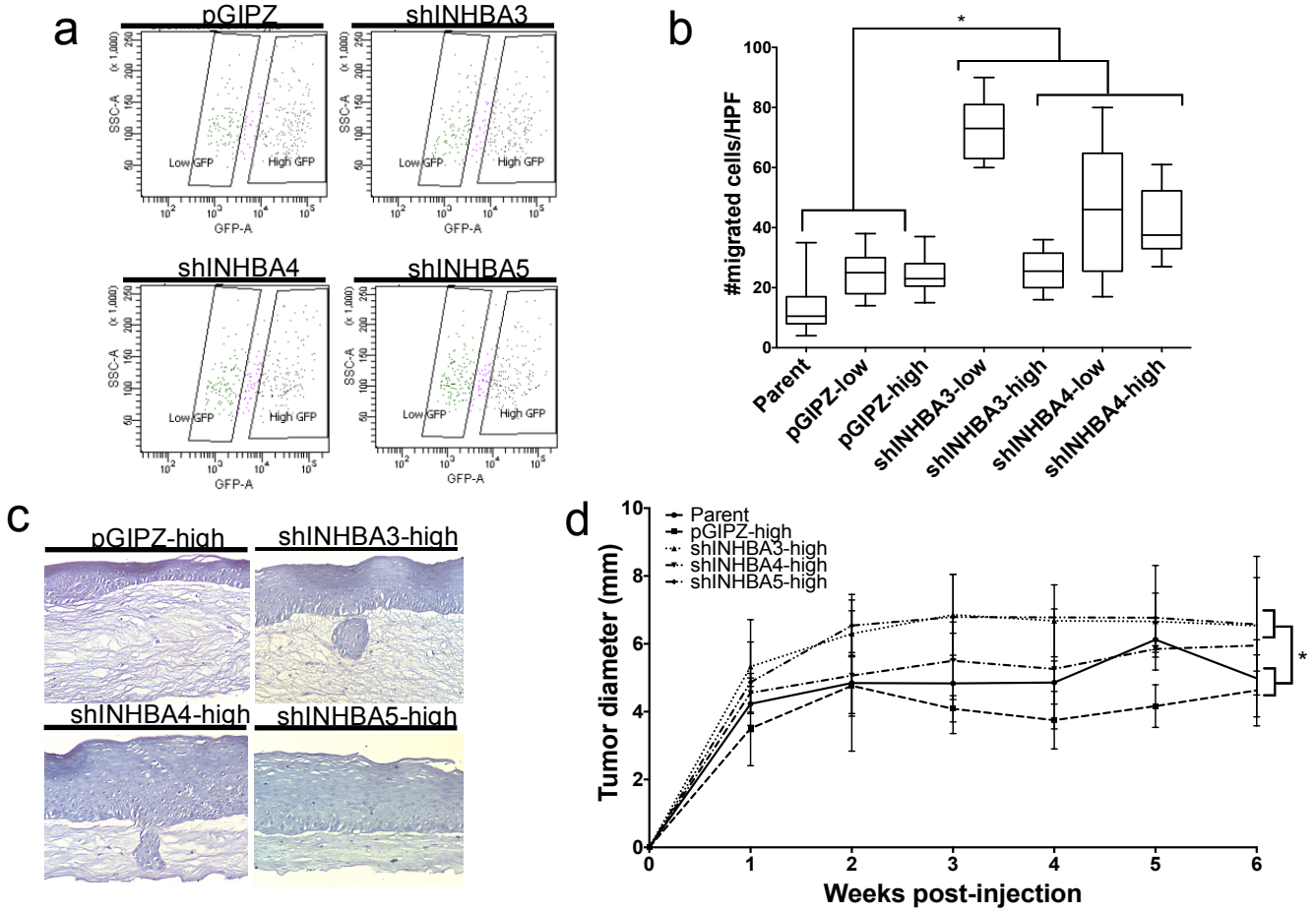

Figure 3-6. shRNA-mediated loss of Activin A promotes epithelial cell migration and invasion. (a) ECdnT cells transfected with shRNA-*INHBA*-GFP constructs (sh*INHBA*3 through 5) were sorted by flow cytometry for positivity and low/high expression (left versus right gating). (b) Boyden chamber migration assays were performed using sh*INHBA*-high and sh*INHBA*-low expressing cells. Both sh*INHBA*-high and sh*INHBA*-low showed overall increased *in vitro* migration compared with parent and empty vector (pGIPZ-high and -low) cells. Migrated cells are depicted as the number of cells counted per high-powered field. Analysis was performed using a one-way ANOVA and subsequent Student's t-test. (c) sh*INHBA* and empty vector control cells (pGIPZ-high) were grown in organotypic cultures with normal fetal esophageal fibroblasts (FEF). sh*INHBA* cultures had increased epithelial cell layer thickness and increased collective cell invasion into the underlying stroma, compared with control cultures. Scale bars, 50µm. (d) NOD/SCID flank injections were performed using sh*INHBA* high and control (parent and empty vector pGIPZ high) cells in co-culture with FEF. After 6 weeks post injection, sh*INHBA* high cells showed overall greater tumor growth, compared to parent and pGIPZ high control cells.

Act A knockdown results in cell invasion and tumorigenesis

Our data obtained in monolayer and organotypic cultures thus far have suggested that the pro-invasive role of Act A was in part driven by the presence of fibroblasts and the induction of MMP expression. Therefore, we next set out to determine if ECdnT cells would exhibit an invasive phenotype without Act A secretion and signaling. To answer this question, ECdnT cells were transduced with three distinct clones to stably express shRNA specifically targeting Act A, sh*INHBA*. Following transduction, the cells were sorted by flow cytometry to select for

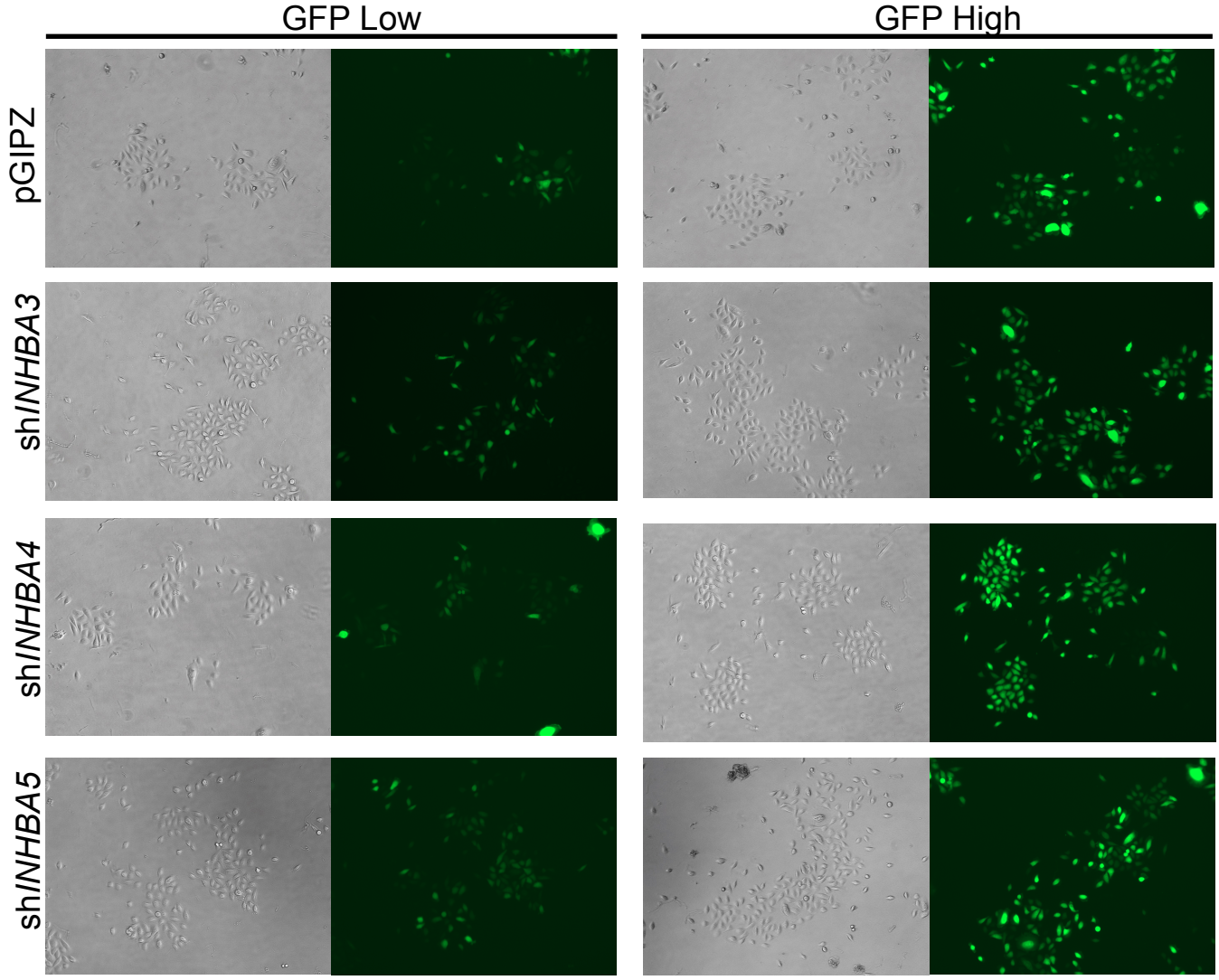

Figure 3-7. Images of ECdnT cells transduced with shRNA against Act A, sh*INHBA*, and empty control pGIPZ vector. Brightfield and corresponding fluorescent images shown had low (left panel) and high (right panel) GFP expression in each of the three clonal cell lines.

GFP-positive populations. To examine if Act A exerts distinct functions at different expression levels, we sorted the stable shRNA and empty vector control clones by GFP-low (sh*INHBA*-low) and GFP-high (sh*INHBA*-high) expression (Figure 3-6a; Figure 3-7). In Boyden chamber migration assays, sh*INHBA*-high and sh*INHBA*-low cells showed increased chemotactic migration compared to parent and empty vector (pGIPZh) control cells (Figure 3-6b; Figure 3-8a). sh*INHBA*-high and sh*INHBA*-low cells showed no proliferation differences compared to controls (Figure 3-8b). Interestingly, sh*INHBA*-low cells showed increased migration compared to sh*INHBA*-high cells, suggesting a dose-dependent effect of Act A signaling. Dose-dependent effects of Act A signaling have been previously described, primarily in embryogenesis (9,28,312). Similarly to the effect observed in the Boyden chambers, organotypic reconstructs of sh*INHBA*-high cells showed thicker epithelial cell layers and increased invasion into the underlying stroma compared to the pGIPZ-high control (Figure 3-6c).

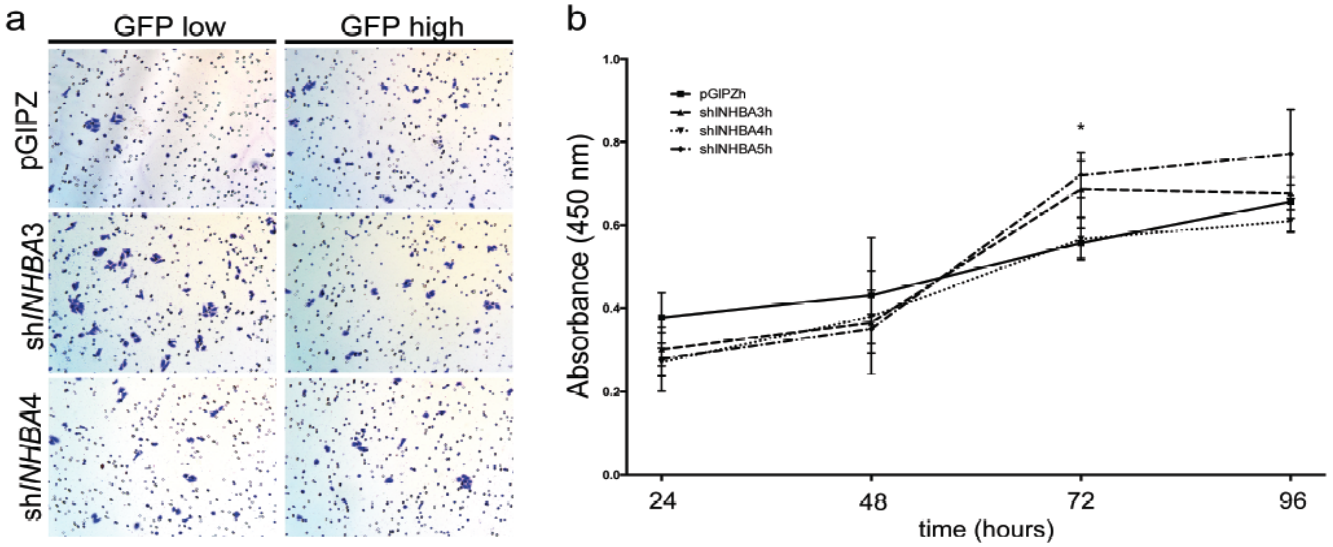

Figure 3-8. Migration, invasion and proliferation of sh*INHBA* cells, compared to pGIPZ control. (a) Images of Boyden chamber migration assays demonstrate increased motility of cells lines transduced with shRNA against Act A, sh*INHBA*. Crystal violet staining of the membranes after cell migration. (b) Proliferation of sh*INHBA* cell lines compared to pGIPZ control vector. WST-1 assays were performed on three different *INHBA* knockdown cell lines and showed no differences in proliferation index compared to empty vector control.

Finally, as we were able to observe an effect after loss of Act A in epithelial cells *in vitro*, we set out to explore functional consequences of Act A suppression *in vivo*. Utilizing a Matrigel-plug xenograft model, we subcutaneously injected sh*INHBA*-high, pGIPZ-high, and parent ECdnT cells with FEF into the flanks of female NOD/SCID mice. After six weeks of growth, mice injected with sh*INHBA*-high cells had larger tumors compared to pGIPZ-high and parental control cells (Figure 3-6d). Therefore, we conclude that Act A function is context- and concentration-dependent and can either promote or inhibit epithelial cell migration, invasion, and tumor growth.

Discussion

Keratinocyte differentiation in the esophagus has much in common with other squamous tissues, such as the interfollicular skin and the oropharynx, making the esophagus an outstanding model to understand squamous epithelial homeostasis and disease (9,28,312). We have previously shown that the coordinated loss of the cell adhesion molecule E-cadherin and TGFβ receptor II is a frequent event in ESCC and results in increased cell invasion (292). Esophageal cancer has a high mortality rate, with 5-year survival remaining static at 19% (313); the current treatment options for patients are limited. The identification of mechanisms that disrupt esophageal homeostasis advances our understanding of the development and progression of pre-neoplastic lesions and has clinical significance.

Little is known about Act A and its involvement in esophageal disease. Act A expression is associated with lymph node metastasis, staging, poor patient prognosis (314), and concomitant upregulation of N-cadherin (315). Act A has been associated with ESCC aggressiveness. This increase in aggressiveness is accompanied by increased proliferation (316) and MMP-7 activity (317). We observed increased epithelial levels of MMP-9 in response

to Act A stimulation, while MMP-2, which is mainly secreted by the fibroblasts in our system (data not shown), remained largely unaffected. While treatment with follistatin and A83-01 enhanced cell invasion, it results in reduced MMP-9 and MMP-2 secretion as well as activity, indicating that other mechanisms are involved in cell invasion independent of MMP-9 and MMP-2 (unpublished data). Analysis of publicly available datasets identified frequent upregulation of Act A, but does not allow us to identify which subcellular compartment (tumor or stromal) is responsible Act A expression or secretion. Our data indicate that Act A exerts paracrine function on the fibroblasts resulting in a feedback loop required for epithelial cell invasion, since depletion of the fibroblasts in the organotypic cultures inhibits Act A-mediated cell invasion.

Our unique opportunity to analyze Act A function using three-dimensional organotypic cultures is highlighted by the differences in Act A signaling regulation as measured by its target ID1 in monolayer and three-dimensional cultures (306). Act A and TGFβ overlap in their intracellular signaling cascades (307) and function, as demonstrated by deletions of TGFβ2, TGFβ3 and the Act A subunit, inhibin β_A, which result in cleft palate defects in the respective mouse models (308-311). Furthermore, analysis of the Act A- and TGFβ-induced transcriptome demonstrate multiple common downstream targets (92). Mutations in the TGFβ/BMP signaling axis are frequent events in different types of cancer. Similar to a 10-bp polyadenine tract within the TGFβ receptor type II gene that results in a frameshift mutation in GI cancers, a biallelic mutation in ACVR2 has been identified in colorectal and pancreatic cancer (89). ACVR1B is mutated in pancreatic cancer (318). In a majority of sporadic colorectal cancers, BMPR2 expression is impaired (319). BAMBI (BMP and Activin Membrane-bound Inhibitor) is upregulated in colorectal cancer, is under direct regulation of the Wnt

pathway (229) and is part of a gene expression profile that predicts metastasis (320).

Interestingly, stimulation of organotypic cultures with TGFβ1 resulted in suppressed ECdnT invasion, while recombinant Act A was unable to inhibit cell invasion. Treatment with Act A or Act A neutralizing antibody had no effect in “normal” organotypic epithelia retaining E-cadherin expression and intact TGFβ signaling. In these normal epithelial cells, only the addition of follistatin induced cell invasion demonstrating the importance of a balance between the Act A-mediated signaling and BMP pathways. Follistatin, while binding Act A with high affinity, can also antagonize BMP signaling, in particular BMP2, 4, 7 and 11, as well as myostatin. BMPs, like Act A, signal through surface receptor complexes containing type I (ALK2, ALK3, ALK6) and type II (BMPRII, ActRII, ActRIIB) receptors. While the BMPs, particularly BMP2 and BMP4, have higher affinity for their type I receptors, they can still stimulate signaling through the shared type II receptors (291). These observations indicate that changes in the balance between Act A- and BMP-mediated signaling can affect the phenotype in a context-dependent manner. ECdnT organotypic cultures, which model a pre-malignant phenotype, showed enhanced invasion in the presence of neutralizing antibody and follistatin, compared to untreated cells. While neutralizing Act A in EC cells suppressed invasion, it failed to inhibit ECdnT cell invasion, indicating that more invasive cells might be less responsive to the invasion-suppressive functions of Act A. Follistatin, on the other hand, increased invasion in all cell lines tested.

Follistatin is an antagonist of myostatin (GDF8), in addition to inhibiting Act A. In an analysis of publically available esophageal and head and neck squamous cell carcinoma datasets (GDS2520, GDS3838), myostatin, unlike *INHBA*, is not differentially expressed in cancer compared to normal tissues. There is no evidence in the literature to suggest that

myostatin contributes to collective invasion or the initiation of ESCC, however myostatin is a key player in later stages of cancer progression through its role in cancer cachexia (321,322).

High serum levels of follistatin have been detected in patients with ovarian (323) and metastatic prostate cancer (324). In oral squamous cell carcinoma, serum levels of follistatin and Act A were not significantly different in the cancer patients compared to control groups (104). Interestingly, follistatin, as well as Act A, was overexpressed in tumor tissues from these patients. In the same study, Act A expression was shown to correlate with poor prognosis overall and disease-free survival, while follistatin was not (104). This observation further highlights that follistatin may have independent roles other than antagonizing Activin A. In other studies, however, follistatin upregulation has been positively correlated with cell motility, invasion and metastasis (325), while other groups have demonstrated that follistatin can also inhibit experimental metastasis in SCID mice (326).

Knockdown of Act A in ECdnT cells using shRNA demonstrated epithelial cell-dependent effects of Act A, whereas the response of the stromal cells remained unaltered. Furthermore, cells with low levels of Act A knockdown were more invasive than with high levels of suppression, indicating a dose-dependent phenotype. As the observations for the use of neutralizing antibody in the different cell lines appear contradictory, a possible explanation is that final Act A activity may vary following neutralization and, therefore, initiate differential cellular responses. Increased tumor initiation and invasion, as observed with ECdnT cells, supports the notion that endogenous Act A may suppress tumor initiation. Based on mouse models targeting Act A expression in the skin, it has been shown that Act A overexpression leads to epidermal hyperplasia (327). However, when Act A-overexpressing mice were bred with transgenic animals carrying dominant-negative deletion of the kinase-domain in the

ActRIB, hyperplasia, hyperproliferation and abnormal keratinocyte organization could be rescued (328). These data elude to the importance of Act A levels in the regulation of epithelial architecture. Interestingly, the same group observed differential results for Act A effects *in vitro* and *in vivo*, not only in regard to epithelial cell proliferation, but also migration. Act A promoted migration in the wounded skin, but showed no pro-migratory effect *in vitro* (323).

These observations, along with our results, indicate that follistatin and shRNA against Act A could increase invasion and tumorigenesis depending on the cellular context. Exogenous Act A treatment in the background of mutant TβRII did not inhibit cell invasion, an additional hint at signal regulation through Act A gradients. Previous research regarding the role of Act A in embryogenesis may allow for the elucidation of the functional consequences of Act A during tumorigenesis, as the reactivation of essential developmental pathways have been found to contribute significantly to cancer initiation and development. *Xenopus* models have shown the dose-dependent effect of Act A in endo-mesoderm development. Act A induces specific gene expression signatures at increasing concentrations, thus inducing distinct cell fates (26,28,329,330). This process occurs through long-range Act A diffusion through the extracellular matrix, and has been shown to signal in a cell-specific manner (330,331). The effect of Act A is further complicated through its intermingling with additional signaling pathways, such

Gene	Function	Expression	Mass Spec
Gremlin 1	BMP inhibitor	upregulated	validated
BMP-2	BMP signaling	downregulated	validated
BAMBI	TGFβ inhibitor	upregulated	N/A
LTBP1	TGFβ inhibitor	upregulated	validated
Follistatin	Activin inhibitor	downregulated	validated
Follistatin-like	Activin inhibitor	downregulated	validated

Table 3-3. Gene expression analysis. Gene expression analysis after laser-microdissection of invasive and non-invasive ECdnT cells compared to E cells demonstrated misregulation of TGFβ, Act A and BMP signaling targets as validated by mass spectrometry.

as Wnt (332). Therefore, based upon ours and previous studies, we conclude that the balance of Act A concentration is crucial to exert its potentially dose-dependent dual function on invasion and growth.

Of note, laser-capture microdissection RNA expression analysis of invasive untreated ECdnT cells identified changes in multiple members of the TGFβ family (Table 3-3). Most of these targets have been validated in mass-spectrometry analysis using conditioned media from ECdnT organotypic cultures. While BMP2 itself was downregulated, Gremlin 1 was found to be upregulated. Similarly, the inhibitory pseudo-receptor BAMBI and latent-binding protein 1 (LTBP1) was upregulated, indicating potential TGFβ signaling suppression. Further, Activin A inhibitors such as follistatin and follistatin-like were downregulated. These observations further highlight the importance of maintaining the equilibrium between intersecting pathways in the regulation of cell invasion and potentially tumorigenesis.

In summary, we showed that overexpression and exogenous Act A treatment result in MMP-dependent invasion requiring the presence of fibroblasts. However, as follistatin induced epithelial cell invasion and knockdown of Act A similarly resulted in enhanced cell invasion and tumorigenesis, we propose that low levels of Act A result in elevated motility or invasive potential, while high Act A levels, depending on the cellular context, results in less invasion. In conclusion, shifting the balance between Act A and follistatin can disrupt epithelial homeostasis.

Acknowledgements and Funding

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CHAPTER IV

ESOPHAGEAL SQUAMOUS CELL CARCINOMA INVASION IS INHIBITED BY ACTIVIN A IN ACVRIB-POSITIVE CELLS

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Preface

In this chapter, we expanded upon the work completed in Chapter 3. In addition to examining the role that Activin A plays in the context of the esophageal squamous cell microenvironment, we looked further into the ligand-receptor interaction that is responsible for the initiation of such a phenotype. To do so, I studied three separate conditions: dysplastic esophageal squamous cells, and ACVRIB-positive and ACVRIB-negative esophageal squamous cell carcinoma (ESCC) cells. I tested these cell types using a three-dimensional organotypic reconstruct model to investigate not only how these dysplastic and ESCC cells respond to high stromal Activin A expression in the presence or absence of ACVRIB, but also the autocrine and paracrine effects of stromal Activin A. The results presented in this chapter ultimately identified a new role for the Activin A-ACVRIB receptor complex in regulating esophageal squamous cell invasion.

Abstract

Background: Esophageal squamous cell carcinoma (ESCC) is a global public health issue, as

it is the eighth most common cancer worldwide. The mechanisms behind ESCC invasion and progression are still poorly understood and warrant further investigation into these processes and their drivers. In recent years, the ligand Activin A has been implicated as a player in the progression of a number of cancers. The objective of this study was to investigate the role of Activin A signaling in ESCC.

Methods: To investigate the role Activin A plays in ESCC biology, tissue microarrays containing 200 cores from 120 ESCC patients were analyzed using immunofluorescence staining. We utilized three-dimensional organotypic reconstruct cultures of dysplastic and esophageal squamous tumor cells lines, in the context of fibroblast-secreted Activin A, to identify the effects of Activin A on cell invasion and determine protein expression and localization in epithelial and stromal compartments by immunofluorescence. To identify the functional consequences of stromal-derived Activin A on angiogenesis, we performed endothelial tube formation assays.

Results: Analysis of ESCC patient samples indicated that patients with high stromal Activin A expression had low epithelial ACVRIB, an Activin type I receptor. We found that overexpression of stromal-derived Activin A inhibited invasion of esophageal dysplastic squamous cells, ECdnT, and TE-2 ESCC cells, both positive for ACVRIB. This inhibition was accompanied by a decrease in expression of the extracellular matrix (ECM) protein fibronectin and podoplanin, which is often expressed at the leading edge during invasion. Endothelial tube formation was disrupted in the presence of conditioned media from fibroblasts overexpressing Activin A. Interestingly, ACVRIB-negative TE-11 cells did not show the prior observed effects in the context of Activin A overexpression, indicating a dependence on the presence of ACVRIB for this phenotype.

Conclusions: We describe the first observation of an inhibitory role for Activin A in ESCC progression that is dependent on the expression of ACVRIB.

Background

Esophageal cancer is the eighth most prevalent cancer and sixth most common cause of cancer-related deaths globally (333,334). The different subtypes of esophageal cancer are esophageal adenocarcinoma (EAC) and esophageal squamous cell carcinoma (ESCC). Though the prevalence of EAC has now surpassed that of ESCC in North America and Europe, ESCC remains the dominant subtype globally (~80%), with the highest incidence and mortality occurring in developing countries in Asia (334-336). ESCC poses a great public health challenge, as little progress has been made in improving diagnosis and outcomes for patients. Approximately 80% of ESCC is diagnosed in late stage and has only a 15% 5-year survival rate; these statistics have remained stagnant over the last 20 years (333-335). Though the list of targeted therapies is constantly growing, therapeutic resistance and recurrence continues to occur (334-337). To address this problem, an examination of new diagnostic and prognostic indicators, as well as the mechanisms underlying progression, is needed to provide further insight into new methods to combat ESCC.

Activin A, a homodimer of inhibin β_A subunits, has been indicated as a key player in ovarian, prostate and breast cancers (338,339). A member of the TGFβ superfamily, Activin A, upon binding an Activin type II (ActRII/B) and type I (ACVRI/B) receptor, subsequently activates the Smad cascade thereby driving transcription of target genes (reviewed in (340)). Activin A was initially discovered as a gonadotroph, where it acts as a potent inducer of cell cycle arrest (7,341). *In vivo* knockdown of inhibin β_A in mice leads to incomplete development

(87) and defects in squamous tissue wound healing (327). Although previously linked to carcinogenesis (342), the mechanism and precise contribution of Activin A to initiation and progression remains to be elucidated. Studies in gastric cancer have shown Activin A to be a potent inhibitor of angiogenesis and inducer of apoptosis (139,343); however, similar to TGFβ, Activin A can act as a tumor suppressor or promoter in different contexts. In breast cancer, some reports have indicated that Activin A can induce cell growth and epithelial-mesenchymal transition (EMT) (344), whereas other studies have demonstrated that Activin A treatment induces cell cycle arrest and inhibits growth (7,345). This “dual role” phenomenon has also been observed in prostate (346) and lung (347,348) cancers.

In the esophagus, clinical and experimental evidence has indicated that Activin A promotes cancer progression. Clinical studies have correlated increased Activin A expression with tumor aggressiveness, differentiation status (349), and poor patient prognosis (317). Several explanations have been suggested to explain the mechanisms induced by Activin A. In ESCC, one such proposal for the Activin A contribution to tumor progression is through the induction of N-cadherin with subsequent loss of E-cadherin, a feature that has been associated with increased tumor aggressiveness (92). Additional evidence has suggested that Activin A can upregulate MMP-7 expression, which has been correlated with gastric and colorectal cancers (350) via the transcription factor AP-1, a non-canonical pathway (89).

Taken together, contrary to its characterization as an inhibitor of angiogenesis and as a growth suppressor, overexpression of Activin A indicates that, during ESCC progression, this signaling pathway switches function from anti-tumorigenic to pro-tumorigenic regulation. Of particular importance is the contribution of the microenvironment in this context, which is investigated here. Activin A has been shown to not only exert functional effects on tumor cells,

but also on stromal cells located within the microenvironment, where Activin A can induce a "wound healing" phenotype (reviewed in (351)).

To address the contrasting roles of Activin A, a growth inhibitor known to be highly expressed in several cancers, we aimed to determine the source of Activin A (stromal versus epithelial-secreted) and to mimic stromal overexpression in an organotypic reconstruct culture system. In this study, we show that, in a dysplastic esophageal microenvironment, fibroblast-secreted Activin A, as one source of stromal Activin A, suppresses cell invasion through the inhibition of epithelial cell proliferation and the regulation of extracellular matrix components. We further observe that esophageal TE-2 tumor cells respond to fibroblast-derived Activin A with inhibition of cell invasion similar to the dysplastic cells, yet this effect was not observed in the Activin receptor IB (ACVRIB)-negative ESCC cell line TE-11. We conclude that during cancer progression, in concordance with upregulated stromal Activin A expression, ACVRIB is frequently downregulated in ESCC cells, allowing an escape from the inhibitory effects of Activin A as a novel mechanism of esophageal tumorigenesis.

Methods

Cell lines and cell culture

Fetal esophageal fibroblasts were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Norcross, GA) and 1% penicillin and streptomycin (P/S) (Gibco) (294). Primary esophageal keratinocytes expressing dominant-negative mutants of E-cadherin and TβRII (ECdnT), established as previously described (292), were cultured in keratinocyte serum-free media (KSFM) supplemented with 40 μg/mL bovine pituitary extract, 1 ng/mL epidermal growth

factor (EGF), and 1% P/S (Gibco). The ESCC cell lines TE-2 and TE-11 were grown in RPMI and DMEM (Gibco), respectively, and supplemented with 10% FBS and 1% P/S (352). The endothelial cell line HMEC-1 were cultured in MCDB131 (Gibco) supplemented with 10% FBS, 10 ng/ml EGF, 1 µg/ml hydrocortisone (Sigma-Aldrich, St. Louis, MO), and 1% P/S (353).

Retrovirus infection

Overexpression of Activin A was performed using a retroviral construct containing cDNA for *INHBA*, the sequence that encodes the inhibin β_A subunit, as previously described (354). The vector backbone pBABE-zeo was purchased from AddGene (plasmid #1766 (355)) and inhibin β_A cDNA was inserted at the multiple cloning site. Virus was generated using Φ-Ampho HEK293T cells (ATCC CRL-3213). Fibroblasts (Fibro-ActA) were then transduced and selected using Zeocin (Life Technologies) at a concentration of 800 µg/ml. Fibroblasts transduced with an empty pBABE-zeo vector (Empty) and untransfected parent fibroblasts (Parent) were used as controls. Activin A overexpression was validated by ELISA.

Cell contraction assay

Cell contraction assay was performed according to the manufacturers' protocol (Cell Biolabs, Inc., San Diego, CA). 3-butanedione monoxime (BDM) was used as a control.

ELISA

Capture ELISAs for Activin A were purchased from R&D Systems (Minneapolis, MN) and performed using conditioned media according to the manufacturers' instructions.

Table 4-1. Antibodies used for immunofluorescence.

Antibody	Dilution	Application	Vendor
Podoplanin	1:500	Immunofluorescence	eBioscience
Vimentin	1:1000	Immunofluorescence	Sigma
N-cadherin	1:500	Immunofluorescence	BD Bioscience
Fibronectin	1:1000	Immunofluorescence	BD Bioscience
E-cadherin	1:1000	Immunofluorescence	BD Bioscience
Vimentin	1:50	Immunofluorescence	Cell Signal Technology
Laminin 5γ2	1:200	Immunofluorescence	Santa Cruz Biotechnology
Ki67	1:200	Immunofluorescence	Cell Signal Technology
Collagen IV	1:200	Immunofluorescence	Cell Signal Technology
αSMA	1:200	Immunofluorescence	Sigma
COL4A2	1:100	TMA	Chondrex
INHBA	1:200	TMA	ProteinTech
CD68	1:20	TMA	R&D Systems
ACVRIB	1:200	TMA	Abcam
Keratin 14	1:50	TMA	Abcam

Organotypic culture

Organotypic reconstruct cultures were performed as previously described (356). Briefly, parental, empty, or Fibro-ActA were seeded into a 3D matrix (75,000 cells/well) containing collagen I and Matrigel (BD Biosciences, Franklin Lakes, NJ) and allowed to incubate for 7 days at 37 °C. Following incubation, ECdnT, TE-2, or TE-11 cells were seeded on top of the fibroblast matrix (500,000 cells/well). Cultures were then allowed to incubate an additional 10 days. Treatments were added to the cultures beginning two hours following epithelial cell seeding and refreshed every two days. A neutralizing antibody against Activin A (nAb; R&D Systems) and A83-01, a chemical inhibitor of TGFβ/Activin A/BMP type I receptors (ACVRIB/TβRI/ALK7) (Tocris, Bristol, UK), were used for treatment.

Staining

Immunofluorescence of FFPE sections

At time of harvest, organotypic cultures were fixed in 10% formalin and embedded in paraffin. Cultures were cut to 5 μm sections, deparaffinized, and heated for 12 minutes in 1XTE buffer in a pressure cooker to perform antigen retrieval. Sections were blocked with 1XPBS containing 5% bovine serum albumin (1XPBS + BSA; Sigma-Aldrich) for one hour at room temperature. The sections were incubated with primary antibody diluted in 1XPBS + BSA overnight at 4°C. The following day, sections were washed three times with 1XPBS, and incubated with a secondary antibody with a conjugated fluorophore (anti-rabbit Texas Red 1:200, Vector Laboratories, Burlingame, CA; anti-mouse DyLight Alexa488 1:200, Vector Laboratories; anti-rat Alexa594 1:200, Life Technologies), diluted in 1XPBS + BSA, for 1–2 hours at room temperature. Sections were washed three times and mounted using ProLong Gold anti-fade with DAPI (Life Technologies). Sections were imaged on a Zeiss microscope, using Axiocam and AxioVision software (Carl Zeiss Microscopy, Thornwood, NY). Antibodies used for immunofluorescence are listed in Table 4-1.

Endothelial tube formation assay

Growth factor-reduced Matrigel (Corning Inc., Corning, NY) was added to each well of a 96-well plate and allowed to solidify at 37°C for approximately 30 minutes. HMEC-1 cells, in the appropriate conditioned media treatment, were seeded at 15,000 cells/well in triplicate to the Matrigel-containing wells and incubated for 18 hours at 37°C. Following incubation, bright field images of each well were taken and analyzed using Angiogenesis Analyzer for ImageJ (357). This software allows network organization analysis of a skeleton or tree, extremities or nodes

and junctions in a binary image (http://image.bio.methods.free.fr/ImageJ/?Angiogenesis-Analyzer-for-ImageJ&lang=en&artpage=3-7#outil_sommaire_3). Statistical analysis was performed by one-way ANOVA in GraphPad (GraphPad, San Diego, CA).

ESCC tissue microarray

Microarrays were purchased from US Biomax (Rockville, MD). Following immunofluorescence staining, cores were quantified using the "Measure Stained Area Fluorescence" algorithm as part of the Leica Microsystems Tissue IA version 4.0.6 program (Buffalo Grove, IL). Fluorescence area was measured in μm^2. Antibodies used for immunofluorescence are listed in Table 4-1.

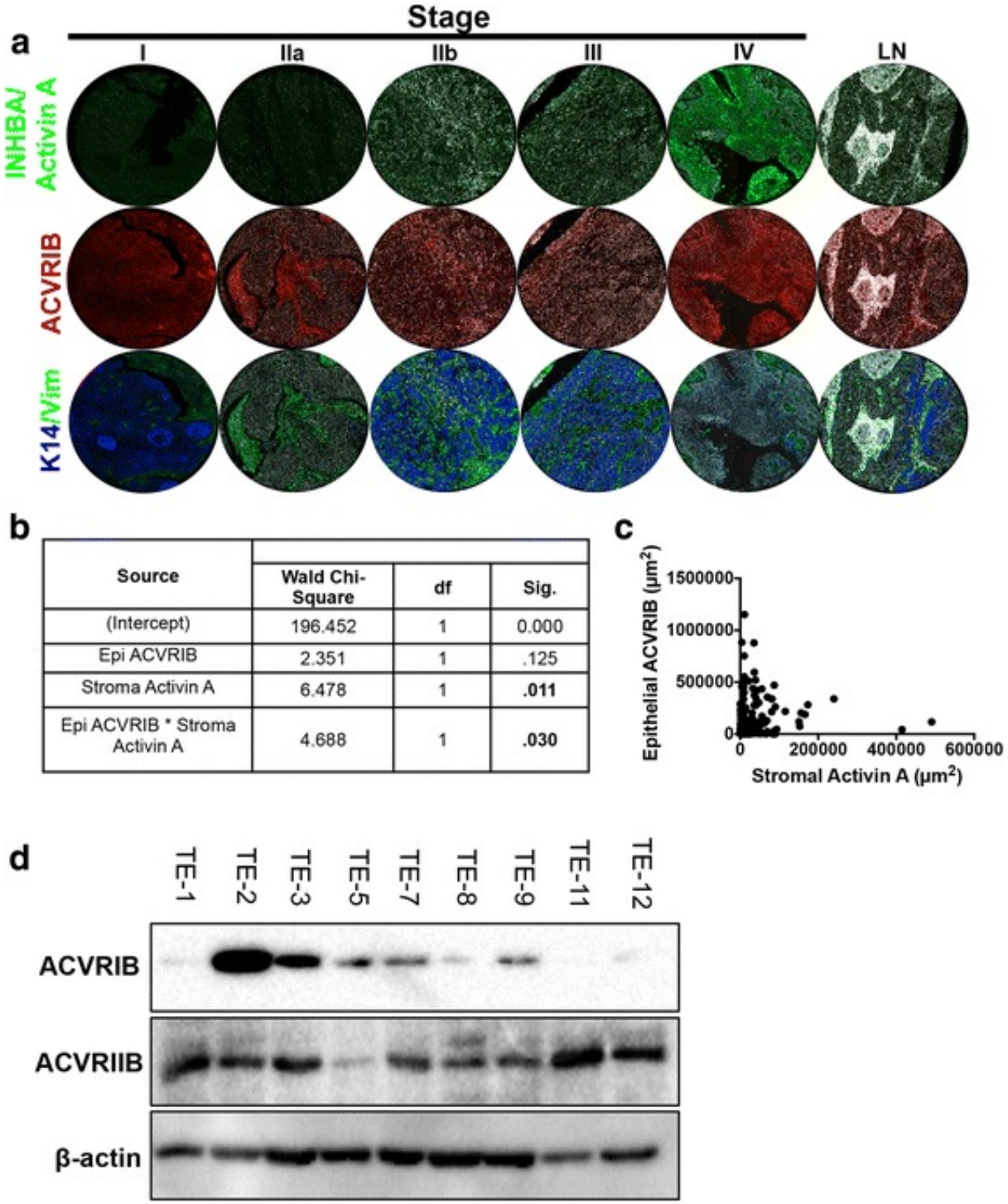
Statistical analysis

Experimental results were analyzed using Student's *t*-test or one-way ANOVA and expressed as the mean +/- standard deviation. Statistical analysis of the *in vitro* experiments was performed in Prism 6.0 (GraphPad). ESCC microarrays were analyzed using linear generalized estimating equations (GEE) or Kruskal-Wallis tests. For matched samples, Wilcoxon signed rank tests were used. Results are expressed as the mean +/- standard deviation. Statistical analysis of the microarrays was performed using SPSS (IBM, Armonk, NY).

Results

Epithelial ACVRIB expression levels are dependent upon expression of stromal Activin A in ESCC

It has been well established that Activin A expression in normal physiology is low, with increased expression occurring in invasive cancer (298,317,358,359). We first aimed to determine the localization and expression level of Activin A in ESCC tissues. Using commercially purchased tissue microarrays, we stained 200 esophageal tissues (cancer adjacent, squamous cell carcinoma, and lymph node [LN] metastases) from 120 patients for Activin A and ACVRIB, the primary type I receptor involved in Activin A signaling (Figure 4-1a). Additional staining for keratin 14 (K14) to determine squamous cells, vimentin (Vim) to identify mesenchymal cells, and collagen as well as CD68, a glycoprotein expressed on monocytes, were used as controls. These markers allowed us to compartmentalize the localization of Activin A and ACVRIB localization to epithelial/tumor or stroma. Data are quantified in Figure 4-2. Using Kruskal-Wallis tests and generalized estimating equations (GEE), we found that Activin A expression alone was not associated with tumor stage in this set of tumor tissues, similarly ACVRIB expression alone varied between stage and was not a predictor of tumor stage (data not shown). However, we identified that after controlling for epithelial ACVRIB expression, Activin A expression in the stroma was, indeed, a significant predictor of stage, when analyzed by multivariable GEE (Figure 4-1b). Therefore, we determined that with increasing stromal Activin A expression, epithelial ACVRIB expression decreased within this patient data set, thereby, promoting a more aggressive ESCC phenotype, as illustrated by increased stage. This relationship is illustrated in Figure 4-1c. We next examined 9 commonly utilized ESCC cell lines for protein expression of ACVRIB

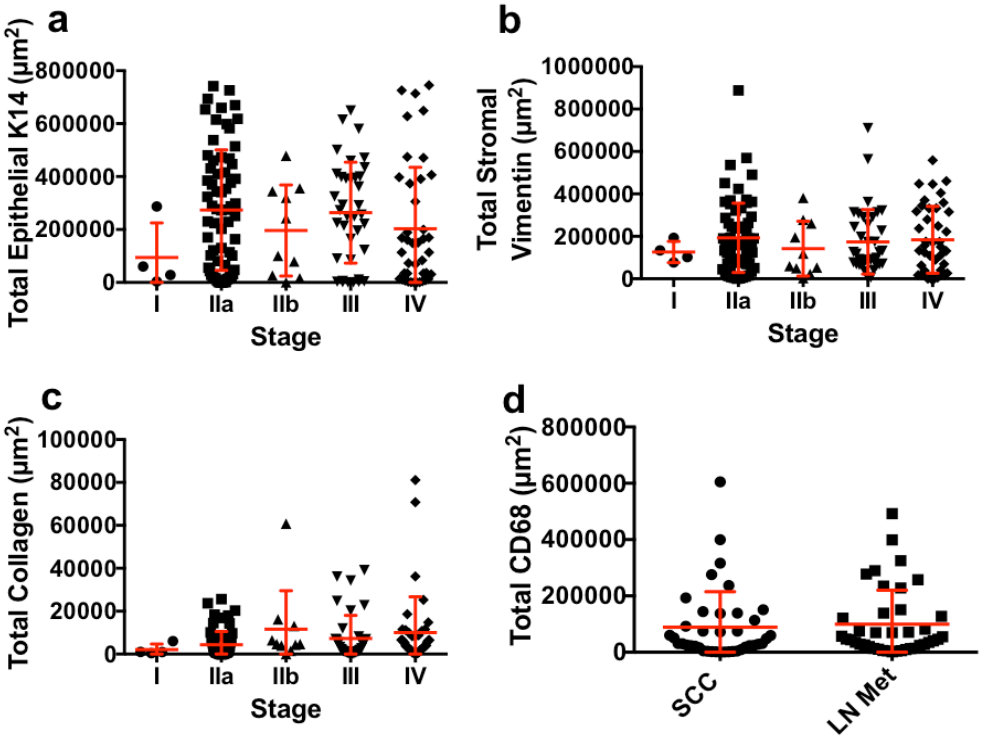


Source	Wald Chi-Square	df	Sig.
(Intercept)	196.452	1	0.000
Epi ACVRIB	2.351	1	.125
Stroma Activin A	6.478	1	**.011**
Epi ACVRIB * Stroma Activin A	4.688	1	**.030**

Figure 4-1. Epithelial expression in ESCC patient samples is dependent upon the expression of Activin A. (a) Representative immunofluorescence staining of microarray samples (whole section image shown of tumor tissues Stage I-IV and lymph node [LN]) for INHBA/Activin A, ACVRIB, K14, and vimentin (Vim). (b) Using a linear generalized estimating equation (GEE), a significant interaction was found between total Activin A expression and epithelial ACVRIB as a predictor of stage. Though epithelial ACVRIB expression was not a predictor of stage alone, we found that stromal INHBA/Activin A and epithelial ACVRIB expression showed a significant interaction. Analysis of immunofluorescence was performed on the whole tissue section. (TMA = 200 cores) (c) Graphical illustration of the relationship between epithelial expression of ACVRIB and stromal expression of ACVRIIB, measured by intensity in μm^2, in the TMA samples. Each dot represents a single patient sample. (d) Western blot of ACVRIB and ACVRIIB of the TE series of ESCC cell lines. β-actin was used as a loading control.

and ACVRIIB, the primary Activin A type II receptor (Figure 4-1d). ACVRIB expression was low in 3 out of 9 cell lines and barely detectable in 4 out of 9, indicating decreased expression in 7 out of 9 ESCC cell lines. On the contrary, ACVRIIB was downregulated in only one cell line, TE-5. To reconcile the high Activin A levels in the context of decreased ACVRIB expression and the functional consequences, we next grew dysplastic and TE-2 and TE-11 cancer cell lines in organotypic cultures.

Figure 4-2. Epithelial and stromal markers do not vary between ESCC patient samples, by stage; control staining and analysis of TMA data. A comparison of ESCC patient samples, using immunofluorescence staining on a prepared microarray, showed that epithelial keratin 14 (K14) (a), stromal vimentin (b), the extracellular matrix protein collagen (c), and monocyte marker CD68 (d) expression did not significantly differ between stage and esophageal squamous cell carcinoma (SCC) versus lymph node metastasis (LN Met).



Fibroblast-secreted Activin A inhibits cell invasion of dysplastic esophageal cells and regulates extracellular matrix protein expression

Fibroblasts play a substantial role in inflammation, wound healing, and extracellular matrix (ECM) deposition (126,360). In the context of cancer, fibroblasts are recruited by cancer cells via epithelial-mesenchymal crosstalk to rearrange the ECM to create a ‘reactive’ stroma, which provides an environment conducive to cell invasion (33,361). Microenvironment-derived Activin

A has been implicated as part of the reactive stroma phenotype (127,362). Considering elevated levels of Activin A are found in the stroma despite the characteristic function of Activin A as an inducer of growth arrest, we first aimed to investigate the role of microenvironment-derived Activin A in a three-dimensional organotypic reconstruct (OTC) model in the presence of dysplastic esophageal keratinocytes (ECdnT) (292). As normal esophageal fibroblasts secrete low to negligible levels of Activin A ((356) and data not shown), we retrovirally transduced

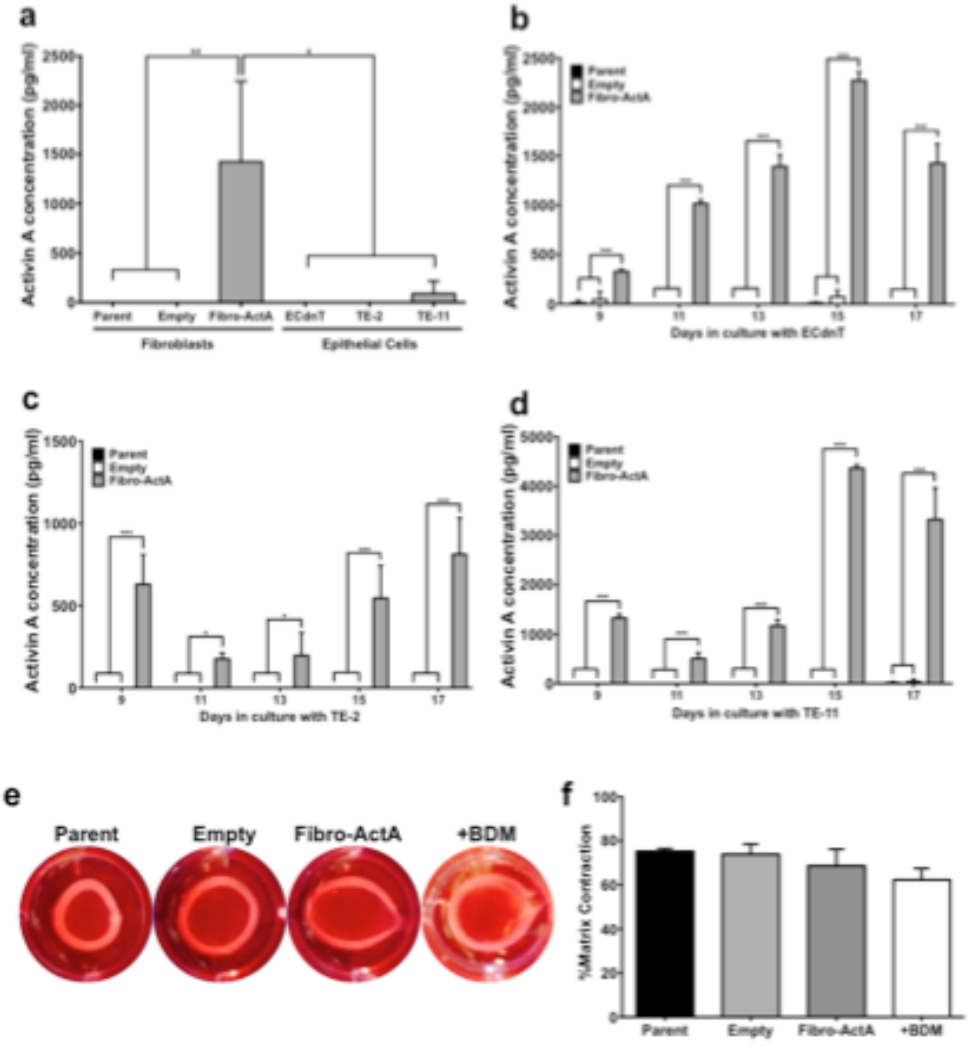


Figure 4-3. Overexpression of Activin A, validated by ELISA, was persistent and did not affect fibroblast contractility; validation of assays by ELISA and cell contractility assay. (a) Overexpression of Activin A was validated following each retrovirus transduction. Levels of secreted Activin A protein, measured in conditioned media, were significantly higher in Act A compared to parent and empty vector control, ECdnT, TE-2, and TE-11 cells in 2D monolayer. Overexpression of Activin A was validated throughout the 17-day organotypic culture with ECdnT (b), TE-2 (c), and TE-11 (d). Fibro-ActA had sustained increased expression of Activin A during this time period. (e) Parent, empty, and Fibro-ActA had comparable ability to contract collagen, indicating that overexpression of Activin A alone did not hinder the fibroblasts ability to contract the extracellular matrix. 3-butanedione monoxime (BDM) was used as an assay control. (f) Quantification (percent) of fibroblast matrix contraction from (e). (One-way ANOVA, *p<0.05).

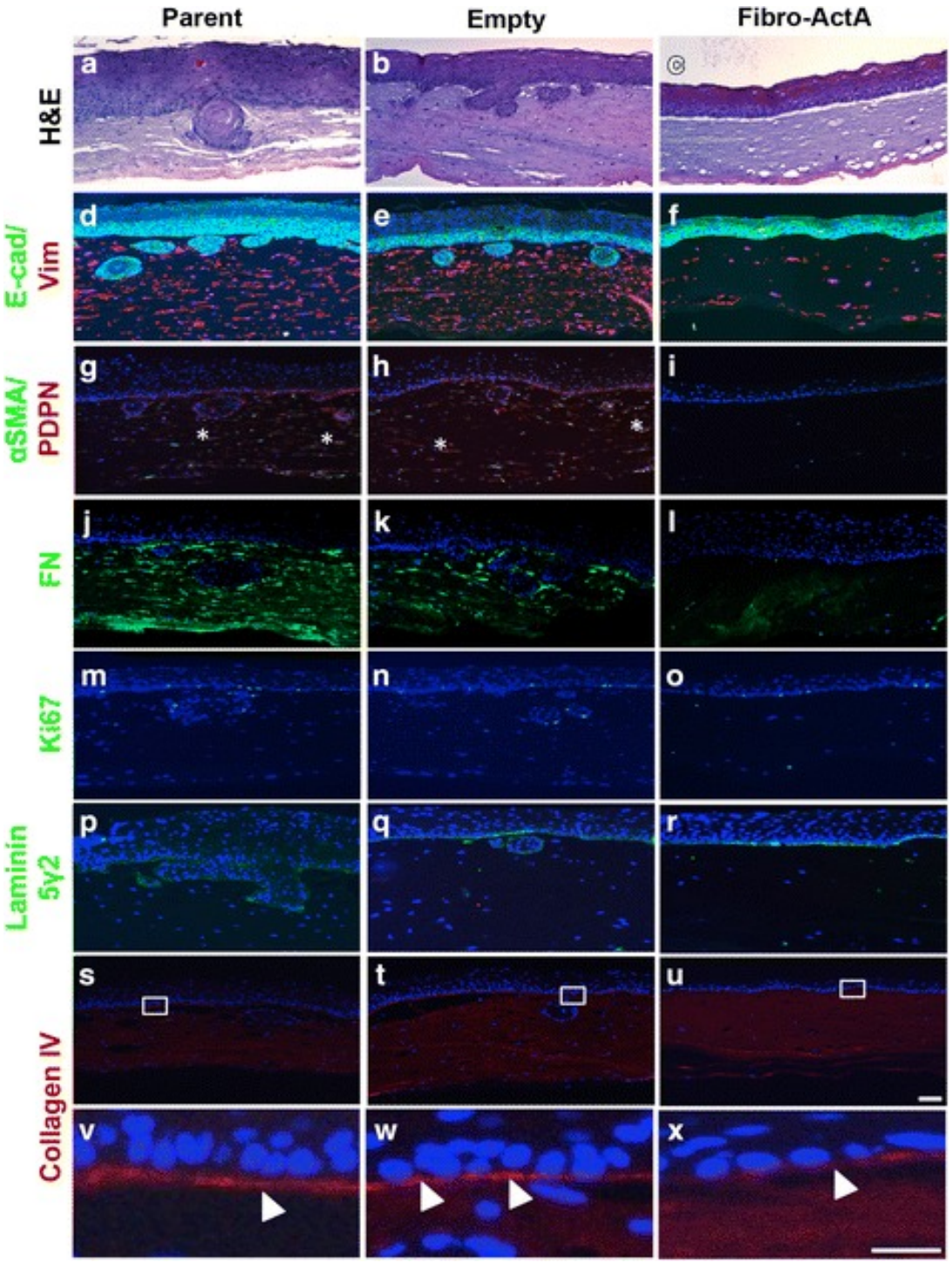


Figure 4-4. Overexpression of Activin A in the dysplastic esophageal microenvironment inhibits extracellular matrix protein reorganization. (a-c) Hematoxylin and eosin staining of parent, empty, and Fibro-ActA organotypic cultures. (d-f) Three-dimensional organotypic Fibro-ActA cultures exhibit no alterations in epithelial ECdnT E-cadherin (E-cad) expression, however vimentin (Vim) is downregulated in the fibroblasts, as examined by immunofluorescence. (g-i) αSMA expression was substantially downregulated in the fibroblasts, while podoplanin (PDPN) expression was downregulated in both epithelial cells and fibroblasts. The asterisks(*) in the parental and empty vector cultures denote co-localization of αSMA and PDPN, a common characteristic of cancer-associated fibroblasts. (j-l) Fibronectin (FN) deposition was decreased in Fibro-ActA cultures compared to parent and empty vector controls. (m-r) Ki67, a marker of proliferation, and laminin 5γ2, a marker of basal cell differentiation, was unchanged between conditions. (s-u) Collagen IV deposition, a primary component of basement membrane deposition, was decreased in Fibro-ActA compared to control. (v-x) Collagen IV staining, higher magnification of boxed region in (s-u). Arrows indicate the collagen IV basement membrane, laid by the epithelial cells. (Short scale bar = 20 μm; long scale bar = 5 μm) (n = 4)

fibroblasts with *INHBA* to achieve Activin A overexpression levels similar to those observed in cancer-associated fibroblasts (127,356). Upon embedding Activin A overexpressing fibroblasts (Fibro-ActA) in the organotypic culture matrix, we validated overexpression and secretion of Activin A by ELISA (Figure 4-3a). Fibro-ActA secreted significantly more Activin A than the tested epithelial cells, ensuring that the majority of Activin A in OTC would be derived from the fibroblasts. To confirm that Activin A overexpression was maintained during the course of each OTC (17 days), we collected media every 2 days and measured Activin A concentrations by ELISA (Figure 4-3b-d). Parent and empty vector fibroblasts were used as controls.

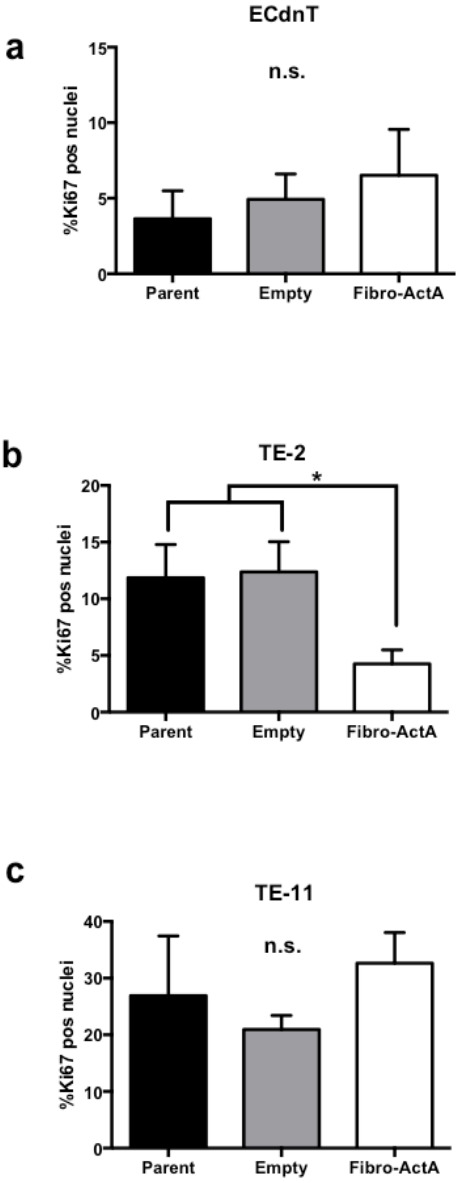


Figure 4-5. Activin A overexpression reduced proliferation of TE-2, but not ECdnT and TE-11 cells. Quantification of Ki67 from ECdnT, TE-2, and TE-11 organotypic cultures in Fig.s 4-4, 4-7, and 4-8. Proliferation of (a) ECdnT, (b) TE-2, and (c) TE-11 esophageal cells, as measured by nuclear Ki67 immunofluorescence staining, in three-dimensional organotypic cultures with parent, empty, and Fibro-ActA. (One-way ANOVA, *p<0.05).

ECdnT cells showed collective cell invasion and keratin pearl formation characteristic of an invasive ESCC when cultured with control parent and empty vector fibroblasts (Figure 4-4a,b). When cultured with Fibro-ActA, ECdnT cell invasion was suppressed (Figure 4-4c). Immunofluorescence staining was performed with anti-E-cadherin (E-cad) antibody to identify the epithelial compartment. An examination of fibroblast protein expression by immunofluorescence

showed that vimentin (Vim), a mesenchymal marker, and αSMA and podoplanin (PDPN), markers of fibroblast differentiation and activation, were downregulated in Fibro-ActA cultures (Figure 4-4d-i). We also observed substantial downregulation of the ECM protein fibronectin (FN) (Figure 4-4j-l). Interestingly, the ability of Fibro-ActA to interact with and contract the ECM was not altered until the epithelial cells were seeded (Figure 4-3e,f), indicating the necessity of epithelial-mesenchymal crosstalk to modify contractility. Epithelial cell proliferation, measured by Ki67 staining, did not change between conditions (Figure 4-4m-o, 4-5a). Interestingly, in all conditions, epithelial cells deposited laminin 5γ2, a squamous epithelium basement membrane marker (363), and collagen IV, a major basement membrane component (Fig. 2p-r) (364). Collagen IV localization to the basement membrane, however, was slightly reduced in Fibro-ActA cultures (Figure 4-4s-x, arrows). These results support the role of Activin A as an invasion suppressor and indicate Activin A-dependent regulation of ECM-associated proteins.

Inhibition of Activin A signaling during dysplasia restores extracellular matrix protein expression

To demonstrate Activin A-dependence and specificity of epithelial invasion inhibition and the expression of several ECM proteins, we used two separate approaches for Activin A inhibition: a neutralizing antibody specific for the Activin A ligand (nAb) and A83-01, a chemical inhibitor of TGFβ/Activin A/BMP type I receptors (ACVRIB/TβRI/ALK7) (365). We have previously shown the ability of nAb and A83-01 to neutralize Activin A signaling in this model system (80,356). Treatment with nAb increased cell invasion in dysplastic empty vector control cells, yet could not overcome the inhibition of cell invasion in the context of Fibro-ActA cultures (Figure 4-6a-d). Similarly, A83-01 treatment, while increasing cell invasion in the ECdnT

cultures with empty vector control, could not restore cell invasion in the Fibro-ActA cultures (Figure 4-6e,f). When we examined ECM protein expression in these cultures by immunofluorescence, treatment with nAb could restore expression of fibrillar FN in Fibro-ActA, similar to that observed in empty vector untreated cultures, yet A83-01 their restoration; we also observed restoration of PDPN (Figure 4-6g-r). This indicates the necessity of Activin A signaling to induce expression of these proteins. Laminin 5γ2 was upregulated in the Fibro-ActA cultures treated with both nAb and A83-01, the

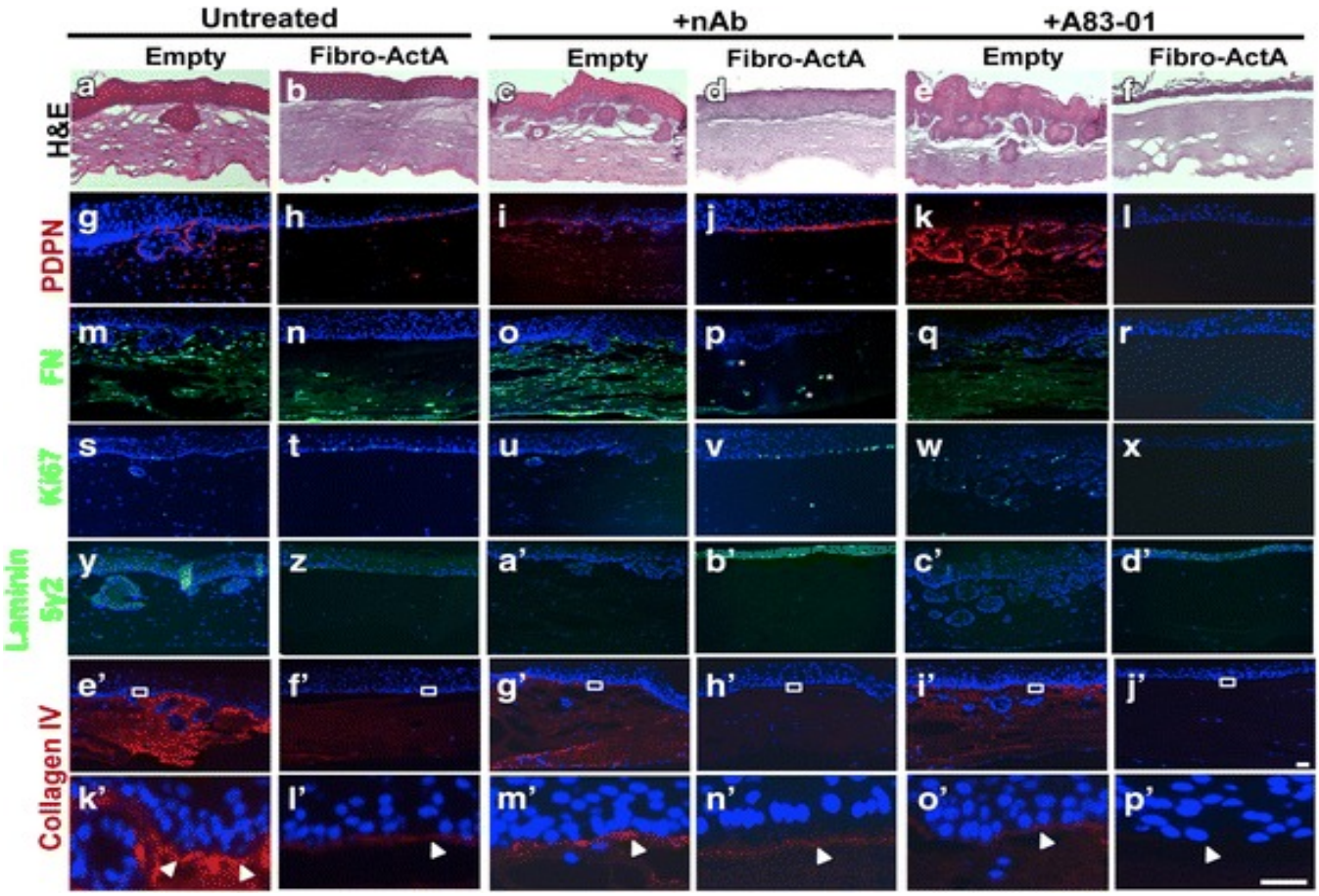


Figure 4-6. Inhibition of the Activin A ligand, but not the receptor, restores extracellular matrix protein expression. (a-f) Hematoxylin and eosin (H&E) staining shows that ECdnT cells did not invade in any of the Fibro-ActA cultures, however invasion was increased in empty vector cultures treated with an Activin A neutralizing antibody (nAb) or A83-01. (g-l) Epithelial cell podoplanin (PDPN) expression was restored to control levels in Fibro-ActA cultures following treatment with nAb. (m-r) Treatment of Fibro-ActA cultures with nAb partially restored stromal fibrillar fibronectin (FN) expression (asterisks) to compared untreated empty vector control levels. This effect was not observed following treatment with A83-01. (s-x) Laminin 5γ2 expression was increased in Fibro-ActA cultures treated with nAb and A83-01. (y-d') Ki67 staining remained relatively unchanged between conditions. (e'-j') Collagen IV deposition at the basement membrane was restored to the expression level of the control upon treatment of Fibro-ActA with nAb. This deposition was not observed in Fibro-ActA cultures treated with A83-01. (k'-p') Collagen IV staining, higher magnification of boxed region in (e'-j'). Arrows indicate the collagen IV basement membrane, laid by the epithelial cells. (Short scale bar = 20 μm; long scale bar = 5 μm) (n = 2)

only tested marker to do so (Figure 4-6s-x). This result indicates that one potential mechanism of regulating laminin 5γ2 expression is through the Activin A-ACVRIB axis. Epithelial cell proliferation, measured by Ki67, was inhibited with the addition of A83-01 to all cultures, but was unaltered in the presence of nAb (Figure 4-6y-d'). Interestingly, when we examined the basal epithelial layer of the Fibro-ActA cultures treated with nAb, we also found collagen IV deposition at the basement membrane (Figure 4-6e'-h'). This was not observed in cultures treated with A83-01 (Figure 4-6i',j'; higher magnification Figure 4-6k'-p'). We, therefore, conclude that the blocking of Activin A-receptor binding is necessary to inhibit the observed ECM alterations, yet not sufficient to induce cell invasion.

Stromal Activin A inhibits TE-2 cell invasion in three-dimensional culture

As we observed that fibroblast-derived overexpression of Activin A inhibited cell invasion in premalignant cells, we aimed to investigate if Activin A had a similar effect on cancer cells. We cultured the ESCC cell line TE-2 with Fibro-ActA. TE-2 cells express the Activin A receptor complex components, ACVR2B and ACVRIB (Figure 4-1d). Similar to the dysplastic model, TE-2 cells were unable to invade into the stromal layer when cultured with Fibro-ActA, compared to controls (Figure 4-7a-c). As in the ECdnT cultures (Figure 4-4), E-cadherin marks the epithelial layer and vimentin labels the fibroblasts in the stromal compartment (Figure 4-7d-f). Interestingly, we observed co-localization of αSMA and PDPN in the control fibroblasts (Figure 4-7g,h, asterisks), not seen in the Fibro-ActA fibroblasts, suggesting differentiation to a myofibroblast lineage in the invasive tumor cultures (366).

Unlike the ECdnT cultures, we observed differences in Ki67 staining between TE-2 Fibro-ActA and control cultures indicating significantly decreased proliferation compared to

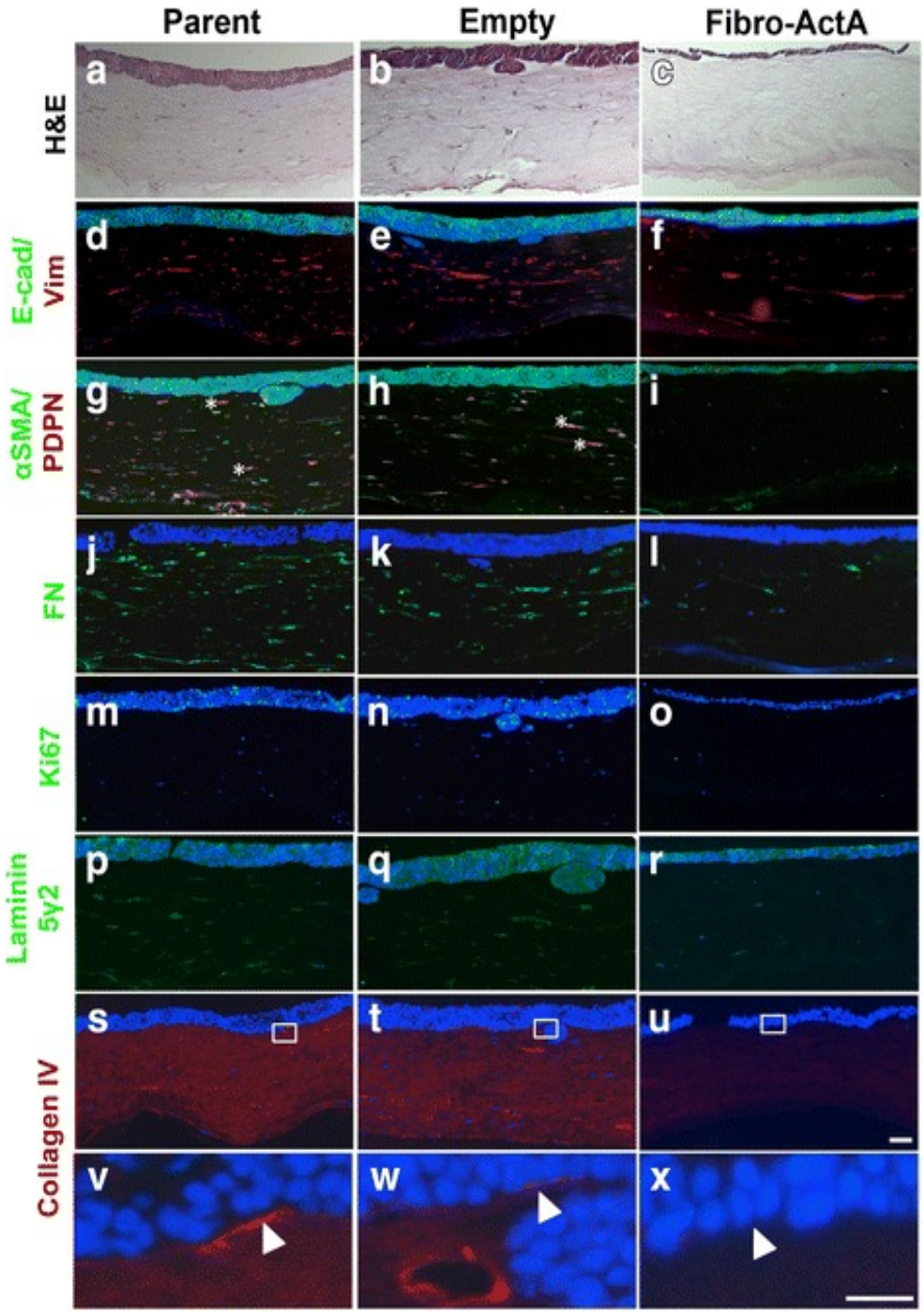


Figure 4-7. Overexpression of Activin A shows similar extracellular matrix protein regulation in ACVRIB-expressing ESCC. (a-c) Hematoxylin and eosin (H&E) staining of parent, empty, and Fibro-ActA organotypic cultures, cultured with TE-2 ESCC cells. (d-f) Three-dimensional organotypic Fibro-ActA cultures exhibit no alterations in epithelial ECdnT E-cadherin (E-cad) expression, however vimentin (Vim) was downregulated in the fibroblasts, as examined by immunofluorescence. (g-i) αSMA and podoplanin (PDPN) expression was significantly downregulated in the fibroblasts and epithelial cells, respectively. The asterisks(*) in the parental and empty vector cultures denote co-localization. (j-l) Fibronectin (FN) deposition was decreased in Fibro-ActA cultures compared to parent and empty vector controls. (m-o) Ki67 staining was decreased in epithelial TE-2 cells cultured with Fibro-ActA, compared to parent and empty vector cultures (quantified in Figure 4-5). (p-r) Laminin 5γ2, a marker of basal cell differentiation, was unchanged between conditions. (s-u) Collagen IV deposition, a primary component of basement membrane deposition, was decreased in Fibro-ActA compared to control. **(**v-x) Collagen IV staining, higher magnification of boxed region in (s-u). Arrows indicate the collagen IV basement membrane, laid by the epithelial cells. (Short scale bar = 20 μm; long scale bar = 5 μm) (n = 2)

controls, as indicated by Ki67 staining (Figure 4-7m-o; Figure 4-5b). Similar to our observations in the premalignant ECdnT model, laminin 5γ2 expression was unchanged between conditions (Figure 4-7p-r). Collagen IV deposition in the basal layer was downregulated in Fibro-ActA (Figure 4-7s-x). Overall, these observations suggest that the TE-2 ESCC cell line responds to the overexpression of Activin A by the fibroblasts in a similar manner to the dysplastic premalignant ECdnT model. This prompted us to analyze the effects of Fibro-ActA on the TE-11 ESCC cell lines, which we determined has low ACVRIB protein expression (Figure 4-1d).

Cell invasion and regulation of the ECM proteins requires intact Activin A signaling

Cancer cells have the ability to adapt in response to environmental factors. One such mechanism of adaptation is cancer cell clonal expansion, where one cancer cell with a particular advantage is able to survive and expand (367). Downregulation or loss of components of the TGFβ signaling cascade, such as TβRII, has been noted in several cancers and associated with increased aggressiveness (292,367,368). Similar observations have been made regarding the members of the Activin A signaling cascade (83). Given the observation that Activin A levels are high in the tumor setting and Activin A acts as an invasion suppressor for TE-2 cells, we used a ACVRIB-negative cell line, which has disrupted Activin A signaling due to the lost receptor complex component, to provide validation and mechanistic insight to the patient sample observations (Figure 4-1). As ACVRIB is the primary signaling kinase for the Activin A cascade, as discussed above, we chose the cell line TE-11 which had low ACVRIB expression (Figure 4-1d) to evaluate the response of ACVRIB-negative cells to fibroblast-derived Activin A. TE-11 cells invaded into the stroma, shown by H&E staining, in

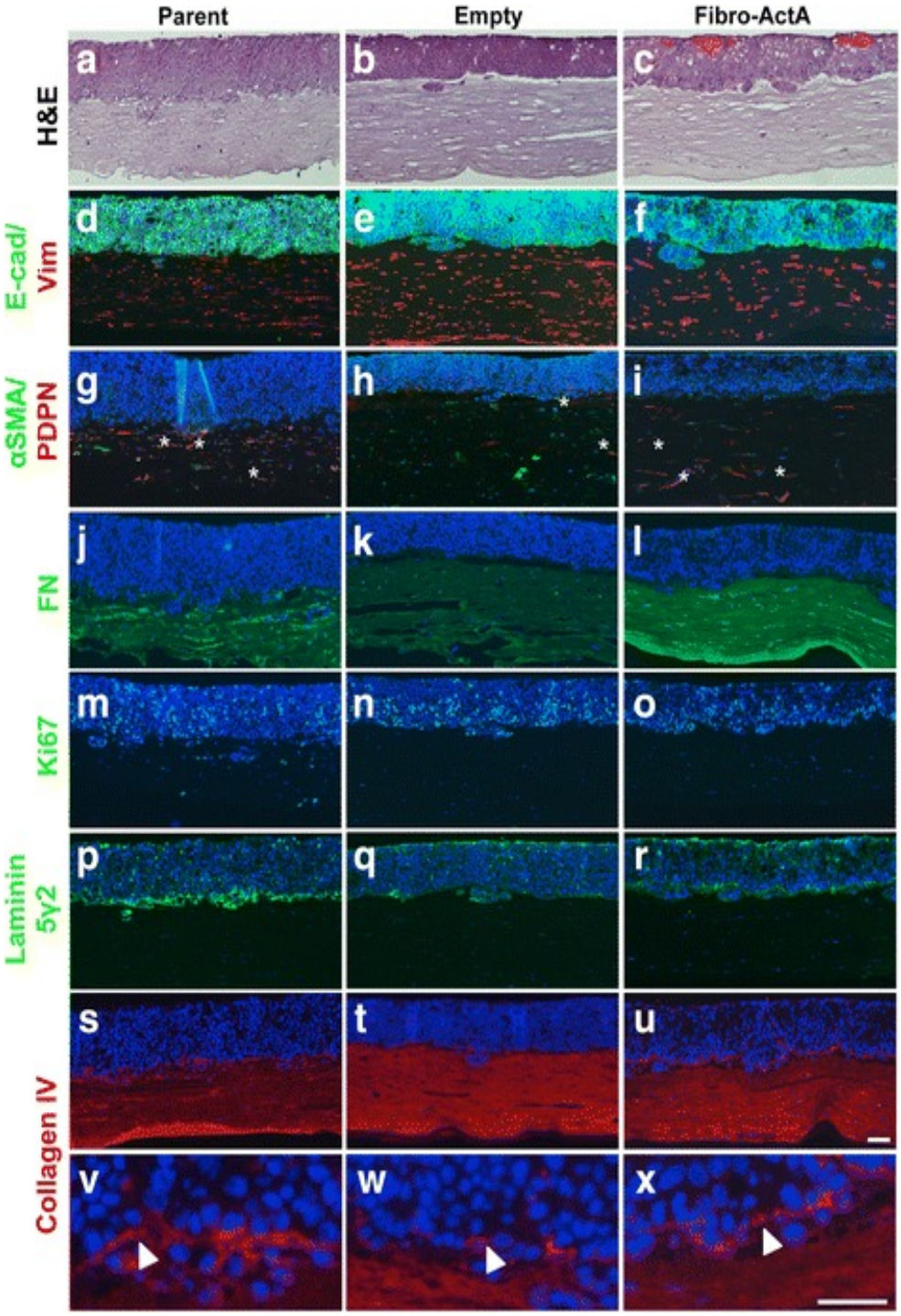


Figure 4-8. ACVRIB-negative ESCC shows epithelial alterations, however extracellular matrix protein expression remains unaltered. (a-c) Hematoxylin and eosin staining of parent, empty, and Fibro-ActA organotypic cultures, cultured with TE-11 ESCC cells. Fibro-ActA cultures show increased keratin deposition in the epithelial layer, compared to parent and empty vector controls. (d-f) Three-dimensional organotypic Fibro-ActA cultures exhibit no alterations in epithelial E-cadherin (E-cad) or stromal vimentin (Vim) expression, as examined by immunofluorescence. (g-i) αSMA and podoplanin (PDPN) expression was unchanged between conditions. Co-localization of the markers is denoted by asterisks(*). (j-l) Fibronectin (FN) deposition, (m-o) Ki67, and (p-r) laminin5γ2 expression was not different between control (parent and empty) and Fibro-ActA. (s-u) Collagen IV deposition, a primary component of basement membrane deposition, was decreased in Fibro-ActA compared to control. (v-x) Collagen IV staining, higher magnification of boxed region in (s-u). Arrows indicate the collagen IV basement membrane, laid by the epithelial cells. (Short scale bar = 20μm; long scale bar = 5μm) (n = 2)

both Fibro-ActA and control cultures (Figure 4-8a-c). By immunofluorescence, we examined the same epithelial, stromal, and ECM markers used in the previous experiments (Figures 4-4; Figure 4-6; Figure 4-7). As expected, in the TE-11 cultures, Activin A secretion by the fibroblasts did not alter the overall expression of vimentin, αSMA, PDPN, and FN (Figure 4-8d-l). Similarly to the TE-2 cultures, co-localization of αSMA and PDPN was observed, however this was noted in all cultures, including Fibro-ActA (Figure 4-8g-i, asterisks). Ki67 and laminin 5γ2 expression was unchanged between conditions (Figure 4-8m-r). Deposition of collagen IV, a common characteristic of cancer cells (369), was increased overall in TE-11 (Figure 4-8s-x) (83). In a related study analyzing Activin A signaling in head and neck squamous cell carcinoma (HNSCC), we observed similar alterations of ECM proteins induced by Activin A stimulation, which could no longer be detected upon utilizing knockout of ACVRIB in the HNSCC lines (data not shown).

Taken together, we show that in contrast to the dysplastic ECdnT cells and the TE-2 ESCC cell line, fibroblast-secreted Activin A could not suppress TE-11 cell invasion nor were ECM components altered, indicating the necessity of intact Activin A signaling to mediate these effects. In the absence of ACVRIB, we presume the tumor cells can escape Activin A-mediated regulation with reciprocal consequences on the ECM itself. This would additionally explain the observation of increased tumor stage in the human tissue set for which stromal Activin A was high in tumors with low ACVRIB expression.

Angiogenesis assessed by endothelial tube formation is significantly inhibited following treatment with conditioned media from Activin A overexpression cultures

Because matrix metalloproteases (MMP) are known to be regulated by TGFβ signaling

pathways and have long been implicated as necessities for epithelial cell invasion, we performed gelatin zymography to examine total and active MMP-2 and MMP-9 expression. We found that expression of pro- and cleaved MMP-2 were reduced in Fibro-ActA cultures from ECdnT and TE-2 cells, yet their expression in TE-11 was not affected by fibroblast overexpression of Activin A throughout the 17-day culture (Figure 4-9a). Overall, MMP-9 expression and activity remained largely unchanged in the different culture conditions, aside from an increase in active MMP-9 in the TE-2 cells at the end of the culture (day 17) in the presence of Fibro-ActA. These results suggest that, even in the presence of MMPs, which promote epithelial cell invasion, Activin A is able to suppress this effect.

As MMPs, particularly MMP-2 and MMP-9, are necessary for tumor angiogenesis

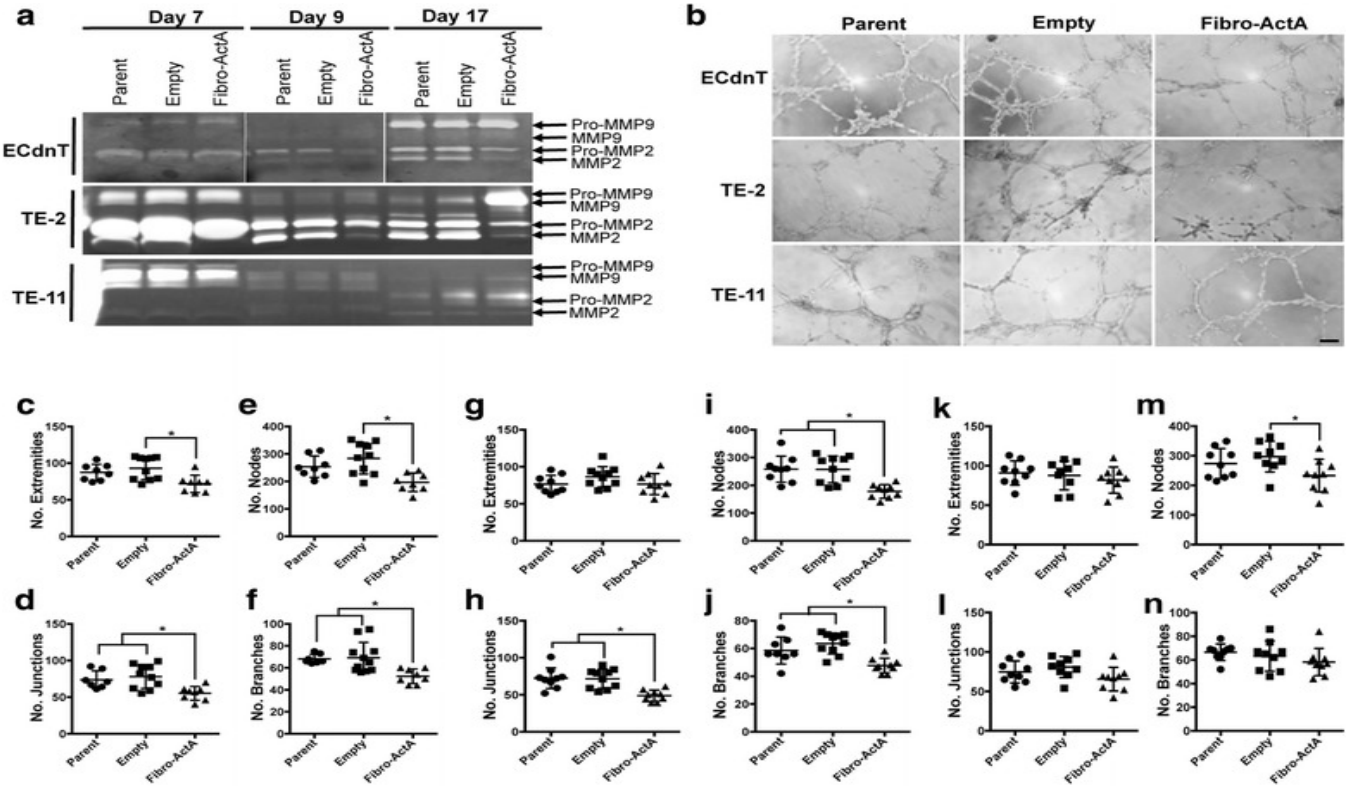
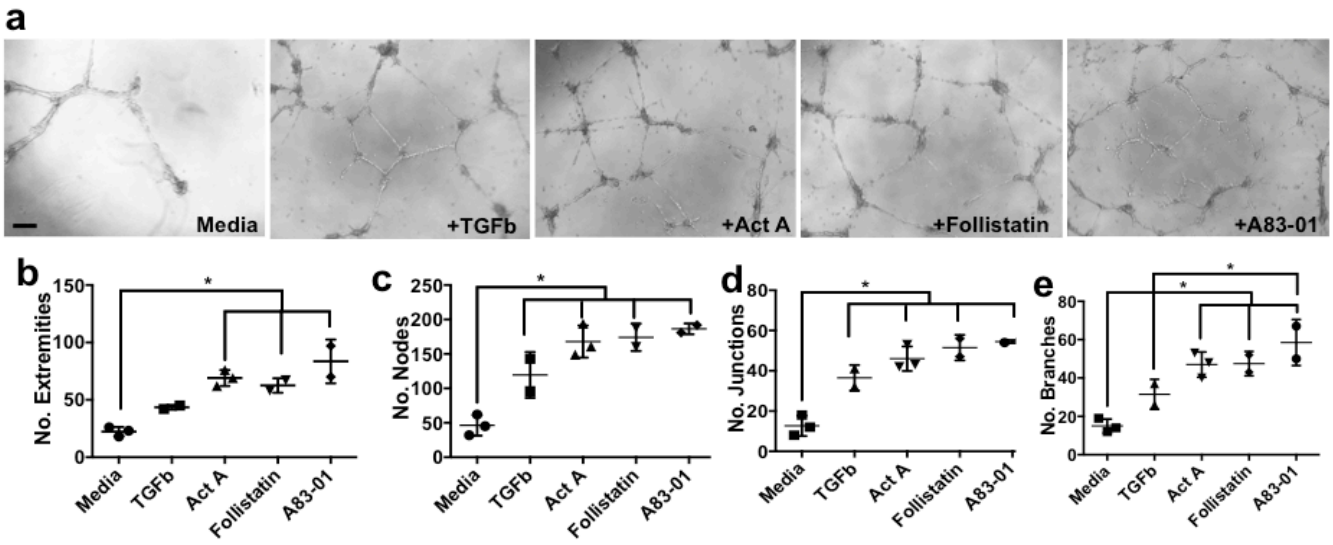


Figure 4-9. Fibro-ActA conditioned media from three-dimensional organotypic cultures inhibits *in vitro* angiogenesis. (a) Gelatin zymography of conditioned media collected from 3D-organotypic cultures at day 7 (fibroblasts alone), day 9 (media collection following the addition of epithelial cells), and day 17 (final collection). Arrows indicate the locations of pro- and cleaved MMP-2 and MMP-9. (b) Representative images of endothelial tube formation assays using HMEC-1 cells. HMEC-1 endothelial cells cultured with day 9 conditioned media from ECdnT/Fibro-ActA and TE-2/Fibro-ActA cultures had less overall tube formation, compared to parent and empty vector controls. This effect was not observed upon treatment with TE-11/Fibro-ActA conditioned media. Quantification of endothelial extremities, nodes, junctions, and branches for the assays with ECdnT (c-f), TE-2 (g-j), and TE-11 (k-n) conditions shown in (b)

Figure 4-10. Endothelial tube formation assays following treatment with recombinant proteins and the chemical inhibitor, A83-01; endothelial tube formation assays and quantification following treatment. (a) Bright field images of HMEC-1 endothelial tube formation assays treated with recombinant protein (TGFβ, Act A, or follistatin) or the chemical inhibitor A83-01. (b) Treatment of HMEC-1 cells with Activin A, follistatin, or A83-01, but not TGFβ, increased the number of formed endothelial extremities. (c-d) Compared to media control, treatment with recombinant protein (TGFβ, Act A, follistatin) or A83-01 increased the number of endothelial tube nodes and junctions, respectively. (e) Treatment of HMEC-1 cells with Activin A, Follistatin, or A83-01, but not TGFβ, increased the number of formed endothelial extremities. (One-way ANOVA, *p<0.05; scale bar = 200 μm).



(reviewed in (370)) and Activin A has been characterized as a potent inhibitor of this process (139,144), we next examined the effect of Activin A overexpressing cultures on endothelial tube formation. We found that treatment of HMEC-1 endothelial cells with conditioned media from dysplastic ECdnT cultured with Fibro-ActA showed reduced tube formation, compared to parent and empty vector control conditioned media (Figure 4-9b). This effect was also observed when using TE-2 conditioned media from Fibro-ActA (Figure 4-9b). Quantitative analysis of the assay images showed that HMEC-1 cells treated with Fibro-ActA conditioned media from ECdnT and TE-2 cultures formed significantly less extremities, nodes, junctions, and branches, compared to parent and empty vector control (Figure 4-9c-j). These results indicate that overexpression of Activin A, and its crosstalk with the epithelial cell compartment in this context, results in the inhibition vessel formation ultimately leading to suppression of tumor cell expansion. Conditioned media from TE-11 cells cultured with Fibro-ActA, however, failed to reduce tube formation and only a significant reduction of nodes was observed (Figure

4-9b,k-n). Interestingly, treatment of HMEC-1 cells with recombinant protein (TGFβ, Activin A, or the Activin A antagonist follistatin) or A83-01 were equally effective at inducing tube formation (Figure 4-10a-e). These results indicate that Activin A alone may not directly inhibit angiogenesis, however it may regulate the expression of several anti-angiogenic proteins to control this process through epithelial-mesenchymal crosstalk.

Discussion

It has been well documented that TGFβ receptors, which are closely related to Activin receptors, are downregulated as a means to evade growth suppression. In colorectal cancer, aside from loss of Smad4, TβRI and TβRII are commonly downregulated or lost (371). Similar observations have been made in laryngeal (372) and gastric cancers (reviewed in (373)). The downregulation of these receptors occurs concurrently with TGFβ upregulation (371). As there are significant similarities between these two pathways, the analogous observations made between the TGFβ pathway, as shown in the literature, and the Activin A pathway, demonstrated here, are not surprising. ACVR2 mutations have been described to attenuate Activin A signaling in prostate cancer (374) and microsatellite unstable colon cancer (375). The mutations identified are similar to the well-characterized frameshift mutations in TGFBR2 (319). Inactivation of ACVRIB so far has only been identified in pancreatic cancer as a consequence of a somatic mutation (229), and homologous deletion is associated with an aggressive phenotype in pancreatic cancer (84). Based on the 120 esophageal squamous tumor tissues we analyzed in this study, we found that ACVRIB can also be lost in ESCC. Furthermore, while ACVRIB loss has not been described to contribute to esophageal cancer, the overexpression of Activin A has been identified previously to be associated with enhanced matrix metalloprotease expression (89) and ESCC aggressiveness (300), partially through

upregulated of N-cadherin (92). We have additionally addressed the loss of ACVRIB in the context of head and heck squamous cell carcinoma and have observed results similar to the data presented here (data not shown).

In our study we found not only that Activin A is increased, but also that its proximity to the stroma is important to its function. When overexpressed in the tumor, Activin A confers differential effects. Some cancers, such as lung and head and neck squamous cell carcinoma, develop insensitivity to the growth inhibitory effects of Activin A, one of the hallmarks of cancer (56,57,101,120). In prior studies, we have described that treatment of premalignant cultures with recombinant Activin A induces esophageal cell invasion (356). In this context, however, signaling was not directional, as both epithelial and stromal compartment were exposed to the recombinant Activin A. In the current study, we mimicked a stromal source of Activin A, which we observed in the patient tissues. The fibroblast-secreted Activin A therefore contrasts stimulation of the whole culture with recombinant protein Activin A, showing inhibition of esophageal cell invasion. This inhibition is a consequence of intact Activin A signaling; however, loss of ACVRIB allows esophageal cancer cells, such as the cell line TE-11, to escape Activin A-dependent regulation.

Though the results of our study provide significant insight into the role of Activin A signaling in cancer progression, limitations remain. The three-dimensional organotypic reconstruct culture system is an efficient way to examine cellular crosstalk *in vitro*, however it is a simplified environment and does not account for additional components of the microenvironment, such as endothelial cells or leukocytes. Activin A may be secreted by and signal within a variety of cell types, including those listed. In our study, we used fibroblasts as a source of stromal Activin A, therefore accounting for Activin A expression alone, but not the

effects within assorted cell types. The integration of different cell types into this system will provide further insight into the systemic effects of Activin A during cancer progression. We aimed to focus on angiogenesis as one example of the complex microenvironment and to identify the functional consequences of fibroblast-derived Activin A overexpression using the endothelial tube formation assay. Activin A has been consistently shown to function as a potent anti-angiogenic factor, contrary to reports indicating its oncogenic role. Treatment of endothelial cells with Activin A showed reduced tube formation possibly through inhibition of proliferation (2,4,9,32-35,61,85,139,140,336). In this study, we described a reduction of tube formation, as measured by the number of nodes, junctions and branches, dependent on intact Activin A signaling. Additionally, we observed that fibroblast-derived overexpression of Activin A downregulates vascular endothelial growth factor expression (VEGF), one of the key components of tumor angiogenesis (data not shown). Previous research identified that the majority of endothelial cells expresses ActRII/IIB and are therefore able to respond to Activin A ligand binding (9,26,28,42,43,56,85,140). However, as we used conditioned media from fibroblast-derived Activin A organotypic cultures, the effects we measured on tube formation may be indirect. *In vitro* co-culture studies of fibroblasts and endothelial cells in the presence of ESCC cells have shown that tumor cell-secreted TGFβ can activate fibroblasts resulting in VEGF secretion and increased formation of endothelial network formation (376). A number of pro-angiogenic factors were downregulated when we performed angiokine arrays (data not shown), which may be under the direct regulation of Activin A. Additionally, Activin A has been found to regulate several secreted factors, such as IL-8, VEGF, GnRH, and PTGS2 (377-379). Therefore, it can be difficult to conclude that Activin A overexpression alone is responsible for all of the observed effects in our experiments, particularly since contradictory evidence has

been previously observed (356). However, our study investigates a long-term stable overexpression of Activin A in a three-dimensional context, rather than the utilization of recombinant Activin A for *in vitro* treatments. Therefore, in this study, we were able to investigate the paracrine signaling effects due to epithelial-fibroblast crosstalk.

In this paper, we showed that Activin A plays a necessary role in controlling mechanisms of esophageal squamous cell carcinoma invasion and, most likely, tumor progression. Activin A signaling from the stroma, e.g. fibroblasts, regulates expression of extracellular matrix proteins and thus signaling pathways involved in cell invasion. It has been found the expression of Activin type I receptors (ActRI and ACVRIB) and type II receptor (ActRII) mediates Activin A effects in collagen gel contraction using human lung fibroblasts (380). In pancreatic stellate cells, autocrine Activin A signaling induces activation and collagen secretion (381). We observed the loss of podoplanin and fibronectin in response to overexpression of Activin A, but also αSMA and podoplanin double positive fibroblasts in the invasive cultures. In the context of fibrosis, which is characterized by the presence of myofibroblasts, Activin A has been shown to regulate myofibroblast differentiation mediated by integrin α11β1, a collagen receptor expressed on fibroblasts (382).

Interestingly, we believe that Activin A, when unable to bind and signal through ACVRIB, is then free to associate with other receptor complexes, such as BMPR2 or BMPR1/ALK2, to propagate a signal (383,384). This data highlights, for the first time, the importance of maintaining an intact Activin A signaling pathway to control ESCC invasion. Previous literature has shown that Activin A can bind with low affinity and transduce a signal through subsequent TGFβ family receptors, of particular note the BMP receptors (385). Substantial research has demonstrated that induction of the BMP pathway can result in

production of collagens, commonly leading to bone formation (386,387). In fact, Activin A has been reported to regulate ECM mineralization, as well as enhance BMP-induced formation of bone and collagen (311,388). This may be a mechanism to control ECM formation aside of ACVRIB-dependent Activin A signaling.

Conclusion

In conclusion, we have demonstrated that in a dysplastic esophageal squamous microenvironment, Activin A works to inhibit cell invasion into the stroma, however when the pathway becomes dysregulated, such as through the downregulation of ACVRIB, cells are rendered unresponsive. Dysregulation of the Activin A pathway may be a way in which cancer cells can adapt to circumvent inhibitory environmental factors.

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CHAPTER V

LOSS OF ACVRIB LEADS TO INCREASED SQUAMOUS CELL CARCINOMA AGGRESSIVENESS THROUGH ALTERATIONS IN ADHESION PROTEINS

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Preface

In Chapter 4, we established the considerable role of the Activin A signaling pathway in regulating cell invasion in ESCC, as well as noting this effect is necessitated by Activin receptor type IB, ACVRIB. Naturally, the work presented in Chapter 4 led us to investigate how the lack of squamous cell carcinoma (SCC) response to Activin A due to the absence of ACVRIB alters cell behavior and overall function. To begin this investigation into Activin A-ACVRIB signaling, I first used the head and neck SCC (HNSCC) cell line OSC-19 to generate a CRISPR/Cas9 cell line with genetic deletion of ACVRIB (ACVRIB-KO) to investigate the long-term, stable effects of loss of ACVRIB. Following validation, I tested these cells in a variety of *in vitro* assays (proliferation, migration, invasion, organotypic culture) to examine the biological effects of ACVRIB deletion. Small interfering RNA (siRNA) was used an additional cell line, KYSE520, to validate my observations. Interestingly, I found that not only does loss of ACVRIB lead to a more invasive, aggressive phenotype in three-dimensional culture, but also that ACVRIB appears to mediate this effect through the regulation of cell-cell and cell-ECM adhesion. The results of this chapter suggest a novel role of ACVRIB in the maintenance of cellular adhesion.

Abstract

Squamous cell carcinomas of the head and neck (HNSCC) and esophagus (ESCC) pose a global public health issue, particularly in less developed regions. Unfortunately, over the course of the last several decades, little progress has been made in improving overall HNSCC or ESCC outcomes. This may, in part, be due to our lack of understanding the mechanisms that drive HNSCC and ESCC progression. Therefore, greater effort needs to be invested in identifying these processes. In recent years, the Activin A signaling pathway has been implicated in a number of cancers. The role of the Activin A signaling pathway, on its own, remains poorly understood. We have previously discovered that the ligand Activin A acts as a tumor suppressor when epithelial ACVRIB remains intact in ESCC. However, the tumor suppressive effects of Activin A are lost when squamous cell ACVRIB expression is downregulated. Therefore, we decided to further investigate the function of ACVRIB in the regulation of HNSCC and ESCC migration and invasion. To investigate the role of ACVRIB in SCC biology, cell lines with ACVRIB knockout (ACVRIB-KO) or knockdown (siACVRIB) were first generated using CRISPR/Cas9 or siRNA, respectively. These cells lines were then used in an array of *in vitro* assays to study the functional effects of loss of ACVRIB. Finally, we examined the protein expression profiles and signaling pathways thought to mediate the observed functional effects by immunofluorescence and Western blot. Interestingly, we found that ACVRIB-KO and siACVRIB cells show a greater capacity to proliferate, migrate, and invade in two- and three-dimensional cultures. Additionally, ACVRIB-KO cells grew in foci rather than the typical cobblestone morphology of squamous cells and exhibited an altered actin cytoskeleton, leading to increased formation of laemellopodia. Based upon these observations, we examined the protein profiles of these cells and found variant expression of

cell-cell and cell-extracellular matrix adhesion proteins, such as E-cadherin, αv integrin and β4 integrin, as well as differentiation proteins. These effects may be mediated through ACVRIB-independent signaling through downstream Smad1/5/8 and MAPK/ERK signaling. Therefore, we present a novel mechanism for Activin A in the absence of ACVRIB in HNSCC and ESCC, suggesting a previously unknown flexibility of this ligand.

Introduction

Activin receptor-like kinases (ALKs) have a long-standing role in organism development and cancer. ALKs are a family of seven type I receptors that share high sequence homology (40-60%) and structural similarities, including an extracellular glycosylation site, transmembrane domain, and cytoplasmic tail with a serine/threonine kinase domain (reviewed in (389)). ALKs operate as the primary signal transducing receptor for the superfamily ligands, among which include TGFβs, Activins, and BMPs, which will be further described in detail. As there are greater than 45 ligands in the TGFβ superfamily with only seven type I receptors, there is ligand-receptor overlap (30).

ACVRIB, also known as ALK4, is an example of the ligand-receptor overlap noted in the ALK family, as it is the primary type I signaling receptor for several TGFβ superfamily ligands, including growth and differentiation factor (GDF) 1, GDF3, Activin A, Activin B, GDF8/myostatin, and GDF11 (133,390-394). ACVRIB participates in ligand-dependent signaling when a ligand, such as those described above, binds to a type II receptor (i.e. ActRII or ActRIIB) homodimer, and then phosphorylates a downstream Smad (Smad2/3 or Smad1/5/8), which forms a complex with Smad4. The Smad complex translocates to the nucleus to drive gene transcription. The above described pathway is considered the canonical

ACVRIB pathway, however there are non-canonical pathways in which ACVRIB has been shown to participate, including MAPK/ERK, Akt/PI3K, Wnt/β-catenin (30,340,395). Complicating our understanding of the downstream signaling activation is the fact that pSmad1/5/8 (also delineated pSmad1/5/9), which is a bona fide target of BMP signaling, can also be phosphorylated in response to Activin A stimulation (217). This leads to the assumption that possible complexes can be formed between different type I and type II receptors, as demonstrated in some recent reports (396-399).

ALKs have been found to play critical roles in both development and cancer. Existing evidence has demonstrated participation of ALKs in vasculogenesis, angiogenesis, osteogenesis, chondrogenesis, gastrulation, germ cell differentiation, and folliculogenesis in development (166-168,182,183,199,218,222,238,239,242,243). ACVRIB, in particular, has been implicated in specific processes, such as morphogenesis and differentiation (400). The essential function of ACVRIB has been additionally demonstrated in mouse models, as global knockout of Acvr1b results in embryonic lethality (218). Therefore, several conditional knockouts were developed to assess the role of ACVRIB (225,401). Conditional deletions of Acvr1b in squamous tissues (i.e. oral cavity, esophagus, and skin) result, primarily, in alterations in hair follicle cycling. Amongst these mice, several phenotypes were observed, including stunted growth, hair loss, and increased proliferation in the epidermis, however no alterations of the oral cavity or esophagus were noted (225). The results of these models indicate that ACVRIB is necessary for proper embryonic and post-natal development.

Aside from the aforementioned roles of ACVRIB and the other ALK family members in development, ALKs have been described in cancer initiation and progression in a variety of cancers, including breast cancer, melanoma, colorectal cancer, esophageal carcinoma, head

and neck squamous cell carcinoma, pituitary adenomas, and pancreatic cancer, among others (170,190,210,227,229,230,233,254). Interestingly, in the case of ACVRIB, loss of this receptor is associated with cancer progression in pituitary and pancreatic cancers. In pituitary cancer, splice variants of ACVRIB have been detected. These variants lack the kinase domain and cannot, therefore, propagate signal even in the presence of ligand and following receptor complex formation (227,228). In pancreatic cancer, loss or deactivation of ACVRIB occurs in approximately 2% of cancers (229,402). Though not as prevalent as Smad4 loss, which occurs at a frequency of approximately 45%, identified receptor loss suggests a role for ACVRIB as a tumor suppressor (229,403,404). Similarly, ACVRIB mutations and loss of protein, respectively, have been documented in head and neck and esophageal cancers (133,405)

The focus of this study was to induce ACVRIB loss and analyze the subsequent functional consequences for SCC based on our previous observation that ACVRIB loss occurs in advanced ESCC (133). We have previously shown that there is a coordinated upregulation of stromal *INHBA*/Activin A with loss of epithelial ACVRIB expression across stage, suggesting that ACVRIB is responsible for mediating the tumor suppressive effects of Activin A (133). As described in Chapter 4, we demonstrated in the ESCC cohort using immunofluorescence staining that *INHBA* is upregulated. This has been additionally shown in ESCC patient samples where approximately 59% had upregulated *INHBA* compared to normal tissues (406). In both ESCC and oral squamous cell carcinoma (OSCC), overall increased Activin A expression is associated with tumor prognosis, lymph node metastasis, and poor prognosis (85,89,92,104,225). While Sobral and colleagues have shown that stromal myofibroblast-derived Activin A promotes OSCC proliferation and migration, another study in HNSCC has suggested that one potential mechanism for the promotion of migration and invasion could

occur is through the induction of RUNX2 (127,407). However, neither study determined that these effects were mediated by ACVRIB. Based on our findings in ESCC, we hypothesized that loss of ACVRIB leads to tumor progression as the absence of growth regulation by Activin A induces alterations in cell-to-cell adhesion and cell invasion. In this study, we deleted or knocked down ACVRIB in ESCC and HNSCC cell lines to examine the role of this type I receptor in cell migration and invasion. We found that not only do cells with reduced expression of ACVRIB demonstrate increased *in vitro* proliferation, migration, and invasion, but also that this occurs through the regulation of proteins involved in cell-cell and cell-ECM interactions. Overall, the results of this study indicate a novel role for ACVRIB in the maintenance of the epithelial and stromal compartments.

Materials and Methods

Cell culture

The head and neck squamous carcinoma cell line OSC-19 and oral cancer-associated fibroblasts (CAF) were cultured in DMEM, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin (P/S) (Gibco, Grand Island, NY), as previously described (408,409). The esophageal squamous cell carcinoma cell line KYSE520 were cultured in RPMI and supplemented with 10% FBS and 1% P/S (Gibco), as previously described (410).

CRISPR/Cas9 cell line generation

Knockout of ACVRIB in OSC-19 cells was generated using the Genome-Wide knockout kit purchased from Origene (cat. no. KN206920) and performed according to the manufacturer's protocol. Following transfection, OSC-19 cells were selected with puromycin (2 μg/ml) and single clones isolated. Clones were screened by Western blot and validated by flow cytometry.

siRNA transfection

KYSE20 cells were seeded in a 6-well plate at a density of 200,000 cells per well. The following day, KYSE520 cells were transfected with 10 nM ON-TARGETplus siRNA SmartPool or non-targeting control (GE Dharmacon, Lafayette, CO) diluted with Lipofectamine RNAiMax (Life Technologies, Carlsbad, CA) in OPTI-MEM (Gibco). Cells were either trypsinized and reseeded for assays after 24 hours or harvested for RNA or protein after 48 hours.

Flow cytometry

ACVRIB flow

Flow cytometry experiments were performed by the Vanderbilt Medical Center Flow Cytometry Shared Resource. To discern the ACVRIB-KO population, OSC-19 cells were first trypsinized, washed with 1xPBS and resuspended at 5x10^6 cells/ml in 1xPBS. In a separate tube, 100 µl of the cell suspension was transferred and ACVRIB antibody (cat. no. ab109300; Abcam, Cambridge, UK) was added to a final concentration of 1:100. Cells were incubated for 30 minutes at room temperature, then washed with 1xPBS and centrifuged. The anti-rabbit secondary antibody Alexa647 (Life Technologies) was added at 1:1000 and incubated at room temperature for 20 minutes. Cells were washed with 1xPBS, centrifuged, and resuspended in flow cytometry buffer. Unstained cells and ACVRIB-positive cells were used to set gates.

Propridium iodide cell cycle analysis

Cell cycle analysis, determined by propridium iodide (PI) staining, was performed as follows. OSC-19 and KYSE520 cells were trypsinized, washed with 1xPBS, and resuspended at 2x10^6 cells/ml in ice cold 1xPBS. Following resuspension, 9 ml of ice cold 70% ethanol was

added and cells were incubated at -20°C overnight. Cells were washed with cold 1xPBS, centrifuged and resuspended in 500 µl PI staining solution (0.1% Triton X-100, 2 mg RNAse A, 400 µl 500 µg/ml PI in 1xPBS) overnight at room temperature. The following day, cells were analyzed in the Vanderbilt Medical Center Flow Cytometry Shared Resource on a 5-laser BD LSRII. Cell cycle analysis is depicted using BD FACSDiva 8.0 software (BD Biosciences, San Jose, CA).

Western blot

Cells were plated in 6-well plates at an initial density of 200,000 cells/well. Prior to beginning treatment, cells were serum-starved overnight at 37°C. Following overnight starvation, cells were pre-treated with 250 nM DMH1 (ALK2i, Tocris, Bristol, UK) or kept in serum-free media overnight at 37°C. The next day, cells were treated with Activin A (ActA: 10 ng/ml, R&D Systems), follistatin (FST: 100 ng/ml, R&D Systems), or kept in serum-free media for 30 minutes. Cell lysates were collected and Western blots performed as previously described (356).

Hematoxylin and eosin staining

Hematoxylin and eosin staining was performed by the Vanderbilt University Medical Center Translational Pathology Shared Resource.

Immunofluorescence

Formalin-fixed, paraffin-embedded sections

Staining of formalin fixed paraffin-embedded sections were performed as previously described (133).

Phalloidin

Phalloidin staining was performed using CytoPainter Phalloidin-iFluor 488 (Abcam), according to the manufacturers' instructions, and mounted with ProLong Gold Anti-fade with DAPI (Life Technologies).

Proliferation

EdU

ACVRIB-KO or control cells were seeded at low density on 0.02% gelatin-coated coverslips in growth media. Following adherence, coverslips were washed with 1xPBS and incubated with 5 μM EdU (Abcam) in growth media. Next, cells were fixed for 30 minutes with 4% paraformaldehyde at room temperature and washed three times for five minutes each with 1xPBS. Cells were permeabilized for 30 minutes using 0.5% Triton-X 100 diluted in 1xPBS. Subsequently, cells were incubated with the EdU development cocktail (100mM Tris-buffered saline pH 7.6, 4mM CuSO4, 5μM sulfo-cyanine 3 azide, 100μM sodium ascorbate). Finally, cells were rinsed with 1xPBS, three times for five minutes each, and mounted on slides using ProLong Gold anti-fade with DAPI (Life Technologies). Slides were imaged on an Olympus BX61WI (Center Valley, PA) upright fluorescent microscope, using Volocity Imaging Software (Perkin Elmer, Waltham, MA).

Cell counting

To further investigate proliferation, cells were first seeded in 6-well plates at a density of 10,000 cells/well (day 1). At 48 hour intervals, cells were dissociated using 0.25% trypsin (Gibco) and counted using a hemacytometer and 0.4% Trypan Blue solution (Gibco). Fold-change, relative to day 1, was calculated.

Magnetically attachable stencil (MAts) migration assay

MAts assays were performed as previously described (411). Briefly, MAts were fabricated and coated with 1% pluronic solution diluted in 1xPBS. Prior to use, MAts were rinsed, sterilized under UV light, and added to a gelatin coated 12-well plate. Then, 200,000 or 250,000 cells were seeded in each well. After cells adhered to the plate, they were washed and serum-starved overnight. The MAts were then removed using a forceps. Images were taken upon MAts removal and at 12-hour intervals. Results were calculated using TScratch (NIH, Bethesda, MD), as previously described (411).

Boyden chamber assays

Migration

Boyden chamber assays were performed in 24-well format according to the manufacturer's instructions. Briefly, cells were trypsinized, centrifuged, and resuspended at 100,000 cells/ml. A chemoattractant (growth media [DMEM with 10% FBS] or cancer-associated fibroblast conditioned media [CAF CM]) was added to the bottom chamber at 750 µl/well. 500 µl (50,000 cells) were added to the top chamber. The plates were incubated at 37°C for approximately 24 hours. Cells were then washed with 1xPBS, the top chambers swabbed with a cotton-tipped

applicator to remove non-migrated cells, and fixed using 100% methanol at -20°C for 10 minutes. Chambers were immediately moved to a 0.1% crystal violet solution and stained, while rocking, overnight. The next day, the chambers were removed from the crystal violet solution, washed with water, cleaned again with a cotton-tipped applicator, and allowed to dry. When the membranes were dry, they were carefully removed with a scalpel and mounted on slides using Permount (Fisher, Hampton, NH). Slides were allowed to dry overnight before being imaged. Cells were counted, presented as number of cells per high-powered field (hpf), and data analyzed by Student's *t*-test.

Invasion

Boyden chamber invasion assays were described as above, with the exception that chambers were rehydrated with serum-free media for 2 hours at 37°C prior to cell seeding.

Adhesion assay

Twenty four-well plates were coated overnight with various extracellular matrix substances at the following concentrations: bovine serum albumin (BSA; 5% in 1xPBS), gelatin (0.02% in 1xPBS, Sigma, St. Louis, MO), collagen I (1 mg/ml, Corning), fibronectin (FN; 1 µg/ml, R&D Systems), vitronectin (VTN; 10 µg/ml, R&D Systems), osteopontin (OPN; 2 µg/ml, R&D Systems). The wells were then aspirated, washed with 1xPBS, and blocked with 5% BSA. Cells were seeded at 50,000 cells per well and imaged at intervals to observe cellular adherence.

Organotypic reconstruct cultures

Organotypic cultures (OTC) were performed as previously described (356). In addition to the referenced protocol, OTCs were treated with several recombinant proteins or chemical inhibitors beginning at day 7 of the OTC protocol. Treatments (10 ng/ml Activin A, R&D Systems; 1 μM A83-01, Tocris; 250 nM DMH1, Tocris) were refreshed every two days during the treatment period.

Biostatistical Analysis

Experimental results were analyzed using Student's *t*-test or one-way ANOVA and expressed as the mean +/- standard deviation. Statistical analysis of the *in vitro* experiments was performed in Prism 6.0 (GraphPad, San Diego, CA).

Results

Loss of ACVRIB initiates a proliferative phenotype in OSC-19 cells

As discussed above, loss of ACVRIB has been documented in a variety of cancers, including pituitary, pancreatic, head and neck squamous, and esophageal squamous cell cancers, and has been suggested to act as a tumor suppressor (133,229,403,405,412). We have previously

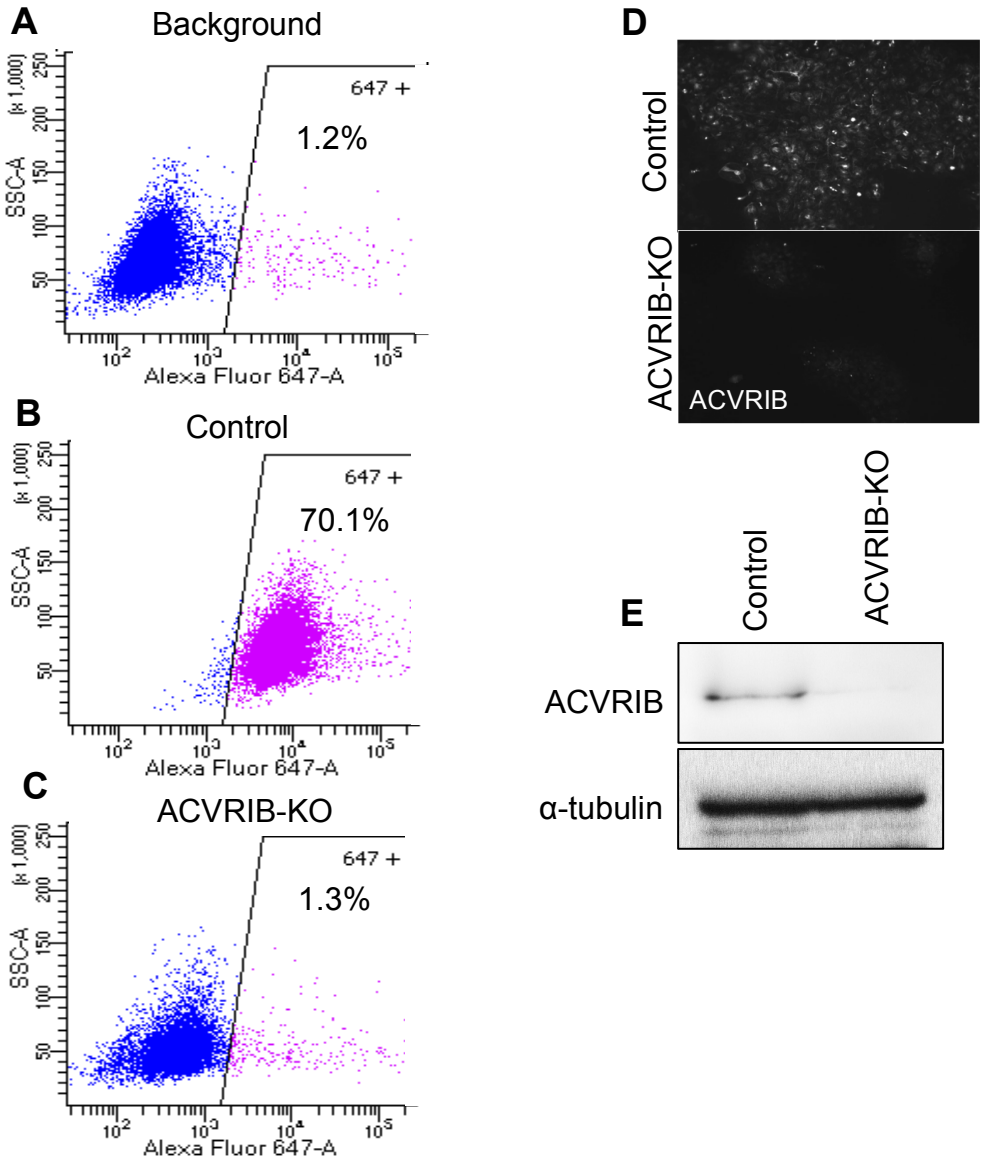
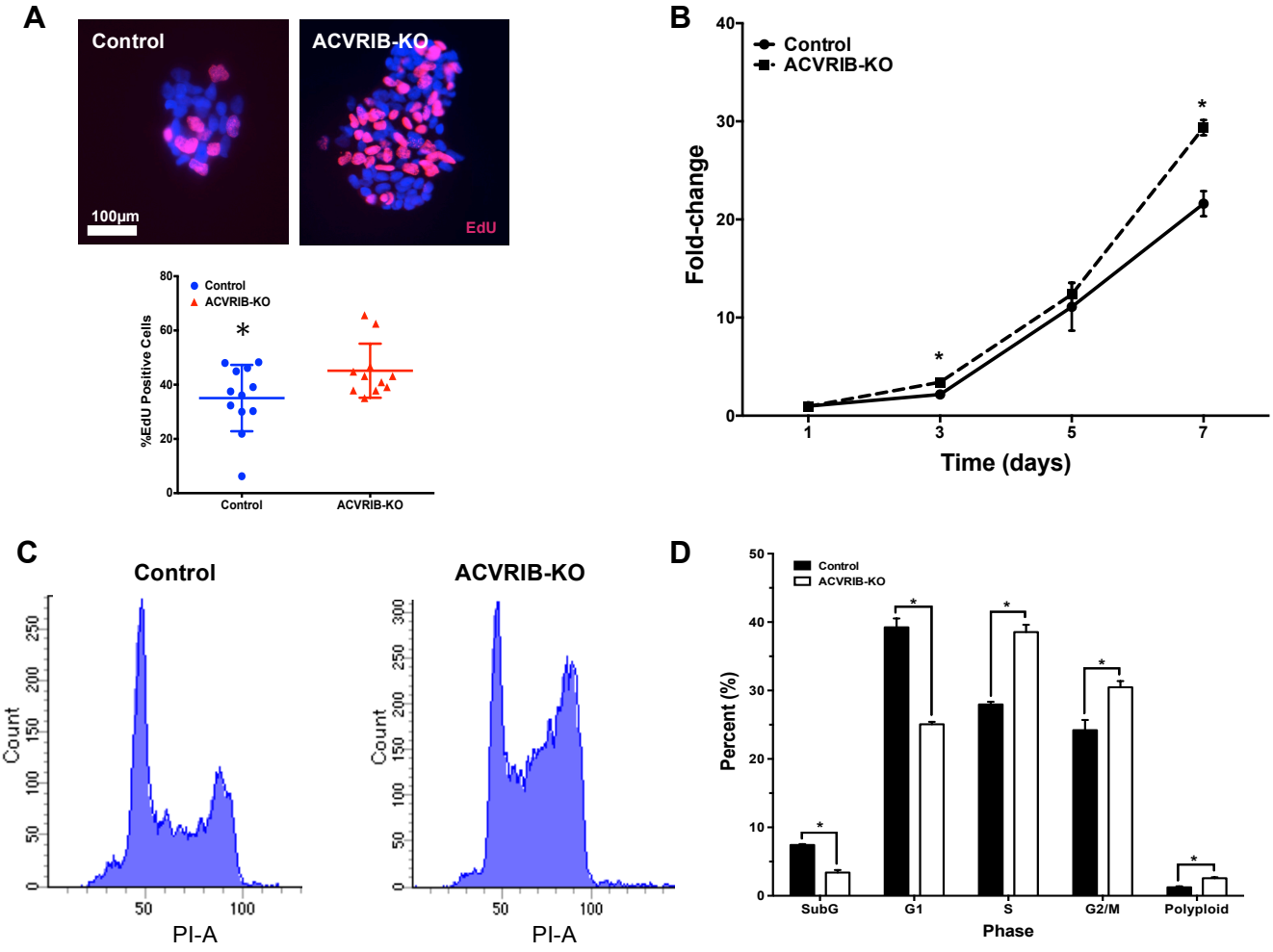


Figure 5-1. Validation of ACVRIB knockout in OSC-19 head and neck squamous cells. Cells were validated by several methods. Flow cytometry showing (A) background stain (B) control and (C) ACVRIB-KO populations. Immunofluorescence staining of ACVRIB in (D) control and (E) ACVRIB-KO cells. (E) Western blot of control and ACVRIB-KO OSC-19 cells. α-tubulin was used as a loading control.

Figure 5-2. ACVRIB-KO cells exhibit increased proliferation. Proliferation of ACVRIB-KO and control OSC-19 cells was examined by several methods. (A) Staining of OSC-19 cells demonstrated increased incorporation of EdU into ACVRIB-KO cells, compared to control. (B) Validation of EdU staining was performed by cell counting, using 0.25% Trypan Blue to exclude dead cells. By day three of culture, ACVRIB-KO cells were showing substantially increased proliferation compared to control. (C) Cell cycle analysis by propridium iodide staining in flow cytometry. (D) Statistical analysis of flow cytometric cell cycle analysis. ACVRIB-KO cells had significantly less cells in G1, while more were observed S and G2/M; *p<0.05.



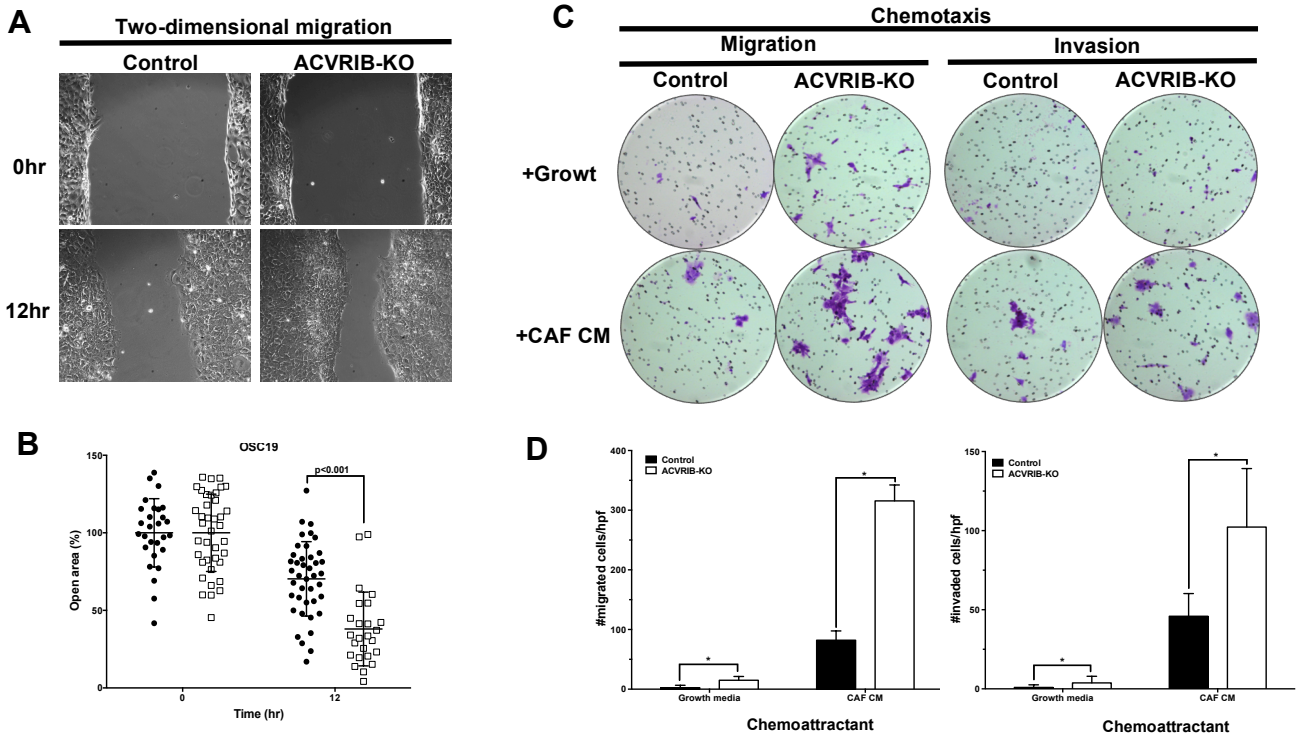
shown that there is a coordinated upregulation of stromal *INHBA*/Activin A with loss of epithelial ACVRIB expression across stage, suggesting that ACVRIB is responsible for mediating the tumor suppressive effects of Activin A (133); however, the functional effects of loss of ACVRIB have yet to be fully elucidated. Therefore, we decided to investigate the consequences of loss of ACVRIB, initially in the HNSCC cell line OSC-19. Following validation of a CRISPR/Cas9 model of ACVRIB loss (ACVRIB-KO) in OSC-19 cells by flow cytometry, immunofluorescence, and Western blot (Figure 5-1), we examined the proliferative capabilities

of these cells. Several groups have shown that the Activin A signaling axis via ACVRIB works as a growth inhibitor (413). Here, we validated this phenotype in ACVRIB-KO cells through utilizing 5-ethynyl-2'-deoxyuridine (EdU) incorporation and Trypan Blue cell counting, showing increased proliferation of ACVRIB-KO cells compared to controls (Figure 5-2A,B). Interestingly, ACVRIB-KO cells, compared to control, had smaller nuclei, grew in clusters and developed foci (Figure 5-2A). To further our investigation into the proliferative phenotype of ACVRIB-KO cells, we next performed cell cycle analysis by propridium iodide (PI) staining in flow cytometry (Figure 5-2C). Intriguingly, we found a significantly decreased number of cells in G1 and an increased number of cells in S and G2/M phases, further confirming the proliferative phenotype observed by EdU and cell counting (Figure 5-2D). These results suggest that loss of Activin A signaling through ACVRIB allows for increased SCC proliferation and clonal expansion, however whether this is through the upregulation of pro-growth proteins, such as cyclins or p21, or the downregulation of cell cycle inhibitors is not yet known.

Cell motility is increased in the absence of ACVRIB

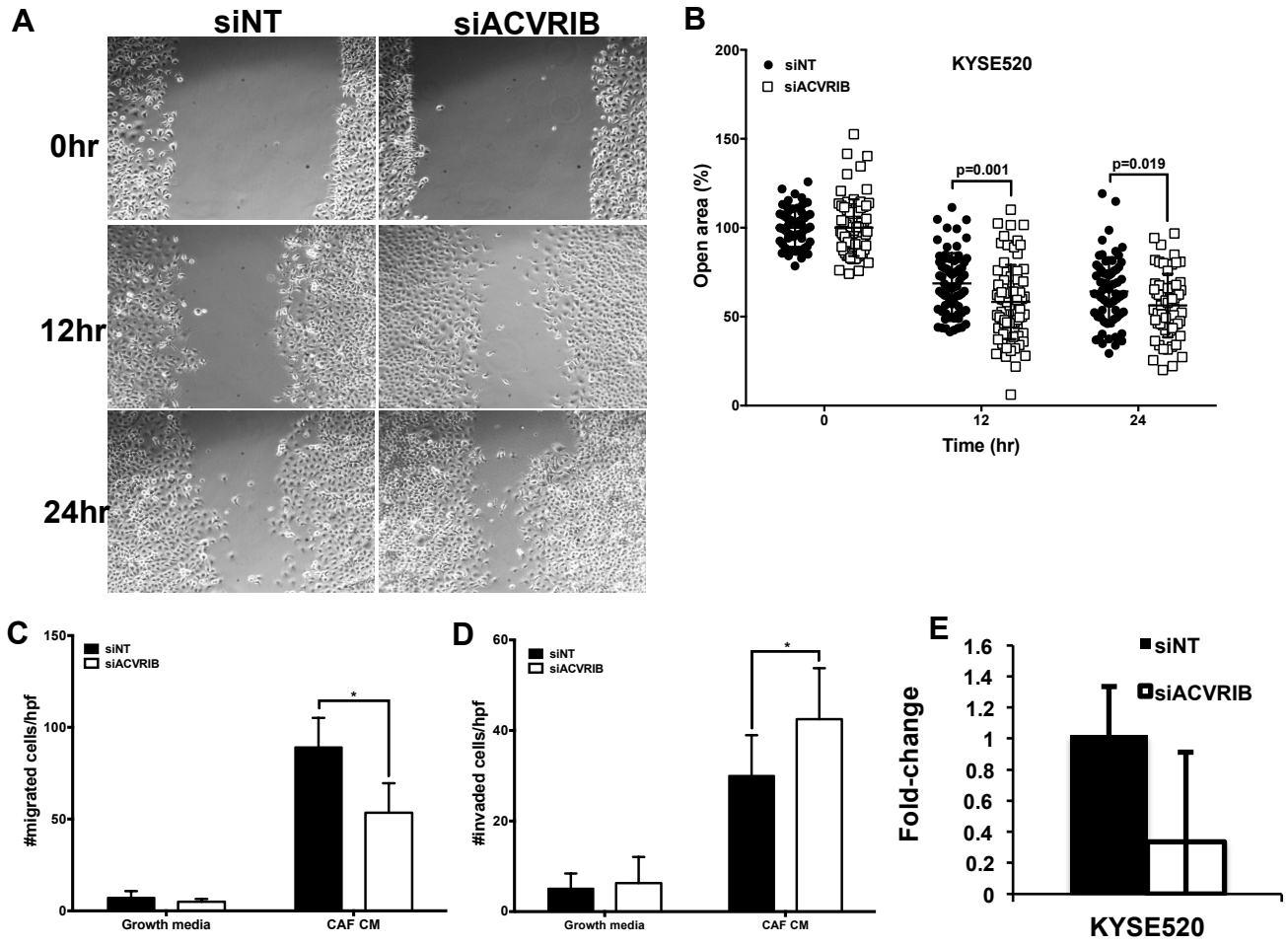
As we have confirmed enhanced proliferation in the absence of ACVRIB, we now sought to investigate the migration and invasion capabilities of these cells. Several studies have suggested that Activin A signaling can drive migration and invasion. For example, Yoshinaga and colleagues showed that overexpression of Activin A in ESCC cell lines had increased *in vivo* tumorgenicity than mock transfected cells, and Howley and colleagues demonstrated increased EMT in inhibin β_A-upregulated mammary epithelial cells, however these studies focused on the immediate action of the Activin A ligand, not the signaling complex (300,414). In contrast, we have shown that Activin A overexpression in ESCC with intact Activin A

Figure 5-3. Migration and invasion capabilities of OSC-19 cells are enhanced with ACVRIB loss. (A) Directional migration, investigated by MAts assay and assessed over the course of 12 hours, was enhanced in ACVRIB-KO cells (left), compared to control (right). Cells were seeded on a gelatin-coated surface. (B) ACVRIB-KO cells significantly reduced the open area of the MAts by 12 hours, compared to control (Student's *t*-test, p<0.001). (C) ACVRIB-KO cells demonstrated increased chemotactic migration (left) and invasion (right), using growth and cancer-associated conditioned media (CAF CM) as chemoattractant in Boyden chamber assays. (D) Statistical analysis, per high-powered field (hpf) of (C) (Migration - left; invasion – right; Student's *t*-test, $p<0.05$).



signaling cascade inhibits cell migration and invasion in three-dimensional organotypic culture (133). Therefore, we decided to investigate the mobility (migration, invasion) of ACVRIB-KO cells to mediate these observations. Using magnetic attachable stencils (MAts) on gelatin-coated plates, we first examined the ability of the loss of ACVRIB to perform directional migration. ACVRIB-KO cells had less open area in the MAts void at 12 hours, compared to control cells (Figure 5-3A, quantified in B). After 24 hours, the void was completely closed for both ACVRIB-KO cells and controls (data not shown). We additionally tested directional migration using MAts in the esophageal squamous cell carcinoma cell line KYSE520 with small interfering RNA (siRNA) knockdown of ACVRIB (siACVRIB*)*. Similarly to the OSC-19 ACVRIB-KO, KYSE520 cells with siACVRIB significantly decreased the open void area by 12 and 24 hours, compared to control transfection (siNT) (Figure 5-4A,B).

Figure 5-4. Knockdown of ACVRIB by siRNA in KYSE520 enhances motility. (A) By 12 hours and 24 hours, siACVRIB knockdown cells close more open MAts area than non-targeting control (siNT). (B) Quantification of MAts assay shown in (A) (C) Quantification of number of migrated KYSE520 siACVRIB or siNT cells per high-powered field (hpf). Cells were stained with 0.1% crystal violet and counted. There were less KYSE520 siACVRIB cells that migrated through the Boyden chamber cmpared to siNT. (D) Similar to (C), quantification of number of invaded KYSE520 cells per high powered field. KYSE520 siACVRIB cells invaded more in Boyden chamber assay than siNT. (E) qRT-PCR of ACVRIB in KYSE520. *p<0.05



Next, we tested ACVRIB-KO and control OSC-19 cells in Boyden chamber migration and invasion in the presence of growth media, which was used as a positive control, or oral cancer-associated fibroblast conditioned media (CAF CM, Figure 5-3C). ACVRIB-KO cells showed increased ability to migrate and invade in the presence of both growth and CAF CM (Figure 5-3D). Interestingly, CAF CM was a more potent chemoattractant for the ACVRIB-KO and control cells, suggesting a substantial role for epithelial-mesenchymal crosstalk in this process (reviewed in (415)). A similar effect was observed with the KYSE520 cells. Though the

siACVRIB condition did not exhibit significantly more migration in the presence of CAF CM compared to control, siACVRIB showed more potent invasion when CAF CM was used as a chemoattractant (Figure 5-4C,D). Validation of siRNA knockdown of ACVRIB is shown in Figure 5-4E. Overall, our data suggests that loss of ACVRIB function, through either deletion or knockdown, enhances the ability of SCCs to migrate and invade.

As we observed increased migration and invasion in ACVRIB-KO and siACVRIB cells, we decided to further examine the morphology and structure of these cells. An examination by bright field microscope showed that the control cells made an epithelial layer with cobblestone-like morphology, characteristic of squamous epithelium (Figure 5-5A,B) (416). Upon loss of ACVRIB, OSC-19 cells form small clusters with lamellopodial projections (Figure 5-5C,D, arrowheads). This led us to examine the composition of the ACVRIB-KO actin cytoskeleton by immunofluorescent phalloidin staining. Compared to control cells, which showed high actin density along the cell surface, ACVRIB-KO cells had protrusions at the cell surface (Figure 5-5E-H). This data suggests that ACVRIB is involved in the composition of the actin cytoskeleton, which impacts cellular motility.

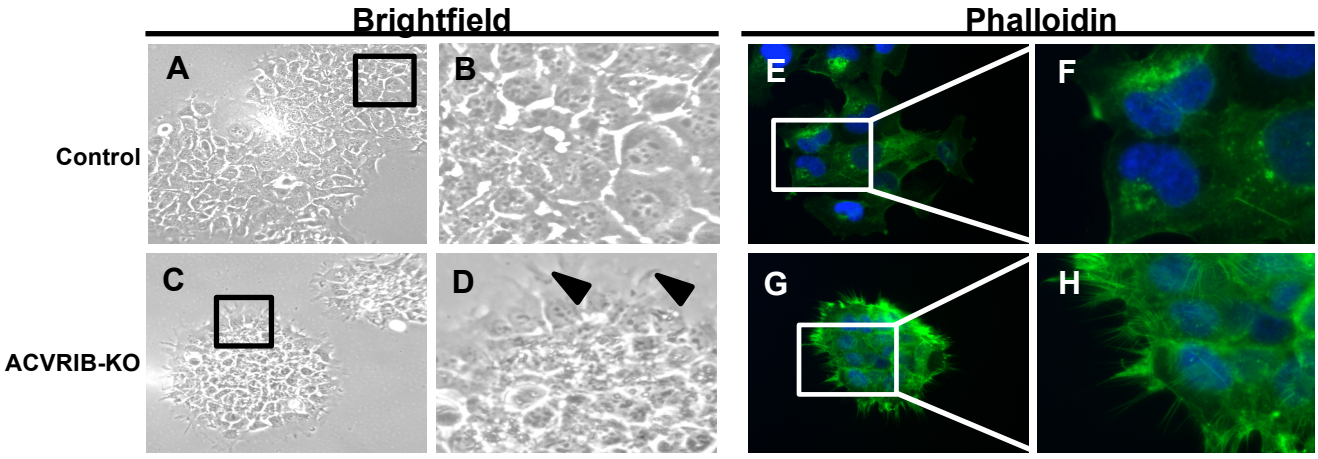
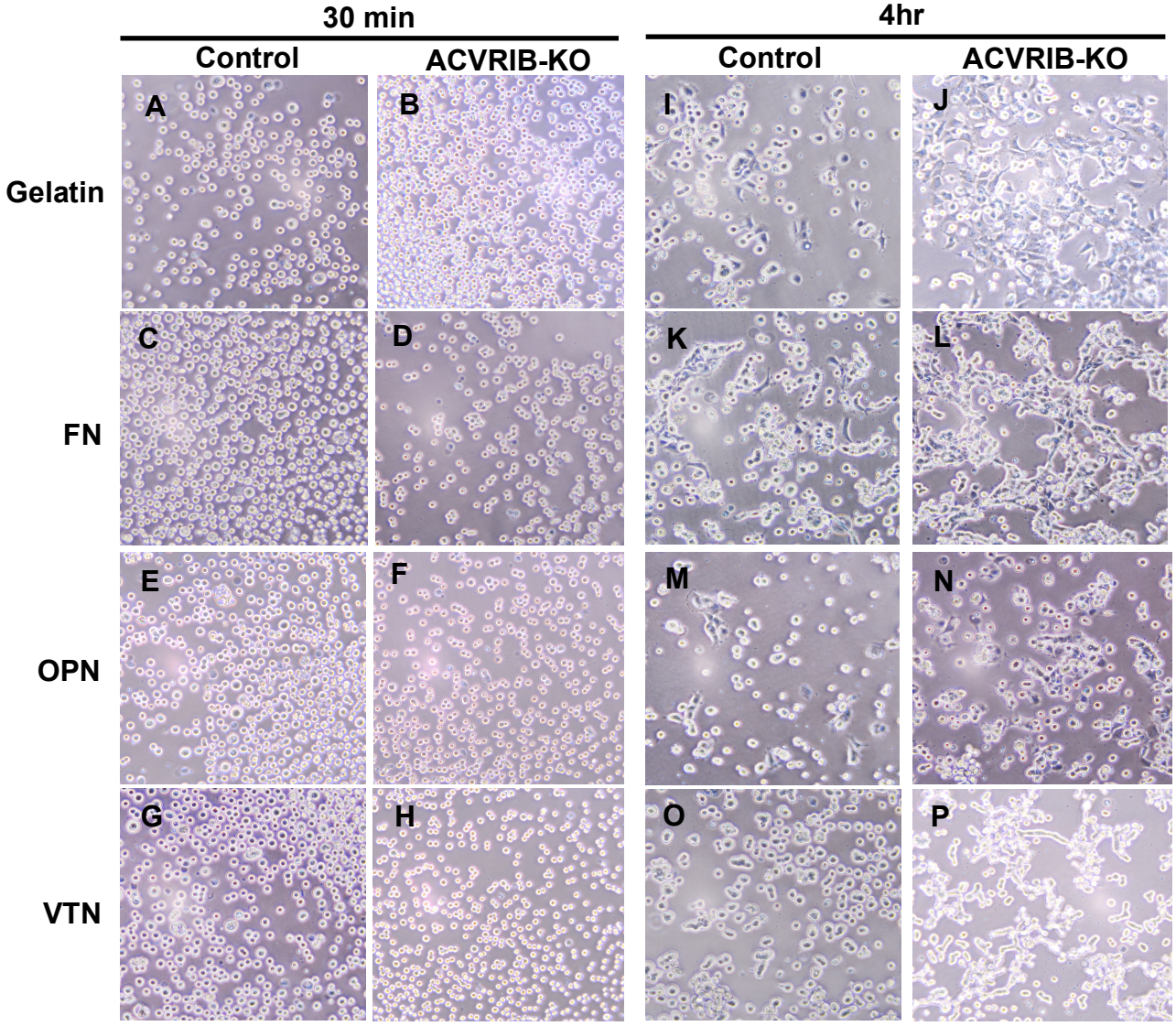


Figure 5-5. OSC-19 with deletion of ACVRIB exhibit morphological alterations in the actin cytoskeleton which are associated with migration. (A) Control OSC-19 cells have a characteristic squamous cell morphology, showing a cobblestone structure. (B) Higher magnification of boxed area in (A). (C) Following deletion of ACVRIB, OSC-19 cells form small clusters and develop protrusions around the outer edge of the cells. (D) Higher magnifcation of boxed area in (C). (E) Phalloidin stain of control cells. (F) Higher magnification of (E). (G) Phalloidin stain of ACVRIB-KO. (H) Higher magnification of (G).

Figure 5-6. Deletion of ACVRIB alters cell adhesion to extracellular matrix proteins. 24-well plates were coated with various extracellular matrix substrates to determine the adhesive capabilities of control and ACVRIB-KO cells. Plates were coated with gelatin (30 minutes: A-B, 4 hours: C-D), fibronectin (FN; 30 minutes: E-F, 4: hours G-H), osteopontin (OPN; 30 minutes: I-J, 4 hours: K-L), or vitronectin (VTN: 30 minutes: M-N, 4 hours: O-P).



Loss of ACVRIB enhances cellular invasion and adhesion to extracellular matrix substrates

As discussed above, we found that OSC-19 cells with deleted ACVRIB exhibit augmented migration and invasion in Boyden chamber, as well as increased actin projections along the cell membrane. Therefore, we next decided to examine the ability of these cells to interact with ECM substrates, as the constituents of the tumor microenvironment ECM heavily impact

cancer cell motility (369,417). Additionally, research has shown interactions between TGFβ superfamily signaling and cell-matrix (418,419). We focused on several ECM substrates (collagen, fibronectin [FN], osteopontin [OPN], vitronectin [VTN]), which are bound by different cell surface proteins, such as integrins. ACVRIB-KO and control cells exhibited similar adhesive abilities during the first 30 minutes (Figure 5-6A-H). However, interestingly, we found that over a four-hour incubation, ACVRIB-KO cells showed an increased ability to bind to gelatin (collagen), FN, and OPN, but not VTN (Figure 5-6I-P). This suggests an upregulation of expression and activity of integrins that bind to these substrates, which may include α5, αv, β1, β3, or β4 integrins (420,421).

To further investigate these phenomena, we next decided to investigate the ability of ACVRIB-KO cells to invade in a three-dimensional structure, using organotypic reconstruct cultures. An examination of the cultures by hematoxylin and eosin (H&E) staining shows not only increased overall invasion into the underlying stroma, but a combination of collective and single cell invasion (Figure 5-7A,B, insets). This initial assessment of the three-dimensional cultures confirmed our observations in Boyden chamber assays (Figure 5-3C,D; Figure 5-4C,D).

We next decided to use immunofluorescence to assess potential dysregulation of cell-cell and cell-matrix adhesion proteins. Laboratory and clinical evidence has suggested alterations in integrin expression, a main group of cell surface proteins responsible for adherence to the ECM, occurs in SCC. de Moraes et al. found reduced expression of β1, a marker commonly used as a stem cell marker, is correlated with lymph node metastasis in oral SCC, however a separate study found increased expression of β1 in oral SCC compared to

Figure 5-7. Loss of ACVRIB enhances cell invasion in three-dimensional organotypic culture. Hematoxylin and eosin (H&E) staining of (A) control and (B) ACVRIB-KO cultures. Additional immunofluorescence staining for integrins (C-D) α2, (E-F) α5, (G-H) αv, (I-J) β1, (K-L) β3, and (M-N) β4.

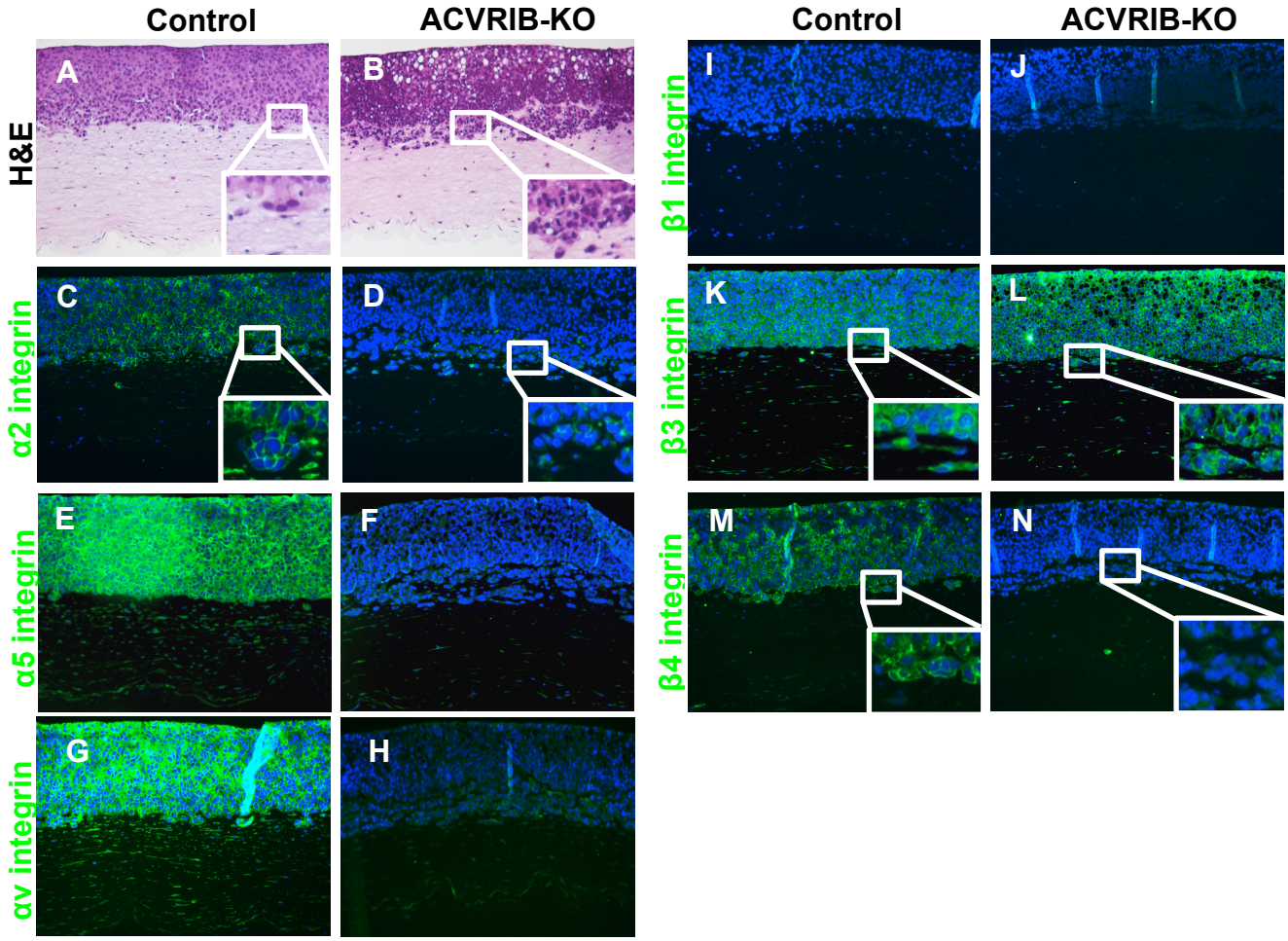
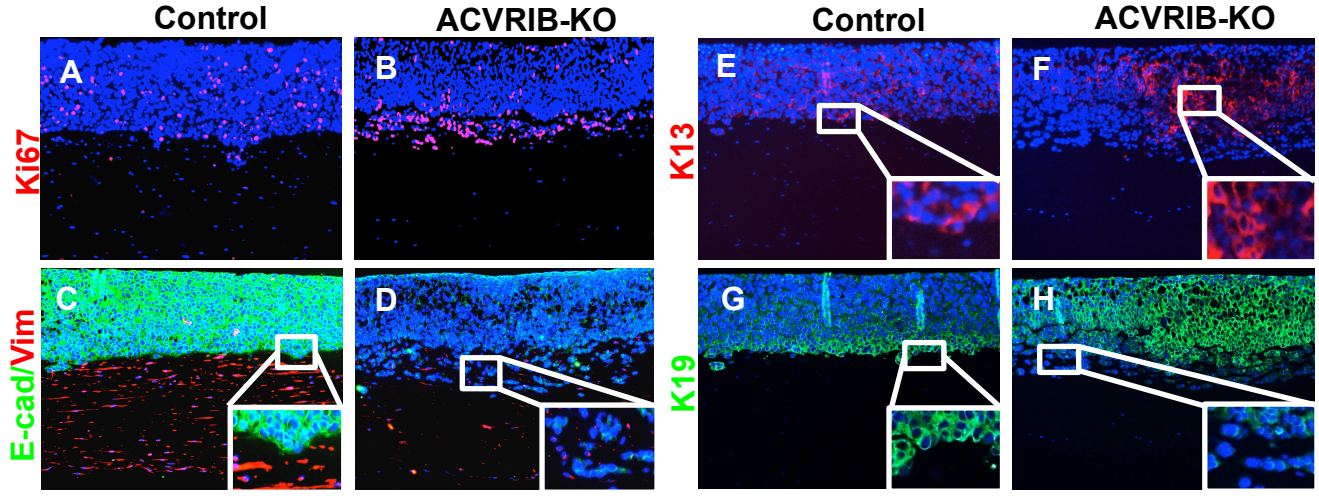


Figure 5-8. Loss of ACVRIB impacts squamous cell proliferation and differentiation status. ACVRIB-KO cells express a different keratin signature compared to controls. (A-B) Ki67, (C-D) E-cadherin/E-cad and vimentin/Vim, (E-F) cytokeratin 13/K13, (G-H) cytokeratin 19/K19.



expression in neoplastic oral mucosa (422,423). Additional integrin pairings, such as αvβ3 and αvβ5, are upregulated and thought to help confer therapeutic resistance in oral SCC (424). Therefore, we decided to examine a panel of integrins suggested to be involved in the progression of SCC. As discussed above, we focused on the expression of integrins implicated in the progression of ESCC and HNSCC. By immunofluorescence staining, we found dysregulation (i.e. loss of expression) of several integrins, including α2, α5, αv, and β4 (Figure 5-7C-H,M,N, insets). β1 integrin was expressed at low to undetectable levels in OSC-19 control and ACVRIB-KO cells (Figure 5-7I,J). However, though there was no change in expression, localization of β3 integrin differed between control and ACVRIB-KO cells, moving from cytoplasmic to membranous expression (Figure 5-7K,L, insets). Intriguingly, those integrins with membranous localization in the control cells (α2, α5, αv, and β4) were downregulated in ACVRIB-KO cells. This result suggests that ACVRIB may regulate cytoskeleton stability, and thus expression of integrins on the cell surface.

Differentiation of OSC-19 cells correlates with invasion phenotype

Integrin expression and epithelial cell differentiation are closely tied processes (425,426). Therefore, as we observed drastic alterations in OSC-19 integrin expression with loss of ACVRIB, we next decided to investigate if the differentiation status of these cells was modified as well. Downregulation of E-cadherin expression and cytokeratins have been associated with various differentiation statuses of squamous epithelial cells. For example, downregulated E-cadherin expression has been noted in several HNSCC studies and was associated with poor prognosis (427,428). This observation has been validated in several other cancers, including ESCC (292). To further examine the differentiation status of these squamous cells, we decided

to also focus on cytokeratin 13 (K13), a marker of differentiated squamous epithelial cells, and cytokeratin 19 (K19), a marker of dedifferentiated squamous epithelial cells. K13 downregulation and K19 upregulation by both RNA and protein has been noted in esophageal and oral SCC (429-432). Prior to exploring E-cadherin and cytokeratin expression, we stained for Ki67 by immunofluorescence. Compared to the control cells, ACVRIB-KO cells had a concentrated number of proliferative cells along the invasive edge of the epithelial layer (Figure 5-8A,B). To see if these proliferative cells corresponded to the basal layer of the squamous epithelium, we stained for E-cadherin, K13 and K19. ACVRIB-KO cells showed a substantial downregulation, however not complete absence, of E-cadherin (Figure 5-8C-D, insets). Within the ACVRIB-KO invasive cells, E-cadherin expression was retained between the cell-cell junctions, but not around the parts of the cell membrane in contact with the ECM. Additionally, the control cells had similar levels of K13 expression throughout the epithelial layer, however K13 was absent in the basal layer of the ACVRIB-KO squamous and upregulated in the middle of the squamous layer (Figure 5-8E,F, insets). K19 expression was concentrated along the basal layer of the control cells, but was located in the medial and upper portion of the ACVRIB-KO squamous epithelium (Figure 5-8G-H, insets). Additional cytokeratins (K14, K15, K18) were examined as well with no discernable differences between conditions (data not shown).

In the absence of ACVRIB, Activin A can induce invasion through alternative signaling

As we had now generated an understanding of the functional changes that occur in the absence of ACVIRB, such as increased migration, invasion, and proliferation, we next sought to investigate which Activin type I receptor could be facilitating this effect. Though ACVRIB is the primary receptor facilitating Activin A signaling, recent evidence has suggested that Activin

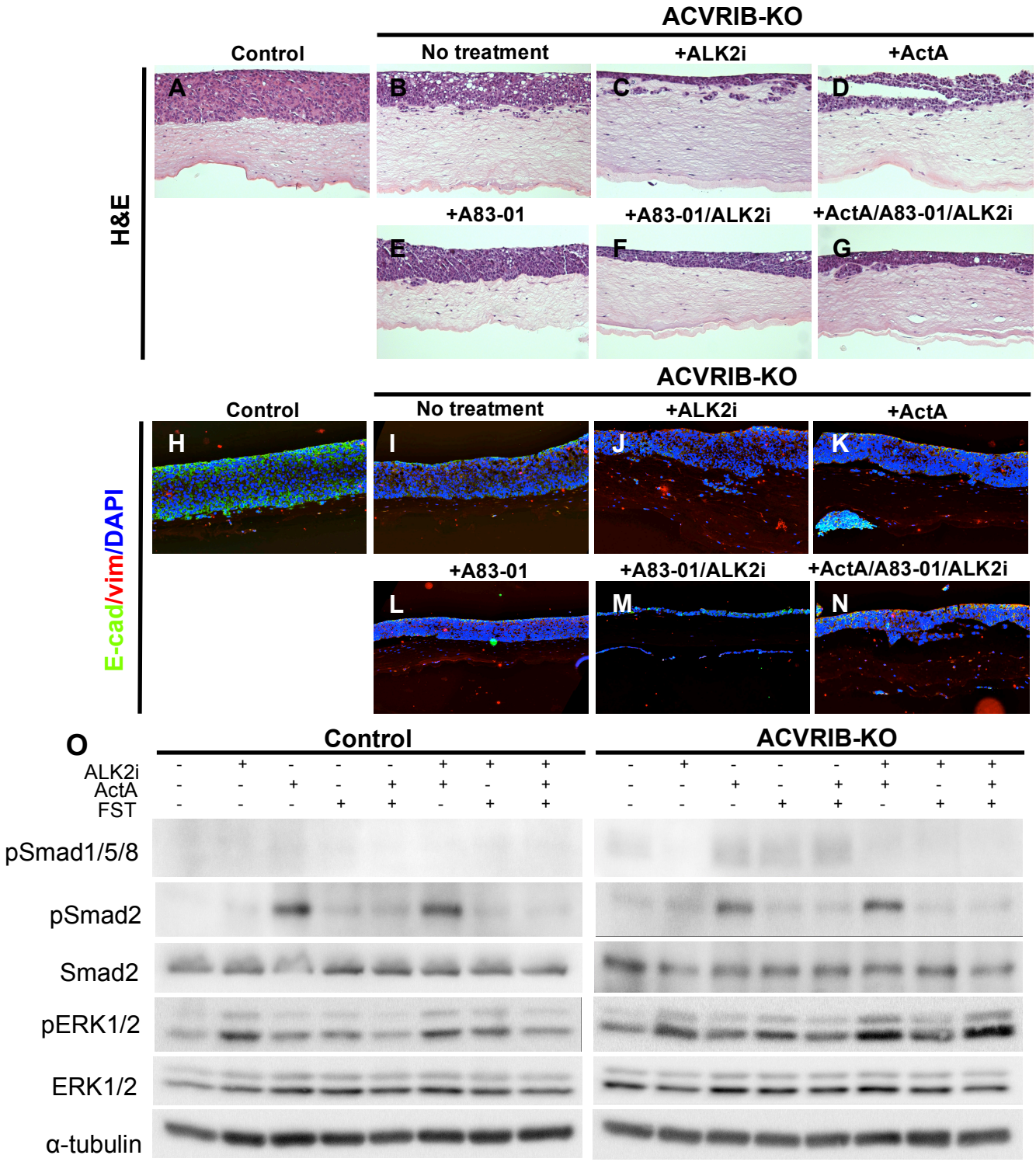


Figure 5-9. Inhibition of Activin type I receptors, by chemical inhibition, alters the invasive capabilities of OSC-19 ACVRIB-KO through the regulation of Smad1/5/8 and ERK signaling. Control OSC-19 cells in organotypic culture (OTC) with no treatment (A,H) was used to compare ACVRIB-KO treatments. Hematoxylin and eosin (H&E) staining of control (A) and ACVRIB-KO (B-G) OTCs, with and without treatments. Treatment conditions were as follows: Control (A), ACVRIB-KO no treatment (No tx) (B), the ALK2/Activin receptor type IA inhibitor DMH1 (ALK2i) (C), recombinant Activin A (ActA) (D), the ALK4/5/7 inhibitor A83-01 (E), A83-01 and ALK2i (F), and combination ActA, A83-01, and ALK2i (G). The same conditions were stained for E-cadherin, vimentin, and nuclear DAPI (H-N). Short-term (30 minute treatment) downstream Smad (pSmad2 and pSmad1/5/8) and ERK signaling following treatment with ALK2i, ActA, follistatin (FST), or combinations was examined by Western blot (O).

A has the ability to propagate signals via other Activin type I receptors with low affinity, such as the BMP receptor ALK2 (ACVRI/ACVRIA). ALK2 and ACVRIB signal through distinct downstream pathways; ALK2 preferentially phosphorylates Smad1/5/8 (Smad1/5/9), while ACVRIB primarily phosphorylates Smad2 (217). However, recent evidence has suggested crossover between Activin A-induced signaling through ACVRIB and ALK2 (433,434). Therefore, we decided to explore Activin A could signal through ALK2 as an alternate receptor in the absence of ACVRIB.

Using a three-dimensional organotypic culture as our model in this setting, we elected to treat the cultures with recombinant Activin A, the ALK2 inhibitor DMH1 (ALK2i), and/or the ALK4/5/7 chemical inhibitor A83-01, and examined E-cadherin and vimentin expression by immunofluorescence (192,365). Similar to that observed previously, control OSC-19 cells expressed substantial levels of E-cadherin, while ACVRIB-KO cells showed increased epithelial cell invasion, minimal E-cadherin staining, and upregulation of epithelial cell vimentin staining, suggesting possible EMT (Figure 5-7C,D; Figure 5-9A,B). Interestingly, none of the treatments or combinations were able to restore E-cadherin expression, suggesting potential intrinsic alterations that occurred within the OSC-19 cells following ACVRIB knockout. However, we did note invasion in the cultures treated with ALK2i, ActA, or ActA/A83-01/ALK2i (Figure 5-9C-D, G). Particularly, the cultures treated with only ALK2i or all three treatments (ActA/A83-01/ALK2i) showed larger invasive clusters compared to the ActA only treatment. With solely the addition of A83-01, not only was there limited epithelial cell invasion into the underlying stroma, but also the stromal layer itself appears thinner (Figure 5-9E). A similar observation occurred within the cultures treated with A83-01/ALK2i, with the additional note that the OSC-19 epithelial layer was also thinner than that of the other conditions, potentially

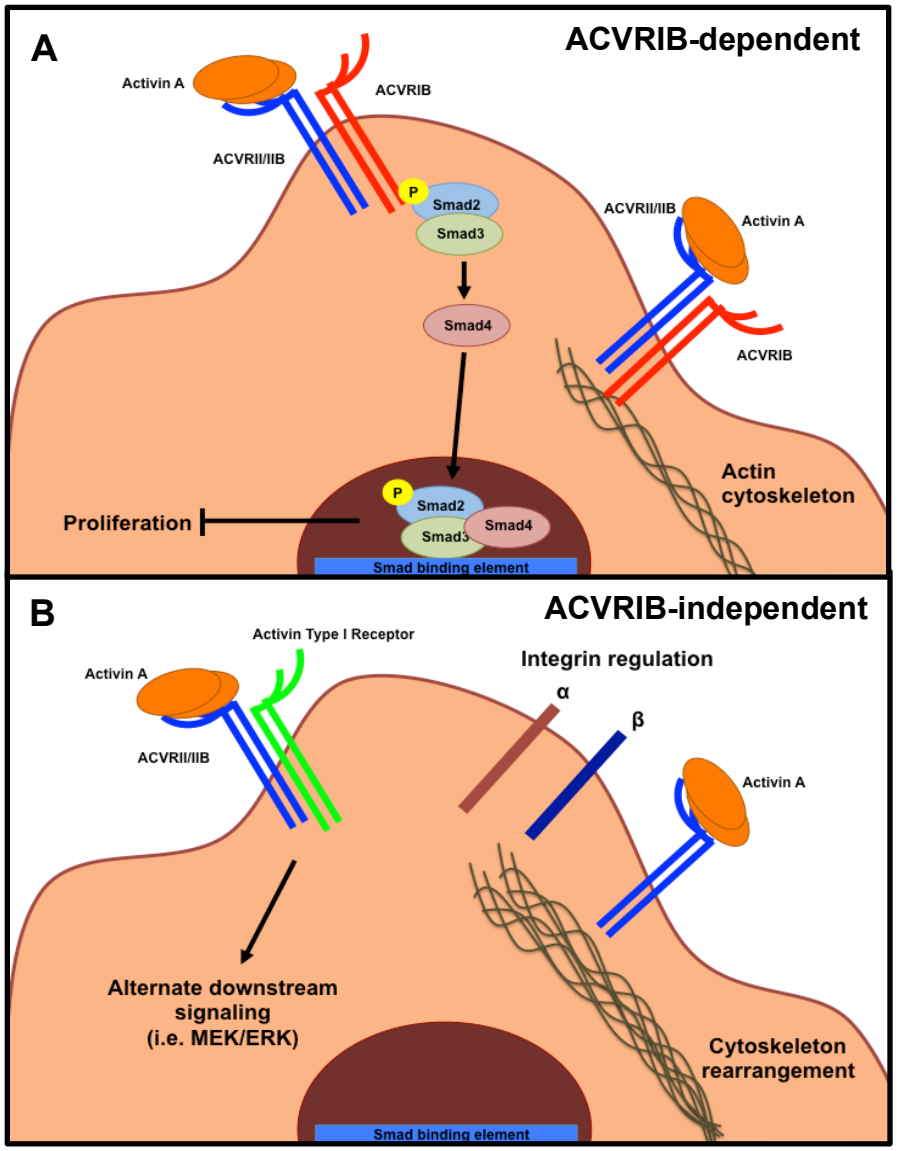
suggesting an effect of TGFβ pathway inhibition in combination with ACVRIB inhibition, as A83-01 can additionally target ALK5 and ALK4 (Figure 5-9F). These results suggest that a balance of the TGFβ/Activin/BMP signaling pathways is required to maintain squamous epithelium integrity and control cell invasion. Additionally, the loss of ACVRIB may upend this balance and, therefore, reroute Activin A to induce other arms of pathways or potentially signal through receptors outside of this family of receptors (Figure 5-9G).

In addition to exploring the functional effects of Activin A signaling independently of ACVRIB, we decided to examine alterations in downstream signaling. As mentioned above, ACVRIB primarily phosphorylates Smad2 to induce downstream gene transcription (7,435). However, we speculated that, in the absence of ACVRIB, perhaps Activin A signaling could induce other signaling molecules, which would then shine light on the functional alterations (i.e. migration, invasion, proliferation) that we had previously observed. To explore this, we treated control or ACVRIB-KO cells *in vitro* with ALK2i, recombinant Activin A (ActA), or the Activin A antagonist follistatin (FST) to block Activin A prior to receptor binding. Control cells had phosphorylated Smad2 (pSmad2) following treatment with ActA and ALK2i, however phosphorylation of Smad2 was blocked when ActA and FST were added together, showing the efficacy of FST as an Activin A inhibitor (Figure 5-9H, left panel). Phosphorylated Smad1/5/8 was unchanged between control conditions. In the ACVRIB-KO cells, ActA treatment induced pSmad2, except when treated in combination with FST (Figure 5-9H, right panel). Interestingly, ACVRIB-KO cells had increased baseline pSmad1/5/8 compared to control cells, indicating active BMP signaling. Upon treatment of these cells with ALK2i, pSmad1/5/8 expression was abolished, suggesting successful inhibition of BMP signaling. Therefore, we conclude from these experiments that Activin A, in the absence of ACVRIB, retains activity through the

phosphorylation of Smad2 and that phosphorylation of Smad1/5/8 occurs in an Activin A-dependent manner via ALK2.

Finally, we examined alterations in MAPK/ERK signaling, as Activin A and ERK signaling are suggested to work together to enhance native pluripotency and promote proliferation (436). Phosphorylation of ERK1/2 (pERK1/2) was unchanged across conditions in the control cells (Figure 5-9H, left panel). ACVRIB-KO cells, however, had considerable alterations across conditions. Though all treatment conditions induced pERK1/2 to some extent, compared to ACVRIB-KO no treatment, treatment with ALK2i/ActA or ALK2i/ActA/FST showed the highest level of phosphorylation (Figure 5-9H, right panel). These results may indicate an interaction between non-canonical Activin A signaling via Smad2, Smad1/5/8 and the MAPK/ERK pathway.

Figure 5-10. Model of ACVRIB-dependent and -independent Activin A signaling. A schematic depicting the ACVRIB-dependent (A) and –independent (B) signaling and its functional consequences.



Discussion

In the current study, we characterized the functional consequences of loss of ACVRIB, and thus canonical Activin A signaling, in HNSCC and ESCC. The Activin A signaling cascade has been long studied for its critical role in development across species (437,438), however its actions in cancer have been widely disputed as the functions of Activin A appear to be almost entirely cell-type and context dependent (reviewed in (340)). To better explore the responsibilities of this pathway in HNSCC and ESCC, we decided to direct our focus to ACVRIB, the primarily receptor for transducing Activin A-induced signals, as we had previous described the inverse correlation between Activin A/ *INHBA* and ACVRIB expression in advanced ESCC (133). By working with a model that eliminated Activin A signaling via knockout of ACVRIB, we were able to discern the functional effects of loss of canonical Activin A signaling. Interestingly, we found that loss of canonical Activin A signaling mediated by ACVRIB results in increased proliferation, migration, and invasion. These effects appear to occur through a combination of pSmad2, pSmad1/5/8, and MAPK/ERK downstream signaling. Ultimately, dysregulation of ACVRIB expression and canonical Activin A signaling led to reorganization of the actin cytoskeleton and, thus, expression of cell surface proteins involved in cell-cell and cell-ECM interaction. We have, therefore, defined a novel role for ACVRIB-dependent Activin A signaling in SCC (Figure 5-10).

The suggestion that type II and type I Activin receptors are involved in the maintenance of epithelial homeostasis is not a unique idea. Our group has previously described that coordinated loss of TβRII and E-cadherin can promote invasion of esophageal keratinocytes in three-dimensional organotypic culture (292). This invasive phenotype could, however, be controlled through the induction of the Activin A pathway, thus restoring homeostatic balance

(133). Additional research has suggested that dominant negative ActRII, achieved through truncation of the protein, can block Activin A signaling in pancreatic cells, providing evidence for the role of this signaling pathway in endocrine and exocrine cell differentiation (439). Among the Activin type I receptors, little is noted regarding their downregulation or absence in cancer (reviewed in (389)). ACVRIB (ALK4) is amongst the most highly studied in terms of Activin type I receptors in cancer. Overall, loss of ACVRIB leads to increased tumorigenesis, particularly of the primary tumor (84,412,440). The role of ACVRIB in metastasis, however, remains to be truly clarified. Research has suggested that though loss of ACVRIB may be beneficial for the primary tumor, retention of cells with expression of ACVRIB could be required for metastasis (440). Collectively, this evidence insinuates the necessity of tumor heterogeneity, as originally described by Gloria Heppner several decades ago (441). In the case of ACVRIB, it appears that downregulation of ACVRIB may be beneficial for the primary tumor, however re-expression/plasticity or retention of the protein in some clones may be necessary for progression. In HNSCC and ESCC, it remains unknown where loss of ACVRIB occurs, whether it is at the genomic, RNA, or protein levels. The ESCC cell line TE-11 lacks ACVRIB protein, however continues to express *ACVRIB* mRNA ((133) and data not shown). Therefore, further examination of the mechanism of ACVRIB regulation is needed, particularly in HNSCC and ESCC, as majority of the evidence regarding ACVRIB loss resides in other cancers.

Targeting ACVRIB *in vitro*, however, remains a challenge. Due to the sequence and structural similarities between the ALKs, developing specific inhibitors for the individual family members is difficult (reviewed in (389)). In the current study, we treated SCC cells *in vitro* using the chemical inhibitor A83-01, which targets ALK4/5/7. Because of the multifaceted

effects of this inhibitor, we cannot say for certain that the phenotype we observed in three-dimensional organotypic was solely the responsibility of ACVRIB inhibition (Figure 5-9). TGFβ signaling, via ALK5, has an established role in cancer and the tumor microenvironment. Therefore, inhibiting ALK5 in our organotypic culture model may affect the makeup of the stroma (442-444). Though not much is known about the functional effects of ALK7, particularly in ESCC or HNSCC, we also cannot rule out the inhibition of ALK7 in this context as well. Unfortunately, at this time no inhibitors specifically target ALK4, as other available inhibitors also target other ALKs (389). Therefore, using the CRISPR/Cas9 system to knockout ACVRIB, as demonstrated here, is the closest that we have come to selectively targeting ACVRIB, though this is currently not feasible in the clinical context.

A main finding of this study was the regulation of the actin cytoskeleton and cell surface proteins. In respect to ACVRIB, little to no evidence has been published exploring such functional effects. Some investigation, however, has described the actions of Activin A during some of these processes, though not in cancer. Riedy and colleagues described that stimulation of rat aortic smooth muscle cells with Activin A leads to migration and the formation of actin stress fibers and focal adhesions, particularly through the phosphorylation of paxillin and $p130^{cas}$ (445). A similar effect was investigated in adipose stromal cells, where stimulation with Activin A could promote differentiation along the smooth muscle cell lineage which exhibit characteristic cytoskeletal actin fibers through the induction of α-smooth muscle actin (αSMA) and calponin. Treatment with an ALK4/5/7 inhibitor could block such effects (446). An additional study investigated a similar phenomenon using NMuNG epithelial breast cells, however F-actin cytoskeleton rearrangements following stimulation with Activin A were not observed; this was only seen following treatment with TGFβ (447). In this context, Piek and

colleagues suggested that the lack of effect may be due to limited availability of Activin A signaling receptors on these cells, reinforcing the theme of cell-type and context dependent Activin A signaling (447).

Though we have unearthed a new role for Activin A-ACVRIB signaling in HNSCC and ESCC, there remain many questions. We have just begun to scratch the surface of non-canonical Activin A signaling in the absence of ACVRIB, potentially through an alternate Activin type I receptor. Therefore, further delineation and investigation into the mechanism of Activin A in this context is necessary to contribute to our deeper understanding of the pathway as well as potential ways to use this information for targeted therapeutics. If it is indeed the case that Activin A, working through an alternate pathway in the absence of ACVRIB, exerts oncogenic effects, the utilization of an Activin A ligand trap, such as follistatin, may aide in improving patient outcome in HNSCC and/or ESCC. In addition, attaining a better understanding of the role of the Activin A-ACVRIB interaction in a more complex physiological context, such as ACVRIB-KO cells in the presence of endothelial cells, immune cells, fibroblasts, neurons, etc., may provide a more complete understanding to how Activin A operates without epithelial expression of ACVRIB. Though Activin A either does not signal or induce signal transduction via an alternate pathway in ACVRIB-absent epithelial cells, this alteration does not impede Activin A signaling in other cells in the surrounding microenvironment. In fact, this may free up Activin A to bind and induce signaling in other cell types. Therefore, investigation into the microenvironmental consequences of ACVRIB-KO may provide a larger, systemic picture of the impact of this signaling pathway.

Conclusion

Despite the remaining questions surrounding the relationship between Activin A and ACVRIB, in this study we provided evidence showing the necessity of ACVRIB-dependent Activin A signaling in the regulation of HNSCC and ESCC. When this system becomes disrupted through downregulation of ACVRIB, HNSCC and ESCC become more aggressive. Clinically, this would lead to an ultimately worse overall prognosis for patients afflicted with this deletion. Therefore, further investigation into the consequences and potential therapeutic options revolving around Activin A-ACVRIB may promote better clinical outcomes in HNSCC and ESCC.

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CHAPTER VI

CONCLUSIONS AND FUTURE DIRECTIONS

The work presented here provides valuable insight into the role of the Activin A signaling pathway, particularly that which takes place via the Activin A-ACVRIB receptor complex, in the progression of esophageal (ESCC) and head and neck (HNSCC) squamous cell carcinoma. In Chapters 3-4, we examined the downstream effects of the Activin A ligand in regulating and maintaining homeostasis in the esophageal microenvironment. Towards the end of Chapter 4, we unearthed that these effects are mediated through the preservation of the Activin A-ACVRIB signaling complex. When this signaling complex is disrupted, such as through the downregulation of ACVRIB, the regulatory ability of Activin A signaling is lost. We examined this further in Chapter 5, where we genetically deleted ACVRIB in HNSCC cells using a CRISPR/Cas9 system and downregulated *ACVRIB* using siRNA in ESCC. In these contexts, we found that loss or downregulation of ACVRIB led to increased SCC aggressiveness, demonstrated by increased cell migration and invasion, as well as an alteration of the adhesion protein profile of the cells. Overall, from this work, we have concluded that in cancer alterations of the Activin A signaling pathway, likely through the downregulation of receptor complex components such as ACVRIB, circumvents the cells' ability to respond to the characteristic growth inhibitory signals of Activin A.

Though the work presented here identifies a novel form of cellular resistance in ESCC and HNSCC, there are several questions that remain to be answered. The Activin A field, however not new, paints a complicated picture. First, in the context of Activin A

overexpression, we observed alterations in the behavior of both the epithelial cell and the stromal compartment, i.e. fibroblasts. The primary goal of our work regarding Activin A overexpression was focused on the inhibitory effects on the epithelial cells; however, it is well documented that Activin A not only acts in a paracrine manner, but also in an autocrine fashion. Therefore, when overexpression of Activin A is observed in the stroma, what effect does it have on the fibroblasts? This is a question that can be answered fairly easily in our three-dimensional organotypic reconstruct culture system. Nevertheless, to investigate this question in a more physiologically relevant context, we would need to incorporate other stromal components, such as immune cells (T-cells, B-cells, macrophages, NK cells, etc.), endothelial cells, or smooth muscle cells. Such a question may be answered using a more complicated three-dimensional organotypic model or through the utilization of an *in vivo* model. Second, at what part during the oncogenic cascade (i.e. initiation, progression, metastasis) does receptor downregulation occur? In Chapter 4, we found that Activin A-mediated effects persist in normal tissue and dysplasia. What occurs in the switch between esophageal dysplasia to squamous cell carcinoma to induce receptor downregulation? Parsing this phenomenon apart may shed light on potential ways to keeping this signaling pathway intact, and thus determine a new therapeutic approach.

In Chapter 5, we more thoroughly investigated the effects of loss of Activin A signaling via the loss of ACVRIB. In the absence of ACVRIB, we observed alterations of both the modified epithelial cells and the stromal microenvironment. Our observations suggest that the downregulation of ACVRIB not only alters the makeup of the actin cytoskeletion, but also appears to regulate motility via cell adhesion proteins, such as E-cadherin and integrins. Though the focus of this chapter was on the characterization of loss of ACVRIB in ESCC and

HNSCC, many questions have emerged regarding the molecular mechanisms that facilitated these modifications. In the absence of canonical Activin A signaling through ACVRIB, does Activin A bind and signal through a different type II-type I receptor complex? Though in our hands ACVRIB-KO cells did not secrete significantly more Activin A than control cells (data not shown), even low levels of Activin A will ultimately find a location to bind. One report has suggested that alternative binding is possible, such as through the BMP type I receptor ALK2. Future research should be dedicated to investigating these additional complexes and the downstream signaling pathways that are initiated in response. Moreover, does ACVRIB-induced instability of the actin cytoskeleton influence the integrins present on the cell surface? And did those integrins that were, and now no longer, present on the cell surface undergo degradation? What we have uncovered in regards to the relationship between ACVRIB and integrin expression in a novel finding in the context of cancer and requires additional investigation.

The exploration into the Activin A pathway in cancer has been long and convoluted. Activin A exerts an array of effects, both tumor suppressing or promoting, depending on the context. However, the work that we have presented here perhaps provides some insight into the role of this signaling pathway in the context of esophageal squamous dysplasia, ESCC, and HNSCC. And though we have provided some clarity into the actions of Activin A and ACVRIB, this area of research is still in its infancy with many new avenues to explore.

APPENDIX A

ACTIVIN A SIGNALING REGULATES CELL INVASION AND PROLIFERATION IN ESOPHAGEAL ADENOCARCINOMA

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Preface

Though the bulk of the work presented here has investigated the role of Activin A in esophageal squamous cell carcinoma, we have extended our research to examine the similarities and differences, compared to our previous observations, of Activin A function in esophageal adenocarcinoma. As esophageal adenocarcinoma develops along a spectrum, beginning with Barrett's esophagus and ending at adenocarcinoma, similarly to the development of esophageal squamous cell carcinoma, we utilized a comparable model of Activin A overexpression for this investigation, including overexpression of Activin A and three-dimensional organotypic reconstruct cultures. However, in this context, overexpression of Activin A was derived from the epithelial cells, rather than the stromal compartment. The results of this project indicate that Activin A acts in a cell-type and context-specific manner in esophageal adenocarcinoma; however, in cell types with a mesenchymal phenotype, Activin A

acts as a tumor promoter. This suggests an additional similarity of Activin A to the TGFβ pathway, where the tumor suppressive properties of Activin A undergo a ‘switch’.

Abstract

TGFβ signaling has been implicated in the metaplasia from squamous epithelia to Barrett's esophagus and, ultimately, esophageal adenocarcinoma. The role of the family member Activin A in Barrett's tumorigenesis is less well established. As tumorigenesis is influenced by factors in the tumor microenvironment, such as fibroblasts and the extracellular matrix, we aimed to determine if epithelial cell-derived Activin A affects initiation and progression differently than Activin A stimulation from a mimicked stromal source. Using the Barrett's esophagus cell line CPB and the esophageal adenocarcinoma cell lines OE33 and FLO-1, we showed that Activin A reduces colony formation only in CPB cells. Epithelial cell overexpression of Activin A increased cell migration and invasion in Boyden chamber assays in CPB and FLO-1 cells, which exhibited mesenchymal features such as the expression of the CD44 standard form, vimentin, and MT1-MMP. When grown in organotypic reconstructs, OE33 cells expressed E-cadherin and Keratin 8. As mesenchymal characteristics have been associated with the acquisition of stem cell-like features, we analyzed the expression and localization of SOX9, showing nuclear localization of SOX9 in esophageal CPB and FLO-1 cells.

In conclusion, we show a role for autocrine Activin A signaling in the regulation of colony formation, cell migration and invasion in Barrett's tumorigenesis.

Introduction

Esophageal adenocarcinoma (EAC) is often thought to arise from a clonal stem-like population of cells, which is potentially responsible for its poor prognosis. Transforming growth factor β (TGFβ) and Notch signaling pathways play important roles in regulating self-renewal of stem cells and cell-fate determination. Both pathways are frequently implicated in Barrett's tumorigenesis (448). It has been shown that loss of members of the TGFβ signaling cascade, such as Smad4 and β2 spectrin, can contribute to the initiation of Barrett's esophagus and the progression to esophageal adenocarcinoma through concomitant upregulation of Notch targets Hes1 and Jagged1 (449). Similarly, analysis of a panel of esophageal adenocarcinoma cell lines demonstrated failed cell cycle arrest after TGFβ stimulation, as they did not respond with the expected downregulation of c-Myc or the induction p21 (450). The disruption of TGFβ/Smad-dependent signaling during the progression of esophageal adenocarcinoma was confirmed by a report that showed Smad4 mRNA expression was progressively reduced in the metaplasia-dysplasia-adenocarcinoma sequence by promoter methylation (451). In the same study, the authors demonstrated the loss of TGFβ-dependent induction of p21 and downregulation of mini-chromosome maintenance protein 2 (MDM2) in the majority of Barrett's tumorigenesis (451). Interestingly, in a series of resected adenocarcinomas of the distal esophagus, *TGFB1* mRNA was expressed at significantly higher levels in tumor tissues compared to squamous epithelium and Barrett's mucosa. Additionally, univariant survival analysis has shown that *TGFB1* overexpression was associated with poor prognosis (452).

It is generally assumed that in esophageal metaplasia, the normal squamous esophageal epithelium undergoes transdifferentiation to resemble the columnar epithelium of the gastric tract and the intestine. Bone morphogenetic protein (BMP) 4, a member of the

TGFβ family, has been shown to regulate the processes involved in this metaplastic transformation (453,454). The effects of BMP4 are tightly regulated by its natural antagonist, Noggin, which prevents the BMP-regulated development of the columnar epithelium in the esophagus during embryogenesis (455,456). BMPs, as well as another morphogen, Sonic hedgehog, are typically not expressed in the normal adult esophagus (457), BMP4, however, has been shown to be re-expressed in esophagitis and Barrett's esophagus (453,457). Interestingly, Sonic hedgehog can induce BMP4 secretion in stromal cells with myofibroblast morphology in response to acid injury (458).

Hedgehog signaling and epithelial-mesenchymal transition (EMT) have been implied in the morphogenesis of embryonic and adult tissues. When Hedgehog signaling is blocked, esophageal keratinocyte differentiation and squamous esophageal cancer cell invasion and growth are inhibited (459). These findings suggest that the “mesenchymal gene expression” of undifferentiated cells is maintained or strengthened in cancer cells by Hedgehog-mediated signaling (459). The analysis of other markers of EMT in gastroesophageal junction tumors has shown that the E-cadherin repressors Slug (460), Snail, and Twist (461) are associated with the malignant progression of esophageal adenocarcinomas. TGFβ is known to induce EMT through downregulation of E-cadherin and upregulation of mesenchymal markers (462).

A less studied member of the TGFβ family, the ligand Activin A, has been shown to be upregulated in the progression from Barrett's esophagus to dysplasia and, ultimately, esophageal adenocarcinoma (463). When Activin A signaling was inhibited with siRNA targeting the Activin A gene, *INHBA*, or with the Activin A antagonist follistatin, esophageal adenocarcinoma cell lines demonstrated suppressed proliferation (463). In a previous study analyzing Activin A function in esophageal squamous cell carcinoma, we showed that the

effects of Activin A are largely context- and dose-dependent (356). Herein, we describe the role of Activin A in Barrett's esophagus and esophageal adenocarcinoma cells, especially with respect to cell invasion and crosstalk within the microenvironment.

Results

The Inhibin β_A subunit of Activin A is increased in the progression to esophageal adenocarcinoma

TGFβ and Activin A have both been implicated in the pathology of esophageal adenocarcinoma (EAC) (449,450,463). While TGFβ function is context-dependent, little is known about the role of Activin A in Barrett's tumorigenesis. We first queried publicly available

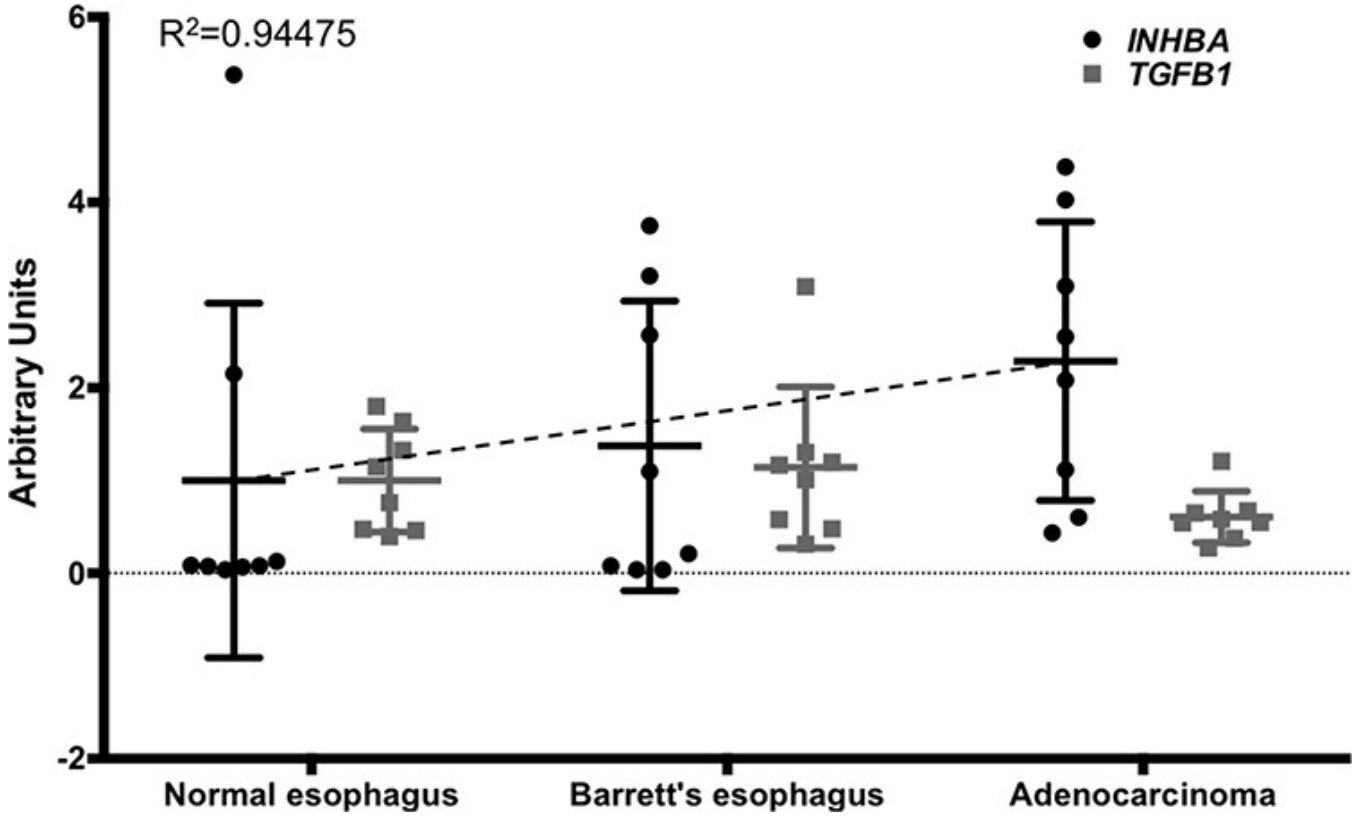


Figure A-1. INHBA expression levels increase during the progression from normal esophagus to Barrett's esophagus and esophageal adenocarcinoma. Comparison of *INHBA* and *TGFB1* expression was based on a publicly available GEO dataset (accession number GDS1321). Values for *INHBA* and *TGFB1* were measured from extracted and purified RNA, shown here as arbitrary units. A trend line for *INHBA* expression (dashed line) was calculated (Y = 0.6436x + 0.2666). P-values for *INHBA* normal vs. BE, p = 0.248; NE vs. EAC, p = 0.932; BE vs. EAC, p = 0.437.

datasets to investigate the expression of Activin A (a homodimer of inhibin β_A subunits), TGFβ, and components of their signaling cascade during the progression from normal esophagus to esophageal adenocarcinoma. Analysis of 24 samples of normal squamous esophagus, Barrett's esophagus, and adenocarcinoma (*n* = 8 per group) for Activin A expression, encoded by the *INHBA* gene, showed a trending increase of expression during the progression to EAC (GDS1321, Figure A-1). Interestingly, although previously shown to be involved in the subsequent metaplastic events, *TGFB1* expression remained unchanged (Figure A-1). Expression of Inhibin A (*INHA*), an Activin A inhibitor formed through inhibin β_A and α subunit heterodimers, and the antagonist follitstatin, were also unchanged (Figure A-2). Analysis of other downstream targets of the signaling pathway showed that Smad2 and

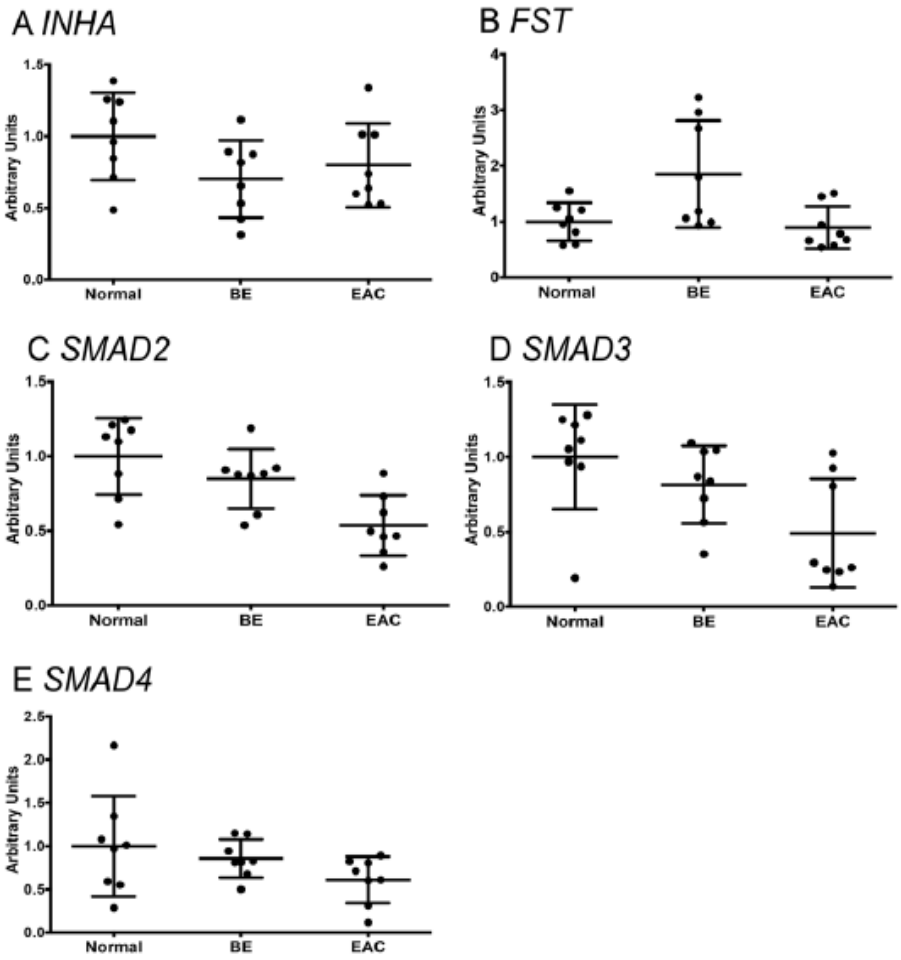


Figure A-2. Analysis of expression levels of components of the Activin A signaling pathways in the progression from normal esophagus to Barrett's esophagus and esophageal adenocarcinoma. Comparison of the expression for *INHA* (the inhibin α subunit that forms heterodimers with inhibin β^A, resulting in the Inhibin A ligand, an Activin A inhibitor), the antagonist *FST* and downstream canonical signaling targets *SMAD4*, *SMAD2* and *SMAD3* based a publicly available GEO dataset (accession number GDS1321). Values were measured from extracted and purified RNA, shown here as arbitrary units. Pearson's correlation coefficients were calculated: *INHA* $y = -0.1002x+1.0347$, $r2=0.43832$; FST $y = -0.0513x +1.3548$, $r2=0.01021$; *SMAD4* $y = -0.195x+1.2125$, $r2=0.97645$; *SMAD2* $y = -0.232x+1.2591$, $r2=0.96066$; *SMAD3* $y = -0.2543x+1.2773$, $r2=0.97617$.

Smad3, as well as the common effector Smad4, were downregulated in the dataset used. While these observations do not exclude TGFβ1 ligand function as an important factor in the biology of EAC, they emphasize the significance of the components of these overlapping pathways and led us to more closely investigate the role of Activin A signaling.

Overexpression of Activin A (*INHBA*) in esophageal model cell lines results in cell type specific alterations of canonical and non-canonical pathways

Gene expression data from human tissue samples rarely allow insight into the cellular source of the RNA. As tumor samples are often comprised of epithelial tumor cells and stroma, the analyzed RNA is derived from both sources. To model epithelial Activin A overexpression, we chose the dysplastic cell line CPB and the EAC cell lines OE33 and FLO-1 and transduced them with an *INHBA* retroviral plasmid (two subunits of Inhibin β_A encoded by the *INHBA gene* result in the Activin A protein). *INHBA* overexpression was validated by ELISA in CPB, OE33 and FLO-1 cells. All three *INHBA*-overexpressing cell lines secreted significantly higher levels of Activin A compared to control (Figure A-3A). Interestingly, when normalized to the number of cells at the end of the collection period (48 hours), Activin A concentration was higher in OE33 *INHBA* cells than CPB *INHBA* cells, while FLO-1 *INHBA* cells secreted the highest levels of Activin A overall. To identify if *INHBA* overexpression affected TGFβ1 secretion levels, we performed ELISA to measure TGFβ1 in the conditioned media. Levels of secreted TGFβ1 significantly increased in the *INHBA*-overexpressing cells compared to control (Figure A-3B). As the function and availability of Activin A can be regulated by secreted factors, such as the antagonists follistatin and Inhibin A, we also measured the concentrations of follistatin (pan-antibody recognizing all three follistatin isoforms FS288, FS300 and FS315)

and Inhibin A in the collected conditioned media by ELISA. The levels of these factors were below the limit of assay sensitivity when compared to the positive controls (data not shown).

To identify which downstream signaling targets were activated in response to *INHBA* overexpression, we collected protein lysates of untreated cells, as well as cells treated with recombinant TGFβ1 as a positive control and FS288. Smad2, a downstream target of Activin A and TGFβ phosphorylated upon signal transduction, was not activated in any of the *INHBA*-overexpressing cell lines, leading us to conclude that continuous exposure

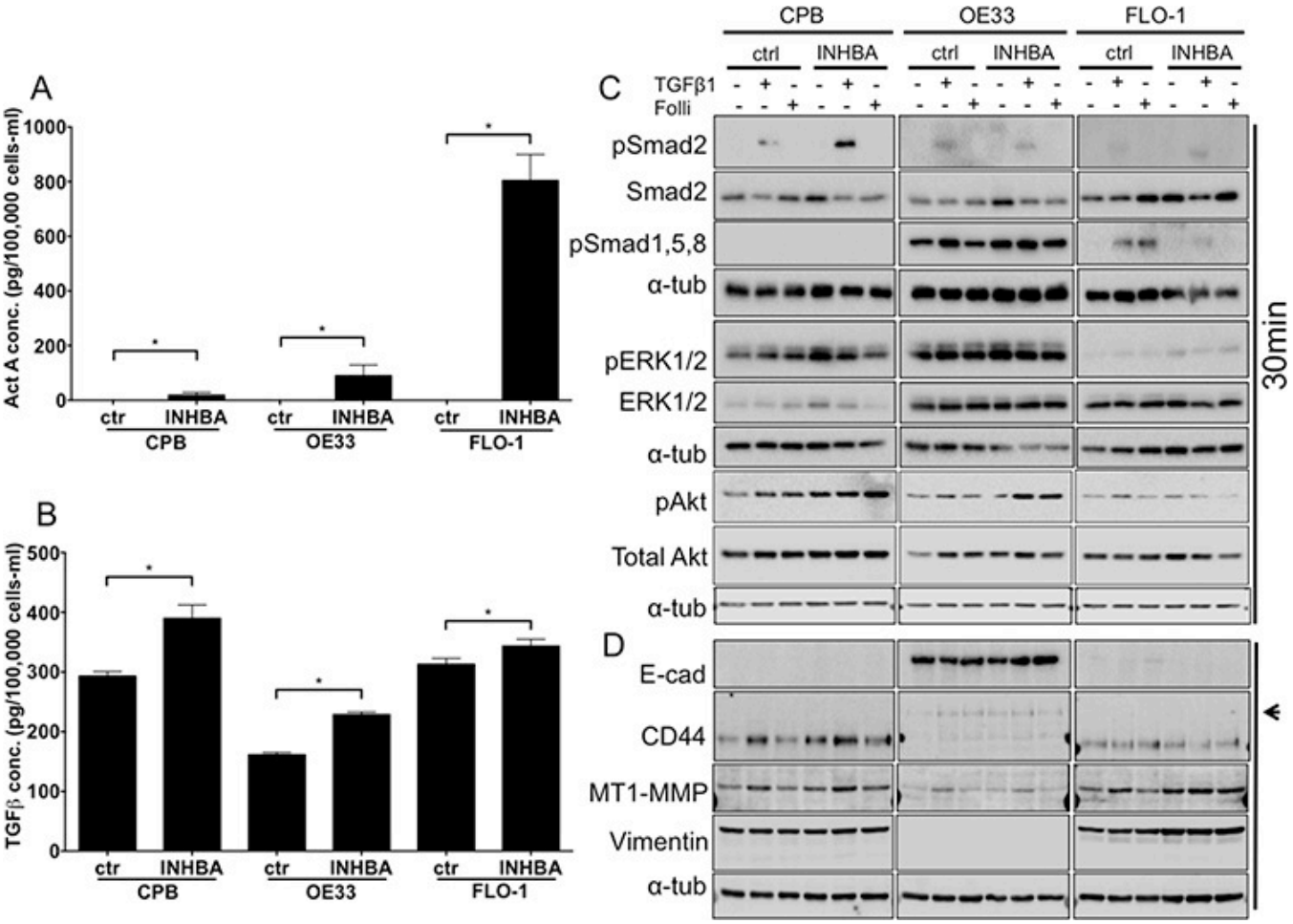


Figure A-3. Overexpression of INHBA in esophageal model cell lines results in cell type specific alterations of canonical and non-canonical pathways. (A) Activin A concentration in conditioned media after overexpression of Activin A (INHBA) compared to empty vector control was assessed by ELISA, and normalized to the cell number at time of collection. Highest overexpression levels were achieved in FLO-1 cells. (B) TGFβ concentration in conditioned media after overexpression of Activin A (INHBA) compared to empty vector control as assessed by ELISA. (C) Protein expression of pSmad2, total Smad2, pSmad1,5,8, pERK1/2 and total ERK1/2, as well as pAkt and Akt was analyzed by Western blot. (D) Antibodies against markers of epithelial-mesenchymal transition showed the expression of mesenchymal markers, such as standard form of CD44, high MT1-MMP and vimentin in CPB and FLO-1. OE33 cells expressed the variant CD44 isoform (arrowhead) and had high E-cadherin (E-cad). * p value < 0.05.

to Activin A desensitizes the cells to signal induction (Figure A-3C). Stimulation with TGFβ1 as a control, however, elicited phosphorylation of Smad2 within 30 minutes. TGFβ1 stimulation resulted in phosphorylation of Smad2 in OE33 and FLO-1 cells. We also analyzed phosphorylation of Smad1/5/8 (Smad1,5,8), which is typically induced by BMP2 and BMP4. Interestingly, OE33 cells, which express more epithelial markers and less mesenchymal markers than CPB and FLO-1 cells (Figure A-3D), had high baseline phosphorylation of Smad1/5/8 (pSmad1,5,8) and pERK1/2. pSmad1/5/8 was increased by TGFβ1 in both control and *INHBA* expressing OE33 cells, and overall *INHBA*-overexpressing OE33 cells showed the strongest signal for pSmad1/5/8 among the three cell lines. In FLO-1 cells, pSmad1/5/8 was suppressed by *INHBA* overexpression, but present in control cells treated with TGFβ1 or FS288. FLO-1 cells showed no activation of the ERK pathway. CPB cells, which showed no pSmad1/5/8 phosphorylation, had a strong signal for pERK1/2. pAkt levels were similar amongst control and *INHBA* overexpressing cells and were not altered with any of the treatments.

Analysis of EMT markers showed a lack of E-cadherin in CPB and FLO-1, which generally exhibit a more mesenchymal phenotype than OE33 cells. OE33 typically show a cobblestone growth appearance and express high levels of E-cadherin. Accordingly, CPB and FLO-1 cells expressed the mesenchymal markers vimentin and MT1-MMP, as well as the mesenchymal variant of CD44. Upon *INHBA* overexpression, we observed increased levels of CD44 and MT1-MMP in CPB cells, which were further enhanced 48 hours after TGFβ1 stimulation. While the mesenchymal variant of CD44 is not expressed in OE33 cells, the standard form is (arrowhead to upper band, (294,464)). MT1-MMP, a membrane anchored matrix metalloprotease, was also increased in response to TGFβ1 in FLO-1 cells and

upregulated in *INHBA*-expressing FLO-1 cells. CD44 was expressed at similar levels in the FLO-1 empty vector and *INHBA* cell lines. *INHBA* overexpression resulted in higher levels of vimentin in FLO-1 cells compared to empty vector control (Figure A-3D). E-cadherin expression levels were largely unchanged in OE33 cells.

To identify if changes in the receptor complex components occur in these cells lines, we

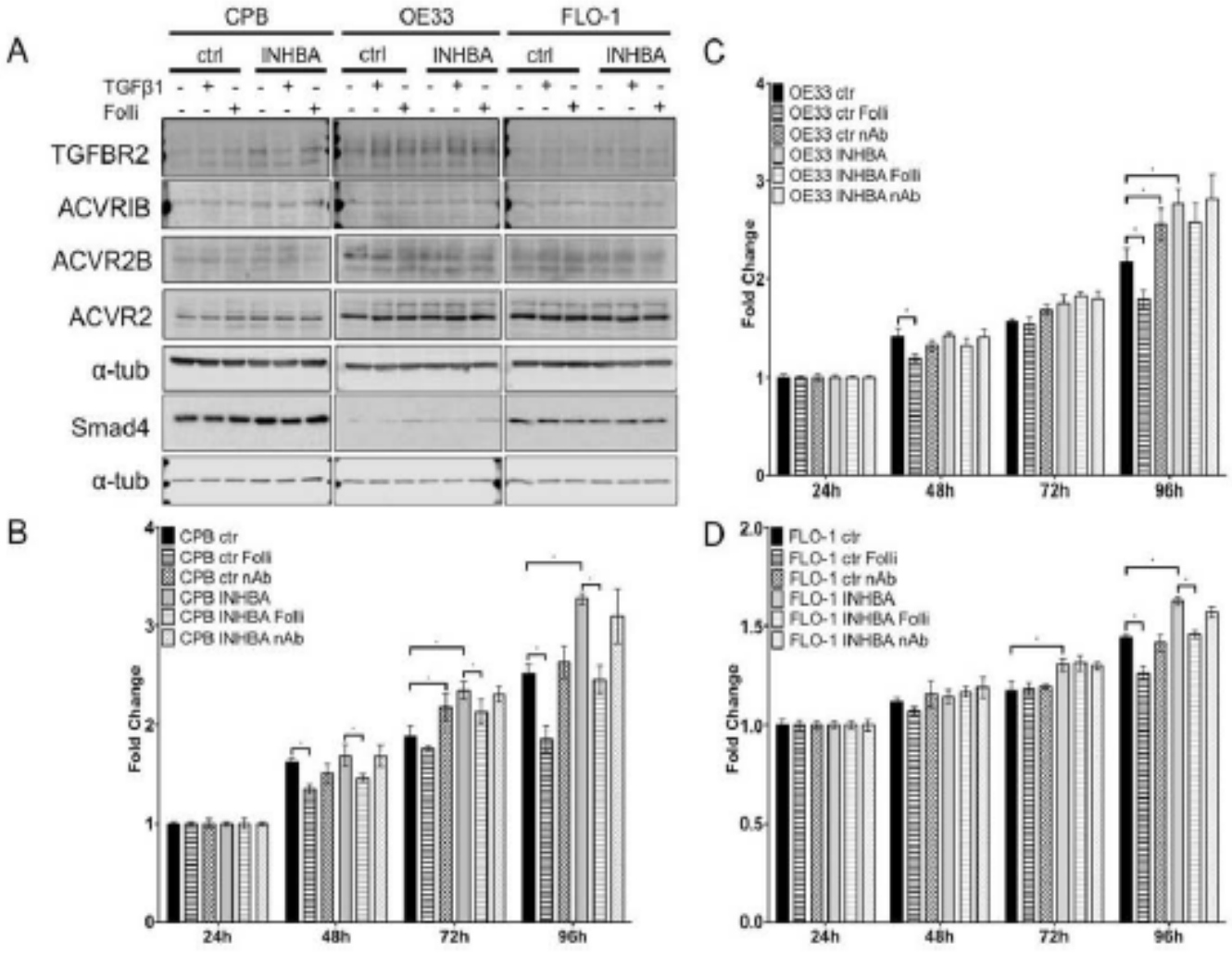


Figure A-4. Protein expression of receptor complex components is unchanged upon *INHBA* overexpression, yet proliferation is altered. (A) Protein lysates were collected after 48 hours after treatment with TGFβ1 or follistatin and used for Western blot. Analysis of components of the Activin A and TGFβ receptor complexes (TGFBR2, ACVR1B, AVCR2 AVCR2B) showed no changes in expression following *INHBA* overexpression. Smad4 expression is lower in OE33 cells than CPB and FLO-1 cells. TGFBR2, ACVRIB, AVCR2B were probed on the same membrane as α-tubulin for Smad4 (bottom panel). Cell viability was measured by WST-1 assay over the course of 96 hours. (B) CPB cells with *INHBA* overexpression have higher proliferation rates, which are reduced in the presence of follistatin, but not an Activin A neutralizing antibody (nAb). (C) OE33 cells and OE33-*INHBA* cells respond to follistatin with reduced proliferation rates, but not nAb. Overall, INHBA overexpression increases cell growth. (D) FLO-1 cells with INHBA overexpression have higher proliferation rates, which are reduced in the presence of follistatin, but not Act A neutralizing antibody. Data from three independent replicates were pooled and two-way ANOVA performed. All proliferation treatment conditions were normalized to their respective 24-hour time-point and the fold change was calculated from this value. *$p < 0.05$.

analyzed the expression of TGFBR2, ACVRIB, ACVR2, ACVR2B and the common downstream target Smad4. Aside from low levels of Smad4 in OE33 and low TGFBR2 in FLO-1 cells, all components were present in the analyzed cells (Figure A-4A).

In conclusion, we assessed the contributions of Smad-dependent canonical and non-canonical signaling targets, and showed phosphorylation of Smad2 only in response to TGFβ1, leading us to assume that *INHBA* overexpression does not alter baseline activation of Smad2. CPB *INHBA* cells had a stronger pSmad2 signal than control cells when treated with TGFβ1. We thus conclude that overexpression of *INHBA* in pre-malignant cells may increase the sensitivity to TGFβ1 signaling. Non-canonical pathway induction was measured by phosphorylation of ERK1/2 and Smad1/5/8, showing pSmad1/5/8 to be activated in OE33 and increased in the presence of *INHBA* overexpression, yet downregulated in FLO-1 *INHBA* cells upon TGFβ1 stimulation as well as with follistatin inhibition when compared with respective FLO-1 vector controls.

INHBA overexpression increases cell invasion in CPB and FLO-1, which exhibit mesenchymal features

Next, we assessed functional alterations of the *INHBA*-expressing cell lines compared to their control cells. To analyze growth rates in response to *INHBA* overexpression, we performed WST-1 viability assays over 96 hours and showed that overexpression of *INHBA* increased proliferation compared to control empty vector cells in all three cell lines (Figure A-4B-D). Addition of FS288 during the incubation period decreased growth by 96 hours. Interestingly, an Activin A neutralizing antibody could not inhibit growth, indicating that the functional effects of follistatin could be mediated by other targets. Folllistatin has been shown to bind with low

affinity to several other members of the TGFβ superfamily, including growth and differentiation facto 9 (GDF9), myostatin (GDF8), and several of the BMPs (465,466). Although follistatin does not bind to TGFβ1 or 2, binding to TGFβ3 has been reported (467).

Using colony formation assays to examine the cell survival and self-renewal capacities of *INHBA*-expressing cells, we detected reduced colony formation in CPB cells; however, no

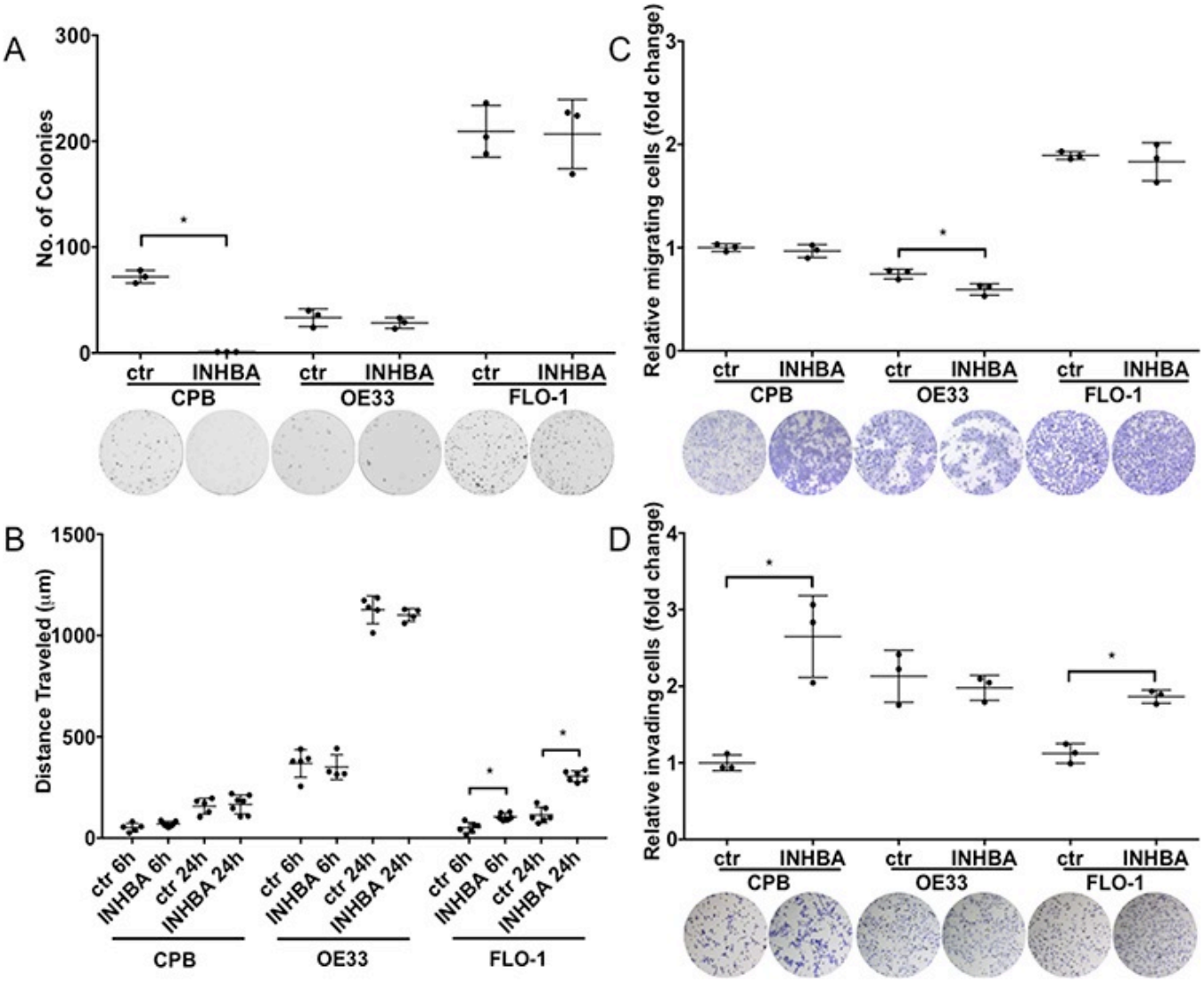


Figure A-5. INHBA overexpression impacts colony formation, migration and invasion potential in a cell-type specific manner. (A) INHBA overexpression inhibited colony formation of CPB cells compared to control (ctr), but not in the esophageal adenocarcinoma cell lines, OE33 and FLO-1. (B) Scratch assays showed high migration capabilities for OE33 cells, independent of INHBA status. INHBA overexpression enhanced scratch closure in FLO-1 cells (FLO-1 INHBA). (C) Migration measured in transwell chamber assays towards a full medium gradient showed inhibition of OE33 migration after INHBA overexpression (OE33 INHBA). (D) CPB INHBA and FLO-1 INHBA had an increased invasion potential compared to empty vector control cells (ctr) when grown in Boyden chamber invasion assays. OE33 INHBA invasion was equal to empty vector control (ctr). Statistical analysis was performed using Student's t-test, *p < 0.05.

effect on OE33 and FLO-1 cells was observed (Figure A-5A). The response of CPB cells to *INHBA* overexpression indicated that premalignant cells or cells at an early stage of EAC progression reduce their capability for cell survival or self-renewal while cancer cell lines evade an Activin A-mediated response.

To investigate the migratory and invasive potential of these cells, we employed two approaches: one that would account for gradient-independent migration - the scratch assay - and the other the chemotaxis-dependent Boyden chamber assays (Figures A-5B–5D). Of the tested cell lines, OE33 cells had the greatest migratory potential in scratch assays, yet no differences between *INHBA*-expressing and control cells were observed (Figure A-5B). However, cell migration in scratch assays was increased in *INHBA*-expressing FLO-1 cells (Figure A-5B). Migration in Boyden chamber assays with full media as a chemoattractant showed no changes in migration for CPB and FLO-1 *INHBA* and control cells. *INHBA* overexpression reduced Boyden chamber migration of OE33 cells (Figure A-5C). Invasion assays, in which cells have to digest a Matrigel matrix in a MMP-dependent process, demonstrated significantly higher invasion in *INHBA*-expressing CPB and FLO-1 cells, but not OE33 cells (Figure A-5D). As we have shown higher levels of CD44 and MT1-MMP in CPB and FLO-1 cells, the increased cell invasion could be dependent on the expression of these mesenchymal markers and the greater capability for digesting the matrix. FS288 and Activin A neutralizing antibody were used as additional controls in this assay to demonstrate the specificity of the function of Activin A in cell invasion (Figure A-6). The antagonist follistatin reduced cell invasion in control and *INHBA*-overexpressing cells, but the Activin A neutralizing antibody had an even greater effect (Figure A-6). This observation is interesting in light of the greater efficacy of follistatin in reducing cell growth compared to the neutralizing antibody

(Figure A-3B-D). We postulate that the corroboration of different pathways controls the diverse functions of proliferation and invasion.

Stimulation with exogenous Activin A results in downstream activation of canonical and non-canonical pathway components

To explore the differences between paracrine and autocrine signaling effects, we next added recombinant Activin A to the culture media. In this set of experiments, we aimed to investigate

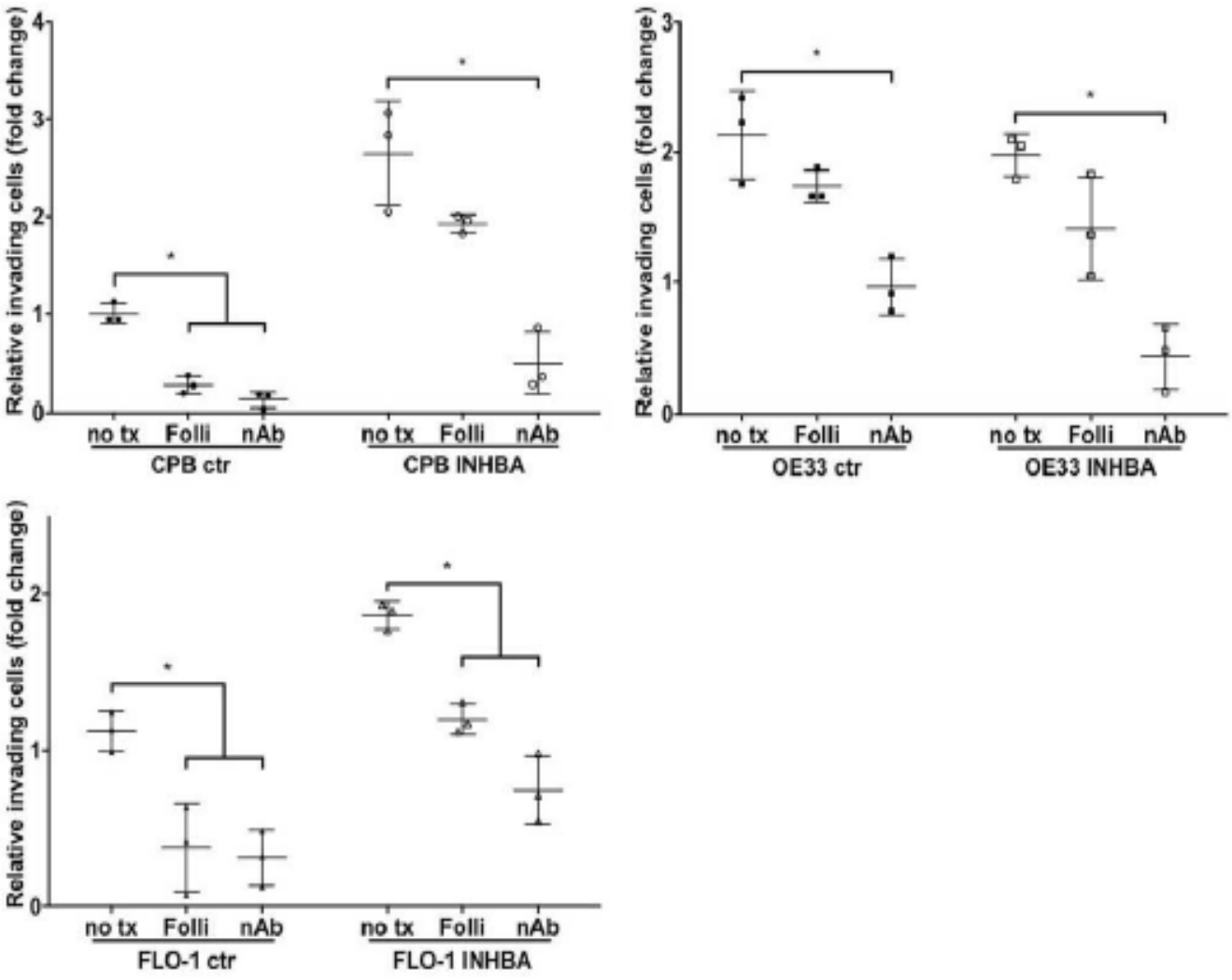


Figure A-6. Cell invasion of control and INHBA overexpressing cells is inhibited by follistatin and neutralizing antibody against Activin A. Empty vector control (ctr) and INHBA overexpressing cells were grown in Boyden chamber invasion assays in the presence or absence of follistatin (Folli) or Activin A neutralizing antibody (nAb) and compared to untreated controls (no tx). Both treatments reduced the invasive capabilities of the cells, yet nAb is more effective than follistatin. Statistical analysis was performed using Student's *t*-test, $*p < 0.05$.

if the model cell lines exhibit differential responses when stimulated with recombinant Activin A, a model of non-epithelial-derived Activin A. Using ELISA, we measured Activin A and TGFβ concentrations in conditioned culture media collected from untreated cells, cells treated with recombinant Activin A, or cells treated with the TGFβ receptor inhibitor A83-01 (Figure A-7A, B). Activin A levels were found to be higher in Activin A-stimulated CPB cells than in OE33 and FLO-1 cells (Figure A-7A). As the concentration of Activin A, when normalized to the amount of cells at the end of the 48-hour incubation period concentration, was much lower than expected in the OE33 and FLO-1 cells, we speculate that the availability is lower either due to decreased stability or increased uptake by the cells. The TGFβ1 concentration in the conditioned media was increased upon exogenous Activin A stimulation in CPB and OE33 cells, but not FLO-1 cells (Figure A-7B). The addition of A83-01 had no effect on TGFβ1 secretion in in all three cell lines.

Western blot analysis showed no phosphorylation of Smad2 in any of the three cell lines tested when untreated (Figure A-7C). Stimulation with Activin A induced phosphorylation of Smad2 in CPB and FLO-1 cells, but not in OE33 cells. TGFβ1, which was used as a positive control for the induction of Smad2 phosphorylation, resulted in robust Smad2 activation across all tested cell lines. pSmad1/5/8 was detected at high levels in OE33 cells independent of Activin A stimulation or inhibition. CPB and FLO-1 cells showed increased pSmad1/5/8 only upon TGFβ1 stimulation. pERK1/2 and pAKT remained unchanged in response to the different treatments. Again, overall pERK1/2 was higher in CPB and OE33 cells compared to FLO-1, similar to our observations in the *INHBA* overexpression model (Figure A-3C). The epithelial marker E-cadherin was expressed in OE33 and increased expression was detected upon inhibition of Activin A with follistatin or TGFβ1 inhibition by A83-01. CPB and FLO-1 expressed

the standard form of CD44, while OE33 expressed the variant form, which is commonly associated with a more epithelial phenotype (arrowhead, Figure A-7D). MT1-MMP had the highest expression in FLO-1 cells. While CPB cells also expressed MT1-MMP, OE33 only showed a signal after TGFβ1 stimulation. Both CPB and FLO-1 had expression of the

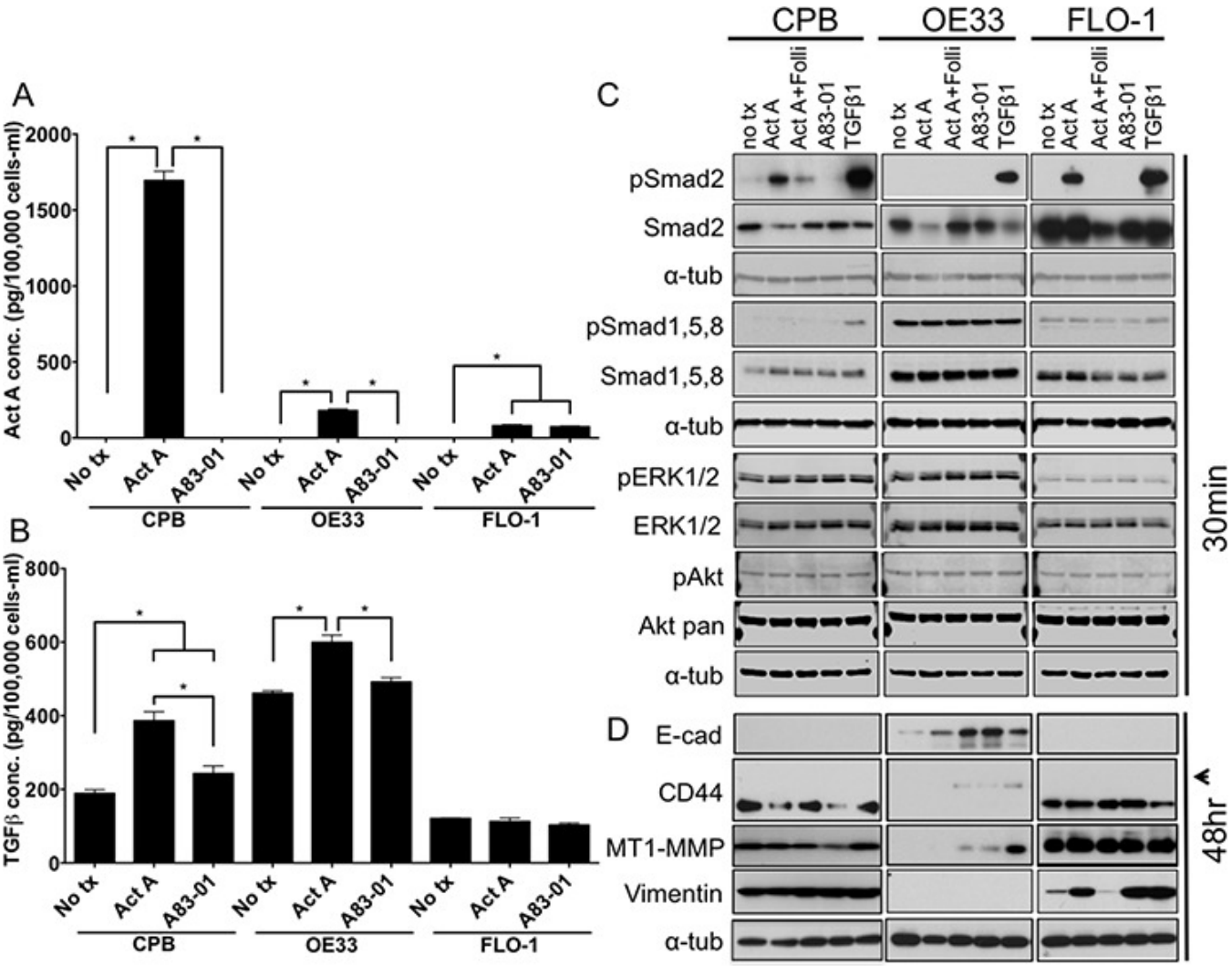


Figure A-7. Stimulation with recombinant Activin A regulates TGFβ1 secretion and induces canonical downstream signaling in CPB and FLO-1 cells. (A) Activin A concentration in conditioned media after stimulation with recombinant Activin A (Act A) or the TGFβ receptor inhibitor A83-01, as assessed by ELISA and normalized per 100,000 cells after 48 hour incubation. (B) TGFβ1 concentration in conditioned media after stimulation with recombinant Activin A or the TGFβ receptor inhibitor A83-01, was assessed by ELISA. TGFβ1 concentration was increased in response to stimulation with recombinant Activin A and reduced with A83-01 in CPB and OE33, but not in FLO-1. Concentrations were normalized to cell number at time of collection. (C) Antibody against phospho-Smads (pSmad2; pSmad1,5,8) and total Smad2, as well as pERK1/2 and pAkt compared to total ERK1/2 and Akt were used for Western Blot analysis. pSmad2 is induced in response to Activin A and TGFβ1 in CPB and FLO-1, but only with TGFβ1 in OE33. (D) Antibodies against markers of epithelial-mesenchymal transition showed increased expression of the variant form of CD44 (arrowhead) in epithelial OE33 cells following stimulation with follistatin (Folli) and Activin A, as well as TGFβ1. MT1-MMP was increased mainly with recombinant TGFβ1 in OE33. FLO-1 cells, which exhibit a mesenchymal phenotype, had a further increase in vimentin expression with Activin A, but also with addition of A83-01 and TGFβ1. Statistical analysis was performed using ANOVA, *$p < 0.05$.

mesenchymal marker vimentin, which was absent in OE33 cells. Interestingly, Activin A increased vimentin expression in FLO-1 cells, which was reduced after the addition of follistatin, demonstrating specificity of the Activin A-mediated effect (Figure A-7D). Analysis of the receptor expression confirmed the low levels of TGFBR2 in FLO-1 cells (Figure A-8A). Overall, TGFBR2 expression levels were unaffected by stimulation with Activin A or by the use of the antagonists and inhibitors, but upon TGFβ1 stimulation, TGFBR2 expression was downregulated in CPB cells, possibly due to receptor internalization or degradation upon signaling induction. The unchanged expression levels for the signaling receptors are not unexpected as the regulation of receptor function depends mostly on phosphorylation events, localization within the cells, and endocytosis upon ligand binding to ensure recycling of the receptors. Yet deletion, mutation, and epigenetic silencing can lead to the loss of expression and, therefore, evasion of a cytostatic response has been reported in the literature (468).

Activin A stimulation increased cell invasion in OE33 cells

As the stroma can be a major source of chemokines and cytokines regulating tumor growth and invasion, we measured the effects of recombinant Activin A treatment on cell viability (Figure A-8B-D), colony formation, cell migration (scratch assay), and chemotaxis-dependent cell migration and invasion (Boyden chamber). When we examined cell growth after treatment with recombinant Activin A, follistatin, an Activin A neutralizing antibody, and combinations of Activin A with the respective antagonists, we observed largely unchanged cell growth with the different conditions (Figure A-8B-D). CPB cells showed an increase after treatment with follistatin, but not neutralizing antibody (Figure A-8B). OE33 cells exhibited a decrease in growth in the presence of Activin A together with follistatin. FLO-1 cells, while unresponsive to

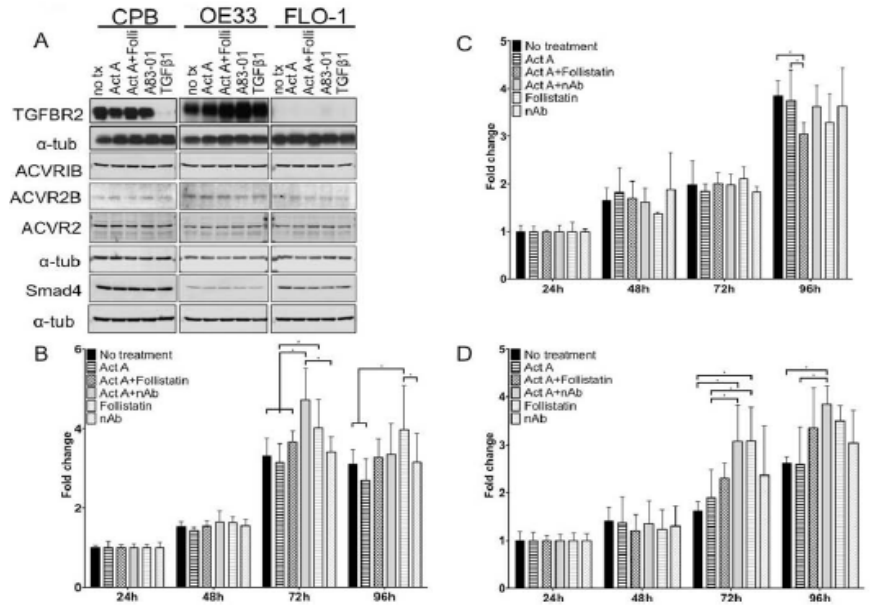


Figure A-8. Protein expression level of receptor complex components are unchanged upon Activin A treatment, however proliferation is altered. (A) Protein lysates were collected after 48 hours and antibodies against TGFBR2, ACVRIB, ACVR2, ACVR2B, and Smad4 used to determine if changes in protein expression occur following stimulation with Activin A, TGFβ (as a positive control), follistatin (Folli), or A83-01. No changes were noted aside from TGFBR2, which was downregulated in CPB cells upon TGFβ stimulation. Membrane for ACVRIB is the same as for Smad4. α-tubulin on the bottom panel serves as ACVRIB loading control. Cell viability was measured by WST-1 assay over the course of 96 hours. Cells were treated with either Activin A (Act A), the combination of Act A and follistatin, Act A and Activin A neutralizing antibody (nAb) or each alone. (B) CPB cells, in response to follistatin showed increased cell proliferation. (C) OE33 cell proliferation was decreased in response to the combination of Act A and follistatin. (D) FLO-1 cells in response to Activin A stimulation showed increased cell proliferation in the presence of Activin A and follistatin or the combination of Act A and neutralizing antibody (nAb). Data from three experiments were pooled and a two-way ANOVA was performed. All proliferation treatment conditions were normalized to their respective 24-hour time point and the fold change was calculated from this value. *$p < 0.05$.

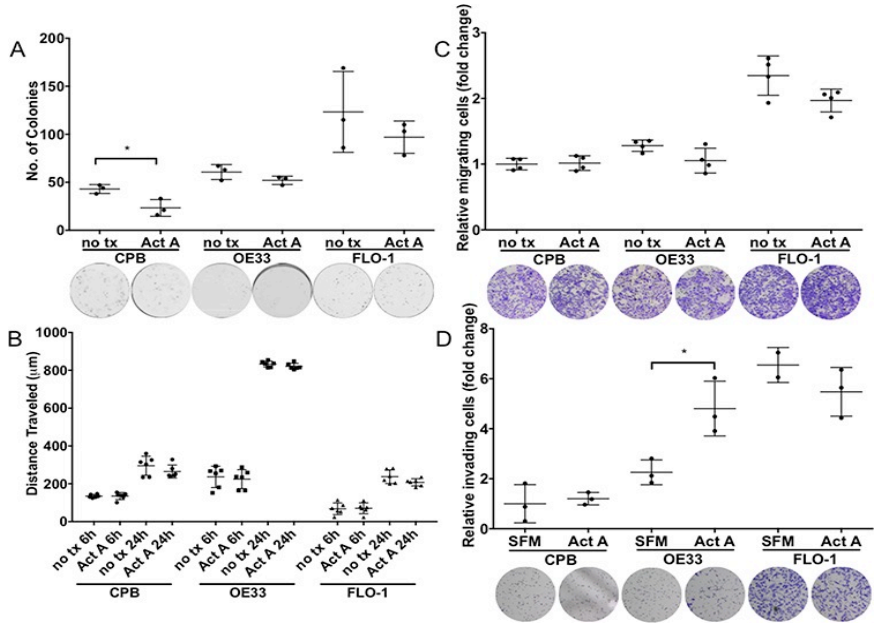


Figure A-9. Stimulation with recombinant Activin A affects colony formation, migration and invasion potential in a cell-type specific manner. (A) Activin A overexpression inhibited colony formation of CPB cells, but not in esophageal adenocarcinoma cell lines, OE33 and FLO-1. (B) Scratch assays showed high migratory capabilities for OE33 cells. (C) Migration measured in transwell chamber assays towards a full medium gradient showed no effect from Activin A stimulation. (D) Using Activin A as a chemoattractant increased the invasive capability of OE33 cells when measured in Boyden chamber invasion assays (SFM: serum-free medium; Act A: serum-free medium with Act A). t-test was used to calculate significance, p-value < 0.05.

Activin A stimulation, showed increased cell growth in the presence of Activin A and its antagonists (Figure A-8D). These results are in contrast to the overall induction of proliferation in all cell lines after *INHBA* overexpression (Figure A-3B). Similar to the data observed with *INHBA* overexpression, colony formation was reduced in CPB cells in the presence of recombinant Activin A (Figure A-9A). Activin A, however, had no effect on the EAC lines OE33 and FLO-1 (Figure A-9A), indicating that Activin A exerts colony reduction in dysplastic but not tumor cells.

When we studied the response to Activin A in scratch assays, we found that while OE33 had the highest migratory potential in the scratch assays, Activin A stimulation had no effect compared to untreated controls (Figure A-9B). No significant differences were observed between untreated and Activin A treated cells in chemotaxis migration (Figure A-9C), however cell invasion assays using Activin A as a chemoattractant increased OE33 cell invasion (Figure A-9D). When follistatin in combination with Activin A was used, FLO-1 cells demonstrated a diminished potential for invasion, indicating a dependence on signaling targeted by follistatin inhibition such as Activin A, and potentially other pathways such as BMP. Other treatments including neutralizing antibody surprisingly had no effect on cell invasion (Figure A-10), possibly hinting at the necessity for additional growth factors to contribute to this phenotype. Fetal bovine serum as a positive control elicited considerable invasion in all three cell lines.

In all, Activin A concentrations in the ELISA assay (Figure A-7A) identified that FLO-1 cells had the lowest measurable Activin A concentration 48 hours after treatment and therefore the lack of response in the functional assays could be dependent on Activin A availability.

Interestingly, Activin A media concentrations were lower for OE33 cells than CPB after Activin A stimulation, yet cell invasion could be induced in OE33 cells.

Overall, comparison of the results for Activin A stimulation and the retroviral overexpression of *INHBA* suggest that the Barrett's cell line CPB is responsive to Activin A as a modulator of cell survival, which ultimately resulted in reduced colony formation. However, only overexpression of *INHBA* enhances cell invasion in CPB and FLO-1 cells, while stimulation with recombinant Activin A does for OE33 cells. A limitation to the approach comparing overexpression as a model for autocrine signaling and stimulation for paracrine signaling is not only the variability of ligand availability and dosage, as can be seen by the differences in concentration by ELISA, but also long-term versus acute exposure to the ligand. It appears, however, that while Activin A stimulation (acute response) can elicit functional changes, only overexpression can result in intrinsic cellular changes, which ultimately becomes the driver for events such as cell invasion after long-term exposure (Figure A-3). While the stroma as a source of Activin A seems to effect migration and invasion less than autocrine functions of tumor-secreted Activin A, the elicited functional responses are potentially influenced by acute versus chronic exposure and crosstalk with other pathways. This hypothesis is supported by the variable regulation of Activin A-dependent functions in the presence of follistatin or Activin A neutralizing antibody, but will require additional experimentation.

Columnar keratins are differentially expressed in organotypic reconstruct cultures

The previous assays allowed for the analysis of epithelial cells *in vitro*, but these methods do not account for epithelial cell crosstalk with matrix components and mesenchymal cell types.

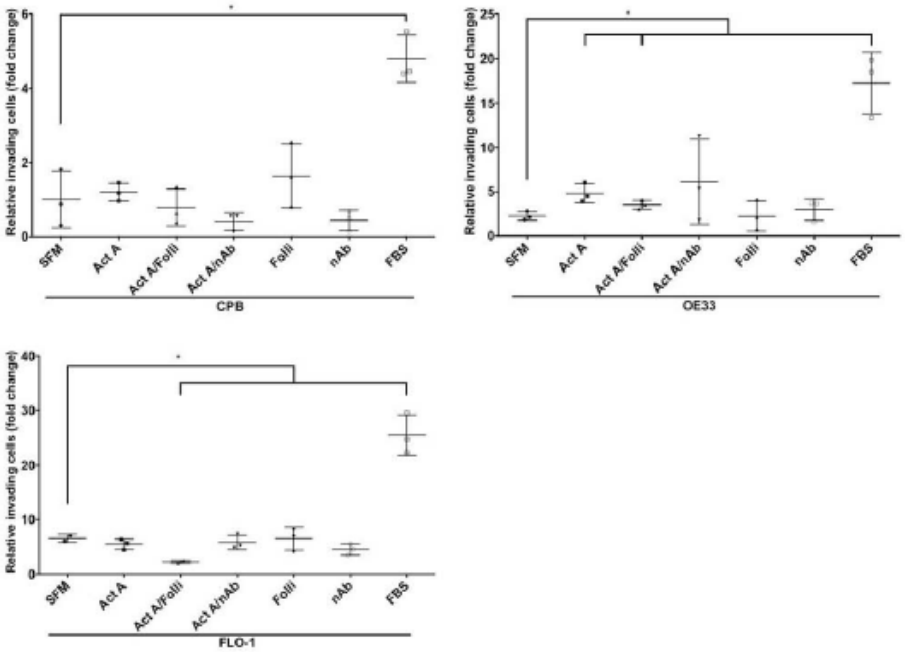


Figure A-10. Cell invasion is inhibited when adding Activin A in combination with follistatin in FLO-1 cells. Cells were grown in serum-free media in Boyden chamber invasion assays with either Activin A (ActA), the combination of Activin A and follistatin (Folli), Activin A and an Activin A neutralizing antibody (nAb) or each alone in the bottom chamber. FBS was used as a positive control. The invasive potential of OE33 cells was increased by the combination of ActA and follistatin. In FLO-1 cells, ActA and follistatin reduced the invasive potential, while FBS increased it in all cell lines. Post-hoc statistical analysis comparing all the conditions was performed using one-way ANOVA. *$p < 0.05$.

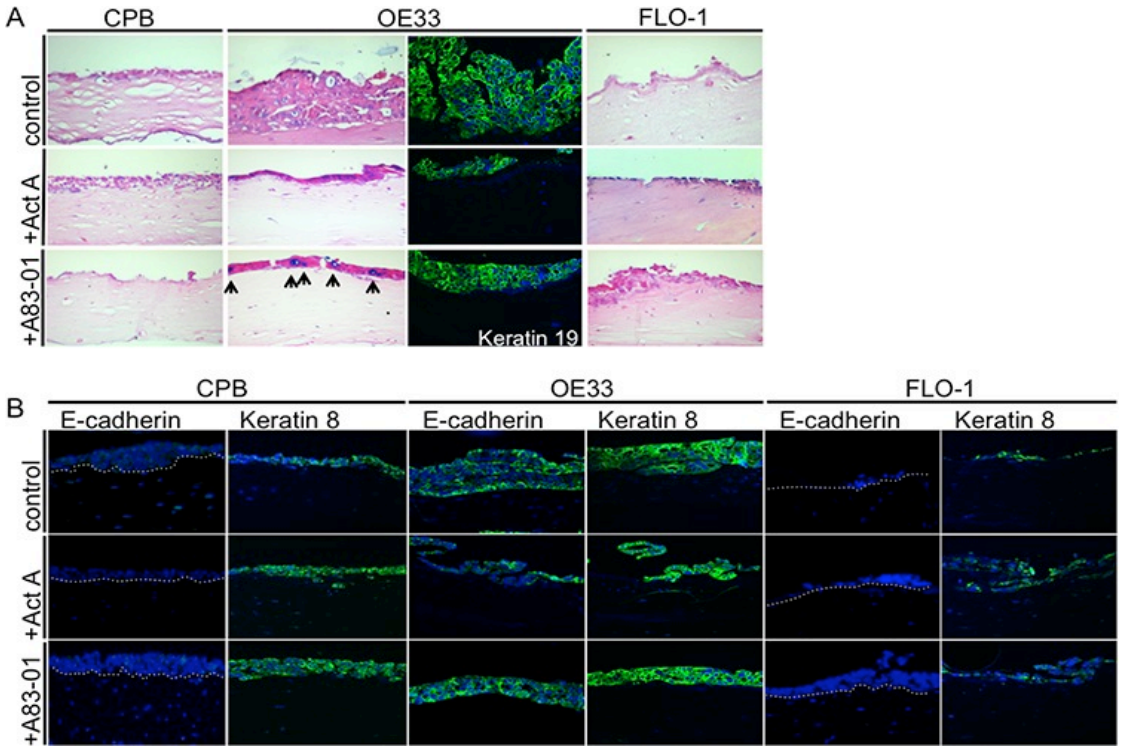


Figure A-11. Barrett's esophagus and esophageal adenocarcinoma cells in organotypic reconstruct cultures. (A) OE33 cells had increased Alcian Blue staining upon A83-01 treatment (arrows). OE33 cells were also positive for the columnar marker cytokeratin 19 (keratin 19). (B) CPB and FLO-1 cells, which have a mesenchymal phenotype, express no E-cadherin, but OE33 showed positive E-cadherin staining by immunofluorescence. Anti-keratin 8 antibody showed that the signal increased in CPB following stimulation with Activin A or treatment with A83-01. Keratin 8 signal was weaker in FLO-1 cells in the presence of A83-01.

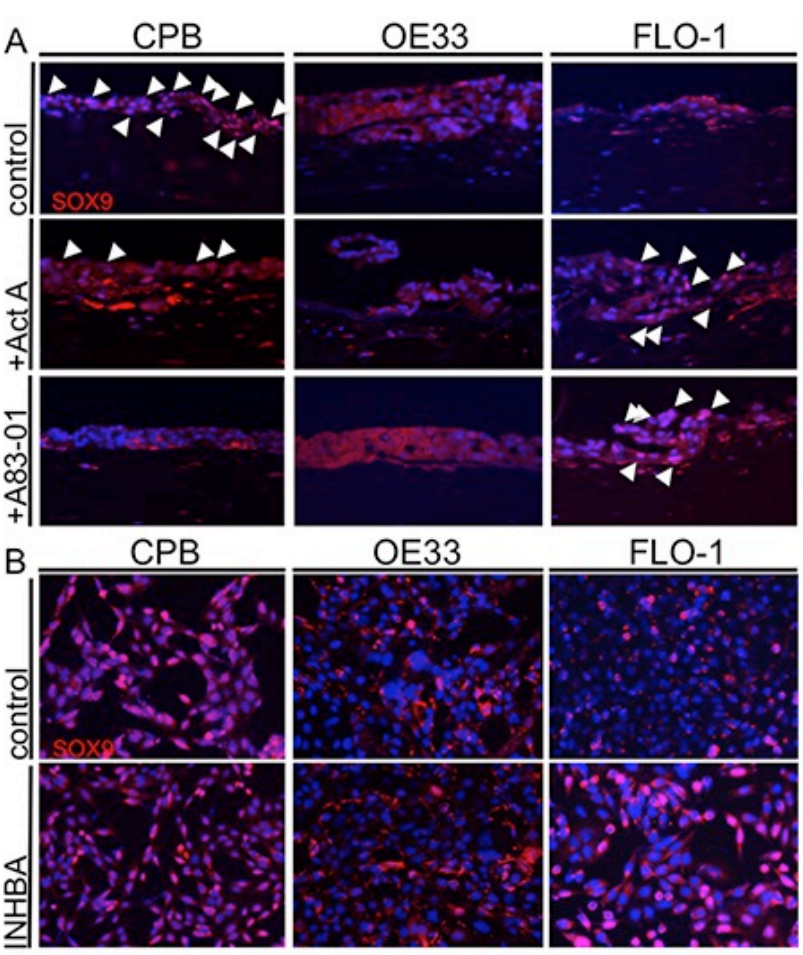
For that reason, we grew CPB, OE33, and FLO-1 cells in the presence or absence of Activin A in organotypic cultures on a collagen/Matrigel matrix with embedded fibroblasts to mimic a physiological stromal context. CPB and FLO-1 cells, which have a mesenchymal phenotype, were unable to form a multilayered epithelium due to the lack of cell-cell adhesion. However, stimulation of the culture with Activin A, as well as inhibition of TGFβ with A83-01, induced overall epithelium formation in CPB and FLO-1 cells. Both cell types were negative for Alcian Blue staining, which was used to detect mucin-secreting cells, and the columnar marker cytokeratin 19 (CK19) (Figure A-11A). The epithelial adenocarcinoma cell line OE33 exhibited Alcian Blue-positive cells, indicating the presence of mucin secreting goblet cells (arrows, Figure A-11A), and positive staining for CK19 (Figure A-11A) in Activin A-treated and control organotypic cultures.

When stained for E-cadherin and for the columnar keratin, cytokeratin 8 (CK8), CPB cells showed no signal for E-cadherin, validating the Western blot data (Figure A-7E). CK8, however, could be detected and increased following treatment with Activin A and A83-01 (Figure A-11B). OE33 cells were positive for both E-cadherin and CK8 independent of the treatment conditions. No E-cadherin staining could be detected in FLO-1 cells, and only a few cells of the epithelium were positive for CK8 (Figure A-11B). Cytokeratin 14, a squamous marker, was not detected by immunofluorescence (data not shown).

Columnar cytokeratins CK8 and CK19 are expressed in glandular, non-squamous epithelium, including Barrett's Esophagus (469), and therefore demonstrate the shift towards a columnar phenotype compared to normal squamous esophageal tissue.

Activin A regulates SOX9 nuclear localization

We further aimed to determine the expression of SOX9 in response to Activin A signaling. SOX9 is known to drive columnar differentiation of the esophageal squamous epithelium (470) and has been described to attribute stem cell-like properties to esophageal cancer cells (471). We hypothesized that because mesenchymal cells have more stem cell-like features, SOX9 should be detected with higher frequency in the nuclei of CPB and FLO-1 cells. To address the



C

Sox 9 positive nuclei/ total nuclei	CPB (%)	OE33 (%)	FLO-1 (%)
Ctrl	88 +/-0.4	4.5 +/-3.3	11 +/-8.2
Act A	82 +/-6.9	4.3 +/-3.5	77 +/-1.5
A83-01	4.3 +/-1.6	4.9 +/-1.5	85 +/-0.5
Ctrl	90 +/-1.7	5 +/-2.4	8.5 +/-1.7
INHBA	63 +/-3.9	4.3 +/-2.5	47 +/-5.8

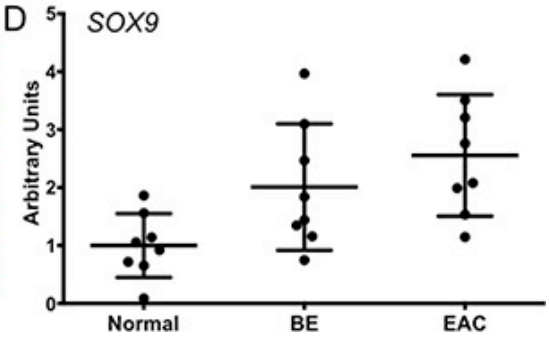


Figure A-12. Activin A induces nuclear SOX9 in FLO-1 cells. (A) In organotypic cultures, CPB cells showed nuclear SOX9 (arrowheads) with and without Activin A (Act A) stimulation; the signal was reduced with A83-01. OE33 cells show no nuclear SOX9 in any of the conditions. The intensity for SOX9 staining was low in FLO-1 control cells with no nuclear localization, but increased with Activin A (Act A) and A83-01, as shown by the white arrowheads. (B) In monolayer, CPB control and *INHBA* overexpressing cells had a nuclear SOX9 signal. OE33 cells showed no nuclear SOX9 in any of the conditions. The intensity for SOX9 staining was low in control cells with no nuclear localization in FLO-1 cells, but increased in FLO-1 *INHBA*. (C) SOX9-positive nuclei were counted and calculated as percentage per total nuclei per field. Four fields per replicate image were counted. (D) The publicly available GEO dataset (accession number GDS1321) was used for analysis of SOX9 expression and showed overall increased SOX9 expression in the progression from Barrett's esophagus to esophageal adenocarcinoma.

expression and localization of SOX9 in response to Activin A and TGFβ signaling, we performed immunofluorescence staining with an anti-SOX9 antibody on the organotypic cultures. In untreated cultures, CPB cells were positive for nuclear SOX9, while few OE33 and FLO-1 cells were positive (white arrowheads, Figure A-12A). Upon stimulation with Activin A, SOX9 expression was unaffected in CPB cells, though the number of SOX9-positive nuclei in the fibroblasts increased. Organotypic cultures of OE33 cells showed increased positive SOX9 signal in the fibroblasts, but not in the OE33 cells themselves. Increased nuclear staining was observed in FLO-1 cells and the surrounding fibroblasts after Activin A stimulation (white arrowheads, Figure A-12A). TGFβ inhibition by A83-01 decreased nuclear SOX9 in the CPB cultures, but did not inhibit SOX9 nuclear localization in the FLO-1 cells (Figure A-12C). Interestingly, when *INHBA*-overexpressing cells were grown on plastic, nuclear SOX9 was also high in CPB and low in OE33 cells, but SOX9-positive nuclei were less frequent in CPB *INHBA* cells than in the Activin A-stimulated cells grown in organotypic cultures (Figure A-12B). In FLO-1 cells, which showed an increased number of SOX9-positive nuclei upon Activin A and A83-01 treatments, we still detected an increase in SOX9-postitive nuclei in *INHBA*-overexpressing cells (Figure A-12B), but not to the same extent as in cultures treated with recombinant Activin A (Figure A-12C). These observations show that expression and nuclear SOX9 localization in CPB cells are TGFβ signaling–dependent, demonstrated by suppression of TGFβ signaling by A83-01 in organotypic culture. Conversely, the *INHBA*-dependent increase of nuclear SOX9 in FLO-1 cells was not reversible through inhibition of TGFβ by A83-01. Differences in numbers between organotypic cultures and plastic indicate a role of the stroma in the regulation of the signaling pathways resulting in SOX9 activation. Using the same dataset as in Figure A-1, we showed an increase in SOX9 expression during the

progression from Barrett's esophagus to esophageal adenocarcinoma (Figure A-12D). Together, we believe that these data indicate the importance of the microenvironment for signal transduction and SOX9 protein expression.

Discussion

Esophageal adenocarcinoma (EAC) has a poor patient outcome, with a 5-year survival of only 14% (472). A recognized risk factor for EAC is Barrett's esophagus, which has been shown to progress from a pre-malignant lesion to EAC in 5–10% of cases (473). EACs can arise rapidly in patients diagnosed with Barrett's esophagus, even under careful surveillance. It is thought that the transformation of non-dysplastic to dysplastic Barrett's esophagus, and ultimately esophageal adenocarcinoma, is driven by a stepwise accumulation of mutations or specific oncogenic events that underlie progression. Yet, most of the commonly mutated genes in EAC—except *TP53* and *SMAD4*—have also been found in non-dysplastic Barrett's esophagus that did not progress towards cancer (474). These findings and the fact that targeted therapies brought little improvement due to reactivation of the targeted pathway, hyperactivation of alternative pathways, and cross-talk with the microenvironment (475) highlight that aside from genomic catastrophes, such as gene inactivation through chromosomal rearrangements and telomere integrity (476,477), other important mechanisms are at play in EAC progression.

We have focused here on the activation of canonical and non-canonical signaling by Activin A to identify the contributions of this TGFβ family member to the pathology of EAC.

Signaling

Activins and BMPs are classified as members of the TGFβ superfamily, and bind to related transmembrane receptors, resulting in overlap in their intracellular signaling cascades and downstream function (87,318,478,479). This has been shown by deletions of TGFβ2, TGFβ3 and inhibin β_A, which all result in cleft palate defects in the respective mouse model. Similar to a 10-bp polyadenine tract within the TGFβ receptor type II gene (*TGFBR2)* that is prone to frameshift mutations in gastrointestinal cancers, mutations in *ACVR2* have been identified in colorectal and pancreatic cancers (319). Additionally, *ACVR1B* is commonly mutated in pancreatic cancer (229), and in a majority of sporadic colorectal cancers BMPR2 expression is impaired (320). Conversely, BMP and Activin Membrane-bound Inhibitor, BAMBI, is upregulated in colorectal cancer and under direct regulation of the Wnt pathway, (321) a component of a gene expression profile that predicts metastasis (322). Exome and whole-genome sequencing of EAC has identified recurrent driver events with high frequency, such as mutations in *TP53* and *CDKN2A*, but also *PTEN* and *SMAD4* (480,481). Interestingly, Activin A, which canonically signals through the Smad cascade, but utilizes a different set of receptors (ACVR2A or ACVR2B), was able to activate Smad2 in CPB and FLO-1 cells, but failed to do so in OE33 cells. OE33 cells, on the other hand, showed overall non-canonical activity of the MAPK/ERK pathway, as well as activation of BMP signaling as measured by pSmad1/5/8, the latter being increased upon *INHBA* overexpression. This could indicate that these signaling pathways are somewhat promiscuous and that ligands can utilize undesignated receptors (384,482). Furthermore, stimulation with TGFβ1 upon overexpression of *INHBA* resulted in activation of the canonical and non-canonical pathways (for OE33 and FLO-1 cancer cells). TGFβ1 has been shown to induce phosphorylation of Smad1/5/8 in endothelial cells mediated

by TGFBR1 and ACVL1 (482). Interestingly, although functional consequences, such as a decrease in clonogenicity, were observed in both acute and long-term exposure settings for Barrett's esophagus cells, we could not measure meaningful canonical and non-canonical signaling activation in *INHBA* overexpressing cells. This could be potentially due to an acquired insensitivity after prolonged exposure, yet we observed increased cell invasion, possibly due to intrinsic cell changes.

Tumorigenicity and invasion

In esophageal squamous cell carcinoma, Activin A has been associated with tumor aggressiveness and increased MMP-7 expression. This increase in aggressiveness is accompanied by increased proliferation (300) and MMP-7 activity (89). However, when the microenvironment is taken into account, the regulation of Activin A through its inhibitor follistatin or a TGFβ receptor inhibitor has differential effects when keratinocytes and fibroblasts were grown in organotypic cultures (356). Our previous study showed that while cell invasion was increased upon Activin A stimulation in a premalignant cell model, inhibition of Activin A and TGFβ1 further enhanced cell invasion. Based on the importance of the balance between Activin A/TGFβ and BMP signaling, squamous epithelial homeostasis appears to be regulated by fine-tuning the concentrations and activity of the different growth factors and their functions (356). Here we show that activation of Activin A signaling results in decreased cell migration of OE33 cells in the context of autocrine signaling (Boyden chamber), yet increased OE33 cell invasion upon stimulation with Activin A, suggesting a stromal contribution. Invasion of CPB and FLO-1 cells was increased upon *INHBA* overexpression, but was unaffected by stimulation. Given the variability in Activin A concentration between overexpression and

recombinant stimulations in the FLO-1 cells, the results could be dependent on a lower concentration of Activin A. Overall, we speculate that the source of Activin A elicits different cellular responses in a context-dependent manner. Aside from the source, an important consideration is the amount of Activin A available, as sudden influx and receptor occupancy in an acute setting will activate signaling; yet during long-term exposure, low-affinity ligand receptor interactions could lead to more promiscuity.

The microenvironment has been recognized as playing an increasingly important role in carcinogenesis. Gene ontology analysis has identified a strong inflammatory component in Barrett's tumorigenesis, and key pathways included are cytokine-receptor interactions and TGFβ (483,484). We extrapolate from our data that the endogenous production of Activin A may result in different phenotypic and functional outcomes than the mimicked paracrine stimulation of the cells. At the same time, similar to the differential effects on multiple cell lines as described above, we found profound variation when analyzing models of EAC progression between dysplastic cells (CPB) and esophageal adenocarcinoma cell lines with an epithelial (OE33) or mesenchymal phenotype (FLO-1) and their responses to Activin A.

Wang et al. have shown that squamous-to-columnar metaplasia can occur when bile-induced injury reactivates latent developmental pathways (485). Hedgehog signaling in squamous epithelial cells upon bile-induced injury stimulated stromal expression of BMP4 by esophageal fibroblasts, resulting in epithelial SOX9 expression (485). Given the activation of pSmad1/5/8, the fine balance between Activin A and BMP signaling might be regulating the context-dependent functional outcomes.

Stemness and resistance

Activin A has been identified as necessary for the maintenance of self-renewal in human embryonic stem cells through the induction of Oct4, Nanog, Nodal, and Wnt3, but, more importantly, through the induction of basic FGF and the suppression of BMP (72). These data indicate the role of Activin A as a mediator of stemness and potentially as a cancer stem cell marker. Suppression of the downstream target ID2 by Activin A and TGFβ is central in the induction of EMT (73), which is antagonized by BMP. Supporting the important role in regulating self-renewal of stem cells and cell-fate determination in the initiation and progression of Barrett's esophagus to EAC, it has been shown that in the normal esophagus, small clusters of Oct3/4-positive cells are nested in the basal cell layer, representing a pool of progenitor cells. Concomitant with the activation of Notch and TGFβ signaling in esophageal adenocarcinoma, an expansion of the Oct3/4 positive cell clusters can be observed (449). SOX proteins, documented as stem cell markers, also exhibited increased expression in esophageal adenocarcinoma cells (449).

SOX9 has been implicated in the induction of a cancer stem cell phenotype in esophageal cancer (449,470). Expression of SOX9 in squamous epithelial cells has been shown to induce the formation of a columnar-like epithelium with the expression of columnar differentiation markers such as cytokeratin 8, demonstrating that columnar dedifferentiation and expression of intestinal markers reminiscent of Barrett's esophagus can be driven by SOX9 (470). The involvement of bile reflux injury in the context of Barrett's tumorigenesis has been shown in acid treatment experiments using normal esophageal squamous, OE33 cells, and a mouse model of bile reflux. Acid or bile exposure led to an induction of stromal BMP4 and epithelial SOX9 resulting in conversion from squamous to columnar epithelium along with

the expression of columnar cytokeratins (486). Nuclear SOX9 was also detected in a surgical model of reflux by esophagojejunostomy (487). Additional support that SOX9 may be an important early event in the development of Barrett's tumorigenesis is seen in the activation of SOX9 following loss of β2-spectrin, which induces a TGFβ signaling switch from tumor suppressor in normal cells to tumor promoter in fibroblasts and EACs (471). Upstream of SOX9, YAP1 has been shown to be a major determinant of cancer stem cell properties in non-transformed and esophageal cancer cells. YAP-induced upregulation of SOX9 was concomitant with the acquisition of stem cell properties (488).

Our data show that induction of nuclear SOX9 is potentially associated with a mesenchymal phenotype, as epithelial OE33 cells are negative for SOX9. Activin A stimulation, as well as its overexpression, resulted in increased nuclear SOX9 localization and may be associated with stem cell-like features, such as the expression of EMT markers. Stem cell-like properties have been attributed to the mediation of therapy resistance (489). Kim Ah et al. (490) reported that, in response to ionizing radiation, TGFβ downregulates *c-Myc* mRNA expression and inhibits the growth of OE33 EAC cells *in vitro*. While TGFβ enhanced radioresistance of OE33 cells, it did not affect the radiosensitivity of squamous carcinomas KYSE and OE21. The TGFβ-enhanced radioresistant phenotype was associated with induced G0/G1 cell cycle arrest and upregulation of the G1 cyclin-dependent kinase inhibitor p27kip1, as well as downregulation of c-Myc protein expression. Interestingly, conditioned medium obtained from unirradiated OE33 cells enhanced radioresistance compared with fresh medium. This enhancement was abrogated by pre-incubation of conditioned medium with a neutralizing anti-TGFβ antibody, suggesting endogenous TGFβ production by OE33 cells. Given the reports of submucosal metaplasia after Barrett's esophagus radioablation, it remains to be

seen if SOX9 expression coupled with high Activin A serum levels would be useful as an early detection marker.

Taken together, we aimed to determine the role of tumor-derived and stromal Activin A during sequential events of esophageal transdifferentiation promoting Barrett's tumorigenesis. We demonstrated that aside from known mutational or epigenetic alterations, activation of signaling is pleiotropic and context-dependent, thereby highlighting the complex crosstalk with the microenvironment.

Methods

Cell culture

The Barrett's esophagus cell line CPB (CRL-4028) was purchased from American Type Culture Collection (ATCC) and cultured with epithelial cell medium 2 (ScienCell, Carlsbad, CA) supplemented with 5% fetal bovine serum (FBS, Hyclone, GE Healthcare, Pittsburgh, PA) and antibiotics, 100 units/mL penicillin and 100 µg/mL streptomycin (Gibco, Carlsbad, CA). The esophageal adenocarcinoma cell lines, OE33 and FLO-1, were derived by Dr. David Beer (491) and grown in RPMI and DMEM (Invitrogen, Carlsbad, CA), respectively, with 10% FBS at 37°C in 5% CO_2. Fibroblasts were grown in DMEM with 5% FBS (Hyclone), 100 units/mL penicillin, and 100 µg/mL streptomycin (Gibco). For treatment with growth factors 5 ng/ml recombinant human TGFβ1, 10 ng/ml Activin A, 100 ng/ml follistatin-288 (FS288), 100 ng/ml Nodal (all R&D Systems, Minneapolis, MN Systems) or 1 µM A83-01 (Tocris, Bristol, UK) were used. Overexpression of Activin A (*INHBA*) was achieved by retroviral transfection of cells with viral supernatant containing pBABE plasmid with zeocin resistance (Addgene, Cambridge, MA) encoding the *INHBA* gene sequence (Origene, Rockville, MD).

Organotypic culture

Organotypic reconstructs were grown as previously described (294,356) with the exception that each culture was rinsed in 1XPBS and incubated with Epidermalization 3 medium lacking serum for two additional days prior to harvesting. The following treatments were added to the organotypic cultures at the time of epithelial seeding and renewed with every media change: 5 ng/ml recombinant human TGFβ1, 10 ng/ml Activin A, 100 ng/ml FS288 (all from R&D Systems) or 1 μM A83-01 (Tocris).

Scratch assays

Cells were grown to 100% confluence then a scratch was introduced using a 200 μl pipette tip. Measurement areas were marked at six different locations along the scratch. Cells were imaged at 0, 6, and 24 hours post-scratch and distance of cells traveled was measured using the Axiovision software (Carl Zeiss Microscopy, Thornwood, NY).

Colony formation

Colony formation assays were performed by plating 500 cells in six-well plates and maintaining them in complete media for 7–8 days (492). Cells were then fixed with 100% methanol for 10 minutes at −20°C and stained overnight in 0.1% crystal violet at room temperature. Colony counts were assessed using the GelCount™ system and software (Oxford Optronix, Abingdon, UK), courtesy of the Vanderbilt Digital Histology Shared Resource.

Cell migration and invasion assays

Migration and Matrigel invasion chamber assays were purchased from BD Biosciences (Franklin Lakes, NJ) and performed according to manufacturer's direction. After removal of cells from the top of the membrane, cells were fixed in 100% methanol at −20°C for 10 minutes, then rinsed once in 1XPBS. For quantification, cells were stained with 0.3% Janus green (Sigma, St. Louis, MO, cat. no. 201677) for 5 minutes at room temperature. Upon washing, cells were destained with 0.5 M HCl for 10 minutes at room temperature. The HCl solution was collected and transferred to a 96-well plate and absorbance read at 595 nm on a BioTek Synergy 4 microplate reader (BioTek Instruments, Inc., Winooski, VT). Subsequently, cells were stained in 0.1% crystal violet overnight, mounted, and imaged as previously described (356).

Proliferation assays

Cells were plated at 1000 cells per well in a 96-well plate for proliferation assays. WST-1 reagent (Roche, Nutley, NJ) was added to each well at the time points indicated and incubated at 37°C for 1 hour. Absorbance measurements at 450 nm were taken using a BioTek Synergy 4 plate reader (BioTek Instruments, Inc.). Measurements were taken in 24-hour increments.

ELISA

Cells were seeded at 166,000 cells per 6-well insert with full medium. The next day, cells either underwent treatment or culture media were changed to serum-free media before conditioned media was harvested 48 hours thereafter. Cell number was determined and concentration per one ml media was calculated and normalized per 100,000 cells. Capture ELISAs for Activin A,

TGFβ1 and pan-follistatin (FS288, FS300, FS315) were purchased from and performed following manufacturer's instructions (R&D Systems). INHA was measured using an ELISA kit purchased from Cloud Clone Corp (Houston, TX).

Western blot

Western blots were performed as previously described (356). Cells undergoing treatment with Activin A, FS288, A83-01, or TGFβ1 had the individual growth factors added to serum-free cell culture media for 30 minutes or 48 hours, followed by protein lysis. The results are representative of at least three independent experiments.

Immunofluorescence

Organotypic culture tissue, previously fixed in 10% neutral buffered formalin for 24 hours and embedded in paraffin, was sectioned at 5 μm, deparaffinized, and heated in 1XTE buffer in a pressure cooker for 12 minutes for antigen retrieval. Samples were blocked in 1XPBS with 5% Bovine Serum Albumin (Sigma), 1XPBS-BSA, for one hour prior to incubation with primary antibodies in 1XPBS-BSA overnight at 4°C. Tissues were then rinsed three times in 1XPBS and incubated with secondary antibodies in 1XPBS-BSA for one hour at room temperature. After additional rinses with 1XPBS, the sections were mounted with Vectashield mounting medium containing DAPI (Vector Laboratories, Burlingame, CA). Images were taken on a Zeiss microscope, using Axiocam and Axiovision software (Carl Zeiss Microscopy). Alcian Blue Staining was performed by the Translational Pathology Shared Resource at Vanderbilt University Medical Center.

Antibodies and other reagents

SOX2, total Smad 2, phospho-Smad2, phospho-Smad1/5/8, total ERK1/2, phospho-ERK1/2, total Akt, and phospho-Akt were purchased from Cell Signaling Technologies (Danvers, MA), and α-tubulin from Abcam (Cambridge, MA). Anti-TGFBR2 (clone L21) was purchased from Santa Cruz Biotechnologies (Santa Cruz, CA), E-cadherin and Keratin 8 from BD Bioscience, and vimentin from Sigma. Other antibodies used: MT1-MMP (Epitomics, Cambridge, MA) and anti-CD44 clone 2C5 (R&D Systems); SOX9 (EMD Millipore, Rockland, MA); Keratin K13 (Novus Biologicals, Littleton, CO).

Dataset analysis

Dataset GDS1321 was used to query clinical correlations with Activin A, publicly available from GEO Datasets (http://www.ncbi.nlm.nih.gov/gds/). The collected information from each dataset was analyzed and visualized in Prism version 6.00 for Mac (GraphPad software, La Jolla, California).

Biostatical analysis

Biostatistical analysis was performed using Prism version 6.00 for Mac (GraphPad). *In vitro* and *in vivo* experiments were analyzed using Student's *t*-tests, one- or two-way ANOVAs. Statistical significance was set at $p < 0.05$. Pearson's correlation coefficients were calculated. All experiments were done in triplicate with at least three biological replicates.

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