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Activin Upregulation by NF- κ B is Required to Maintain Mesenchymal Features of Cancer Stem-like Cells in Non-Small Cell Lung Cancer

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Abstract

Soluble growth factors and cytokines within the tumor microenvironment aid in the induction of the epithelial-to-mesenchymal transition (EMT). Although EMT promotes the development of cancer-initiating cells (CICs), cellular mechanisms by which cancer cells maintain mesenchymal phenotypes remain poorly understood. Work presented here indicates that induction of EMT stimulates non-small cell lung cancer (NSCLC) to secrete soluble factors that function in an autocrine fashion. Using gene expression profiling of all annotated and predicted secreted gene products, we find that NF- κ B activity is required to upregulate INHBA/Activin, a morphogen in the TGF β superfamily. INHBA is capable of inducing and maintaining mesenchymal phenotypes, including the expression of EMT master-switch regulators and self-renewal factors that sustain CIC phenotypes and promote lung metastasis. Our work demonstrates that INHBA mRNA and protein expression is commonly elevated in primary human NSCLC and provide evidence that INHBA is a critical autocrine factor that maintains mesenchymal properties of CICs to promote metastasis in NSCLC.

Keywords

Activin; non-small cell lung cancer; metastasis; cancer-initiating cells; NF- κ B

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AUTHOR CONTRIBUTIONS

Designed experiments: JJW MK DFA MWM. Performed experiments: JJW MK DFA SHC CMH SJS. Contributed materials and methods: SB DRJ. Performed bioinformatic analysis: SAH XX SB. Analyzed the data: JJW MK DFA SHC CMH SJS SAH XX DRJ SB MWM. Wrote the paper: JJW MK MWM.

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INTRODUCTION

Non-small cell lung cancer (NSCLC) remains one of the leading causes of cancer-related mortality in the Western world. There are three major types of NSCLC, adenocarcinomas (ADC), squamous cell carcinomas (SCC) and large cell carcinomas (LCC). The five-year survival rate is below 17% due to late-stage diagnosis and the frequency of metastasis (1). Progression from carcinoma *in situ* to metastatic disease is a complex, multi-step process that relies on interactions between carcinoma cells and constituents of the microenvironment. Cytokines and growth factors within the NSCLC tumor microenvironment trigger a cellular re-programming event known as the epithelial-to-mesenchymal transition (EMT) (2, 3). Mesenchymal cells acquire resistance to apoptotic cues, upregulate genes that promote self-renewal, and display properties of cancer-initiating cells (CICs) (2).

Transforming growth factor beta (TGF β) is one of the best studied inducers of EMT (4, 5). TGF β , Nodal, and Activins are all members of the TGF β superfamily (6). Although TGF β signaling drives EMT, efficient transition depends on the activation of other co-stimulatory pathways. Tumor necrosis factor (TNF), a pro-inflammatory cytokine, activates NF- κ B to synergize with TGF β in inducing EMT (7–9).

NF- κ B is a pleiotropic transcription factor that promotes oncogenesis by upregulating genes involved in cancer processes (10). In the canonical signaling pathway, transcriptionally competent NF- κ B is comprised of p50 and RelA (11). In unstimulated cells, this heterodimer is sequestered in the cytosol by inhibitor of κ B-alpha (I κ B α). Upon stimulation, I κ B α is phosphorylated by the I κ B kinase (IKK), triggering its polyubiquitination and degradation. This liberates NF- κ B and promotes its nuclear translocation. Posttranslational modifications of RelA regulate chromatin occupancy and recruitment of co-activator complexes required for transcription (12–17).

NF- κ B transcription is commonly deregulated in NSCLC and correlates with metastasis and poor clinical outcome (18). NF- κ B and IKK signaling are known to regulate self-renewal of CICs. Mice expressing a non-functional IKK α protein suppressed mammary tumor development by impairing self-renewal of CICs (19). Moreover, expression of a non-degradable form of I κ B α inhibited NF- κ B activation and delayed mammary tumor development by reducing the CIC population (20). Both the canonical and non-canonical NF- κ B signaling were shown to be required for self-renewal of breast CICs by regulating EMT and expression of inflammatory cytokines (21). Recently, our laboratory showed that NF- κ B activity is required for expression of *Twist1/Twist*, *SNAI2/Slug*, and *ZEB2/Sip1* to drive EMT and CIC phenotypes in NSCLC (9).

Although NF- κ B signaling pathways have been shown to regulate EMT and the maintenance of CICs, molecular targets by which NF- κ B exerts these effects remain poorly understood. This study provides evidence that TGF β /TNF-induced EMT activates NF- κ B to upregulate *INHBA/Activin*, which is required to maintain CICs properties and promote NSCLC metastasis.

MATERIALS AND METHODS

Cell Culture and Reagents

NSCLC ADC (A549 and H522), SCC (H226), and LCC (H460 and H1299), were obtained and cultured as recommended (ATCC). All cell lines were authenticated by ATCC. Three-dimensional (3D) multicellular spheroid cultures were generated as previously described (9). Unless otherwise stated, EMT was induced over 96 hours by stimulation with TNF (Invitrogen, 10 ng/mL) and TGF β -1 (Invitrogen, 2 ng/mL) (9). Reagents include recombinant Activin (Gibco) and the ALK4/5/7 inhibitor SB431542 (Sigma) and the ALK5i (Calbiochem).

Stable Cell Lines

A549 stable cell lines expressing the super-repressor (SR) I κ B α (A549.I) or vector control (A549.V) were created by retroviral delivery of pBABE-Puro (vector) or pBABE-Flag-SR-I κ B α (9). A549 cell lines stably expressing control shRNA or one of three shRNA sequences targeting *INHBA/Activin* were generated using replication-defective lentivirus (Thermo Scientific, Activin.1–3, Supplemental Table S1). Following puromycin (1.5 μ g/mL) selection, 3D cultures were created with each pool and Activin mRNA and protein levels were measured. The shRNA Activin.2 yielded the best knockdown and loss of protein expression (Supplemental Figure 4A and 4B). Three individual clones were isolated and used to create the Activin.2 clonal pool (A549.A). Similar methods were used to generate the shRNA control clonal pool (A549.C).

Primary Human NSCLC Cells

Fresh NSCLC tumors were washed in PBS, minced with scalpels, and incubated with type IV collagenase (Sigma, 1 mg/mL), type V hyaluronidase (Sigma, 100 μ g/mL), and type I DNase (Sigma, 20 μ g/mL) for thirty minutes. Following tissue digestion, cells were pelleted, washed, and resuspended in RPMI/10% FBS/Pen/Strep and then used to generate 3D cultures (9). Human investigations were performed after approval by an institutional review board (Protocol #9508).

Conditioned Media Experiments

Conditioned media (CM) from 2D and 3D cultures either left untreated or stimulated with TNF and TGF β were harvested and filtered through a 0.22 μ m pore. Supernatants were added fresh to naïve 3D (N3D) cultures for 48 hours. After two days, cultures were pelleted, media removed, cultures were incubated with a second batch of fresh CM, and cells were harvested 48 hours later. For neutralizing antibody experiments, filtered CM were pre-treated with either an Activin neutralizing antibody (R&D, 2, 4 μ g/mL) or with an IgG mouse control antibody (Millipore, 0, 4 μ g/mL) for four hours at 37°C with agitation before incubating with N3D cultures.

Microarray Analysis

Monolayer and spheroid cultures were treated with TNF and TGF β or left unstimulated for 96 hours before harvesting. Total RNA was isolated using the RNeasy Total RNA Minikit

(Chemstores) and reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad). Technical duplicates of the experiment were performed, and cDNA from both experiments were labeled and hybridized to a GeneChip Human Genome U133 Plus 2.0 Array (Affymetrix). Normalized, background-adjusted, log₂ expression estimates which account for probe affinity effects were derived using GCRMA (22). Genes that were differentially expressed between 1) 2D treated vs. untreated and 2) 3D treated vs. untreated were identified using the Limma package within Bioconductor (23) by applying a 5% false discovery rate (FDR) cutoff. Data have been deposited in GEO (GSE42373).

Quantitative Real-Time Polymerase Chain Reaction (QRT-PCR) and Immunoblots

QRT-PCR and immunoblot analysis were carried out as previously described (9). Primers used were either previously published (9) or listed (Supplemental Table S2). The antibodies used in this study for immunoblot are E-cadherin (BD), N-cadherin (BD), vimentin (Sigma), α -tubulin (Sigma), and GAPDH (GeneTex).

ELISA

Supernatants from 2D and 3D cultures untreated or treated with TNF and TGF β for 96 hours were harvested and filtered (0.22 μ m). An ELISA kit (R&D) was used to measure Activin levels in the supernatants (100 μ L aliquots), as per manufacturer's protocol.

Transwell Assays and Lung Metastasis

Spheroid cultures were disaggregated by incubation in 0.05% trypsin for ten minutes with intermittent gentle trituration. Transwell assays were performed to measure migration and invasion as previously described (9). For lung metastasis, 4–5 week old female outbred Crl:NU/NU nude mice (Jackson Laboratories) were injected subcutaneously (SC, 1×10^6 cells/site) with cells from disaggregated A549.C or A549.A spheroid cultures left unstimulated or treated with cytokines. After 40 days, animals were euthanized following approved procedures by the UVA ACUC. Lung surface metastatic foci were counted using a dissecting scope 40 days following SC injection.

Immunohistochemistry of Activin

Zinc formalin-fixed paraffin-embedded tissue blocks for ADC and SCC were retrieved from the archives of the UVA Pathology Department and prepared as previously described (49). Alternatively, LCC TMAs were purchased (LC121a, US Biomax). The avidin-biotin immunoperoxidase technique was performed as previously described (49) and slides were counterstained with hematoxylin. Immunohistochemistry (IHC) was performed using Activin antibody HPA020031 (Sigma-Aldrich). Immunostains were examined in an objective semi-quantitative fashion with a score assigned to staining intensity (1 = weak, 2 = moderate, 3 = strong) and a quartile score for percent of tumor cells stained (0=no staining, 1=1–25%, 2=26–50%, 3=51–75% and 4=76–100%). The two scores for each sample were multiplied to arrive at an index score (range 1–12).

Statistics

All data are presented as mean $\pm$ SD of three independent biological experiments. When two experimental groups were compared, values were log₂-transformed, and a one-tailed Student's *t* test in Microsoft Excel was performed. Differences were considered statistically significant when indicated by *p* 0.05 (indicated by *), *p* 0.01 (**), or *p* 0.005 (***).

RESULTS

Mesenchymal A549 cells produce soluble factors capable of inducing EMT

Recently published data from our laboratory demonstrate that TNF and TGF β -stimulated NSCLC spheroid (3D) cultures effectively undergo EMT over a four day period (9). Since the half-life of recombinant cytokines in growth media is short (24), we postulated that A549 cells produce autocrine factors to maintain the mesenchymal state. To test this hypothesis, we harvested conditioned media (CM) from untreated or cytokine-stimulated 2D and 3D A549 cultures. Naïve 3D (N3D) cultures were then incubated with CM for 96 hours. Supernatants obtained from cytokine-treated 3D cultures effectively down-regulated E-cadherin while up-regulating N-cadherin and vimentin (Figure 1A). This effect was not due to residual TNF or TGF β remaining in the media, as supernatant obtained from cytokine-treated 2D cultures did not elicit the same response. Production of autocrine factors required both TNF and TGF β , since CM from spheroid cultures treated with either alone did not induce EMT in N3D cultures, as assayed by downregulation of E-cadherin or increased expression of N-cadherin and vimentin (Figure 1B).

RNA expression profiling identifies *INHBA/Activin A* as an autocrine factor

To identify potential autocrine factors responsible for inducing EMT, we performed microarray and bioinformatic analysis using a “top-down” approach. Microarray analysis performed on 2D and 3D A549 cultures, unstimulated or treated with TNF and TGF β , showed massive and reproducible changes in gene expression (25) (Supplemental Figure 1A). Microarray analysis identified 4064 differentially regulated genes specific to the 3D treated versus untreated cultures. Of these, 1351 genes showed increased expression upon stimulation. This 1351 gene list was then analyzed for overlap with a library of 1636 genes encoding secreted/extracellular proteins (Secreted Protein Database, SPD 26). As illustrated in the Venn diagram in Figure 1C, a 128 gene overlap was identified between differentially upregulated genes in cytokine-treated 3D cultures and the SPD library. Many of the genes in this overlap encode secreted protein ligands that are highly upregulated following the induction of EMT in 3D cultures (Table 1A).

Interestingly, this list included Inhibin β A (*INHBA*). Inhibin β subunits dimerize with each other to form Activins or with the α subunit to make Inhibins (27). Under physiological conditions, Activin A (inhibin β A homodimer) functions as a pluripotent factor for human embryonic stem cells (hESCs) (28,29) and in endoderm and mesoderm formation (30–32). However, in melanoma and pancreatic cancer, Nodal/Activin A signaling has been linked to more aggressive phenotypes and self-renewal of CICs (33,34).

Importantly, in our microarray data, other members of the TGF β superfamily, including *NODAL*, the *TGFBs*, and other Inhibin (α/β) subunits, are not transcriptionally upregulated to the same extent as *INHBA/Activin A* in cytokine-treated cultures (Table 1B). These results were confirmed by QRT-PCR (Supplemental Figure 1B). The unique upregulation of *INHBA* among Inhibin α/β subunits following EMT suggests that these βA subunits predominately homodimerize to produce Activin A, rather than Inhibin or other Activins. In the remainder of the manuscript, we refer to *INHBA/Activin A* as Activin. Thus, our analysis identifies Activin as a potential candidate that may be responsible for inducing EMT in naïve 3D cultures.

Upregulation of *Activin* in cells derived from primary lung tumors

To confirm microarray results shown in Tables 1A and 1B, QRT-PCR was performed on A549 3D cultures following exposure to TNF, TGF β , or both cytokines. Although TGF β alone modestly increased *Activin* mRNA levels, treatment with both TNF and TGF β significantly upregulated *Activin* transcripts (Figure 2A). Importantly, A549 and H1299 cells upregulate *Activin* levels specifically in 3D cultures (Figure 2B). In A549 cells, *Activin* transcription was stimulated within 24 hours of cytokine treatment and steadily increased over a four day period, indicating that cells are capable of maintaining *Activin* expression (Supplemental Figure 2A). The ability of NSCLC cells to upregulate *Activin* levels was not cell line specific, but was observed in cytokine treated 3D cultures for the adenocarcinoma (ADC, H522), squamous cell carcinoma (SCC, H226) and large cell carcinoma lines (LCC, Supplemental Figure 2B). Importantly, the upregulation of *Activin* mRNA levels observed in Figure 2A and 2B corresponded with elevated protein levels in the media, as determined by ELISA (Figure 2C).

Next, CM from cytokine-treated 3D A549 cultures were incubated for two hours with either an Activin-specific neutralizing antibody or with rabbit IgG control. CM were then added to N3D cultures for 96 hours, and immunoblot analysis was performed. Similar to results shown in Figure 1A, N3D cultures incubated with supernatants from cytokine-treated spheres effectively downregulated E-cadherin and upregulated N-cadherin, compared to N3D cultures incubated with CM from unstimulated spheroid cultures (Figure 2D). Neutralizing Activin activity attenuated the ability of CM to induce EMT in N3D cultures, indicating that the secretion of Activin into the media induces EMT. Moreover, treatment of N3D A549 or H1299 cultures with recombinant Activin and TNF induced N-cadherin and vimentin protein levels as effectively as TGF β and TNF (Figure 2E). Experiments in Figure 2D and 2E indicate that Activin is required for full induction of EMT in N3D cultures.

Next, we examined whether NSCLC cells derived from primary human tumors expressed Activin and whether levels increased following cytokine treatment. Two primary human NSCLC ADC and SCC tumors were disaggregated. Spheroid cultures were generated and either left untreated or stimulated with TNF and TGF β . Supernatants were harvested 96 hours later and assayed by ELISA. CM from unstimulated primary human NSCLC spheroid cultures contained high levels of Activin, relative to untreated A549 3D cultures (Figure 2F). Moreover, Activin levels further increased in primary NSCLC cultures following exposure to TNF and TGF β . These data indicate that spheroid cultures from both established and

primary NSCLC cells express Activin, an event that is potentiated following cytokine treatment.

NF- κ B upregulates *Activin* to promote expression of EMT markers

Since we and others have recently shown that NF- κ B is required for EMT and development of CICs in NSCLC (9, 21), we wanted to determine if *Activin* is transcriptionally regulated by NF- κ B. Before addressing this question, we examined the individual and combined contribution of TNF, TGF β , and Activin on NF- κ B and SMAD signaling pathways. While TGF β , and Activin alone resulted in strong constitutive SMAD2 phosphorylation, maximal RelA phosphorylation was only observed following combined treatment with TNF and TGF β (Figure 3A).

Next, A549 stable cell lines were developed that ectopically express the vector control (A549.V) or the dominant negative super-repressor I κ B α (SR-I κ B α , A549.I). A549.I cells fail to induce NF- κ B transcription because expression of the SR-I κ B α prevents nuclear translocation in response to physiological stimuli (9). A549.I cells were unable to fully upregulate *Activin* expression following TNF and TGF β treatment, compared to controls A549.V (Figure 3B). In contrast, the A549.I line demonstrated full transcriptional induction of bone morphogenic protein 2 (*BMP2*), another TGF β superfamily member that is not regulated by NF- κ B. As predicted from the QRT-PCR data, A549.I cells secrete less Activin into the media than the A549.V line (Figure 3C). Moreover, when compared to control cells, the A549.I line was unable to produce autocrine factors capable of inducing EMT in N3D cultures (Figure 3D). Data shown in Figure 3B–D indicate that the loss of NF- κ B transcriptional activity results in the inability of CM to induce EMT in N3D cultures, an effect that correlates with a loss of Activin expression and secretion.

Since results shown in Figure 3D could be due to a failure of A549.I cells to undergo EMT in the absence of NF- κ B activity, we examined whether CM induced EMT through TGF β /Activin/Nodal signaling pathways. To address this question, TGF β /Activin/Nodal signaling was blocked in cultures using SB431542 or ALK5i. SB431542 blocks Activin type I receptor (ALK4), TGF β type I receptor (ALK5), and Nodal type I receptor (ALK7) (36), while the ALK5i inhibitor selectively blocks the TGF β type I receptor (ALK5). CM were isolated and incubated with N3D cultures in either the absence or presence of SB431542 or ALK5i. While ALK5i treated cultures showed a slight reduction in RelA and SMAD2 phosphorylation, compared to vehicle control treated cultures, SB431542 completely abolished signaling (Figure 3E). Importantly, the SB431542 (SB) inhibitor blocked the ability of N3D cultures to respond to cytokine-treated CM as measured by reduced expression of the EMT master-switch transcription factors *SNAIL1/Snail*, *SNAIL2/Slug*, *TWIST1/Twist* and *ZEB2/Sip1*, and mesenchymal markers *CDH2/N-cadherin* and *VIM/Vimentin*, compared to vehicle control treated CM (Figure 3F and Supplemental Figure 3). Although SB431542 blocks signaling in response to Activin, Nodal and TGF β ligands, only Activin is upregulated in A549 3D cultures in response to TNF and TGF β (Table 1B, Supplemental Figure 1B). Thus, the ability of SB431542 to block expression of EMT markers supports the hypothesis that Activin functions to sustain mesenchymal phenotype in NSCLC CICs.

Activin expression is required to maintain EMT in NSCLC

To further examine the importance of Activin, we created stable A549 cell lines expressing either non-targeting shRNA control (A549.C) or shRNA to Activin (A549.A). All three shRNAs to Activin caused a knockdown of mRNA and protein expression, reducing the possibility of off-target effects (Supplemental Figure 4A and 4B). However, stable A549 cells expressing shRNA Activin.2 gave the best knockdown and were used in further analysis. As predicted, cytokine-treated spheroid cultures of A549.A showed a significant knockdown of Activin mRNA and protein expression, compared to A549.C (Figure 4A and 4B). Next, supernatants isolated from A549.C and A549.A cells were harvested and used to treat N3D cultures. As expected, CM from A549.C cells downregulated E-cadherin and upregulated vimentin protein expression in N3D cultures, while supernatants from A549.A were less efficient at modulating these markers (Figure 4C). Importantly, A549.A cells exhibit statistically significantly lower levels of EMT master-switch transcription factors *SNAI1*, *SNAI2*, and *ZEB2*, compared to control cells (Figure 4D). However, the knockdown of Activin had no effect on *TWIST1* expression. Activin was required to sustain expression of *MYCN*, *SOX2*, *KLF4*, and *HMGA2* (Figure 4E), supporting the role of Activin as a maintenance factor required for the expression of genes known to induce self-renewal in CICs (9, 37–40).

Next, we examined whether Activin was also required to sustain expression of gene products that promote cell migration and invasion. The knockdown of Activin resulted in the loss of induction of collagen type XXII (*COL22A1*), matrix metalloproteinases *MMP2* and *MMP9*, and lysyl oxidase (*LOX*) (Figure 5A). Moreover, Activin expression was required for cell migration and invasion of cytokine-treated spheroid A549 cultures, as assayed in transwell assays (Figure 5B and Supplemental Figure 5). The knockdown of Activin resulted in elevated cell migration in unstimulated cells, through an unknown mechanism that may be linked to the fact that low levels of Activin A are required to form Inhibin (27).

Recently, we published that induction of EMT in A549 cells promotes the development of CICs that are highly metastatic under limiting cell dilution (41,42) without the need for subcutaneous (SC) tumor growth (9). To determine the importance of Activin for metastatic potential of CICs, A549.C and A549.A spheroid cultures were treated with cytokines, disaggregated, and injected SC into nude mice. Lung surface metastases were quantified after 40 days. The knockdown of Activin significantly impaired the ability of A549 cells to metastasize to the lung of nude mice when compared to control cells (Figure 5C).

Collectively, our results indicate that TNF and TGF β -induced expression of Activin is a critical step required to sustain mesenchymal properties that promote CIC metastasis.

Elevated Activin in primary NSCLC tumors correlates with metastasis

Since our data thus far indicates that Activin is a key regulator of NSCLC invasion and metastasis, we analyzed expression data to determine whether *Activin* mRNA expression was elevated in lung adenocarcinomas (ADC) and squamous cell carcinomas (SCC), two of the largest cohort of patients diagnosed with NSCLC worldwide. In both types of NSCLC, *Activin* was significantly elevated and correlated with distal metastasis in SCC (Figure 6A and 6B). Next, immunohistochemistry was performed on primary NSCLC tumors. Activin

protein expression was elevated in 13/17 (76%) of ADC and 18/23 (78%) of SCC, while 13/36 (36%) of LCC showed positive IHC staining (Figure 6C). The IHC staining intensities for Activin levels in LCC was lower most likely due to our discovery that fresh-cut TMAs are needed to detect Activin in primary tissues. Similar to a previous study by Seder and colleagues in which ADC were examined (46), we find that Activin expression is elevated in primary tumors ADC, SCC and LCC, and constitutes an important extracellular cytokine capable of promoting EMT in both and autocrine- and paracrine-dependent manner.

DISCUSSION

The fields of EMT and cancer stem cell biology were brought together by the discovery that epithelial mammary carcinoma cells dedifferentiate to exhibit mesenchymal and CIC properties (38, 39). It is now well accepted that spheroid culturing of carcinomas in the presence of growth factors or cytokines enriches for CICs that display self-renewal capabilities and form tumors under limiting cell dilutions (43). Thus, targeting CICs has been proposed as an approach to treat epithelial malignancies (44, 45). Despite this current knowledge, therapies for eradicating CICs have been limited due to our poor understanding of how these cells maintain their mesenchymal phenotype throughout the metastatic process.

Work presented in this study indicates that TNF and TGF β -stimulated NSCLC spheroid cultures upregulate and secrete Activin. This induction was found to occur in both established cell lines as well as tumor-derived primary NSCLC cells. This observation may be highly significant with respect to the pathophysiology of NSCLC, since we find that primary human lung ADC, SCC and LCC show a significant upregulation of Activin expression, compared to non-malignant lung (Figure 6C). Importantly, our findings are in agreement with the study by Seder and colleagues, which originally identified Activin overexpression in primary lung ADC (46). Inhibition of Activin signaling using RNAi or pharmacological inhibition of the ALK4/5/7 receptor indicates that Activin induces the expression of EMT master-switch regulators and self-renewing factors required for CIC phenotypes. As an extension to what is understood about Nodal/Activin signaling as a self-renewing factor required for CICs (33), our studies indicate that Activin promotes NSCLC invasion and metastasis. Overall, our results indicate that Activin signaling is important for induction and maintenance of CICs and that Activin is an important molecular target that could be used to treat NSCLC.

In an elegant study by Lonardo and colleagues, Nodal/Activin signaling was reported to be required for the tumorigenicity of pancreatic carcinoma by supporting the self-renewal capacity of CICs (33). Primary pancreatic spheroid cultures cultured in basic fibroblast growth factor (bFGF) and epithelial growth factor (EGF) express transcripts encoding Nodal and Activin. Although both recombinant Nodal and Activin support CIC self-renewal, the knockdown of Nodal is sufficient to suppress this phenotype. Therefore, Nodal, not Activin, is the predominant TGF β family member driving self-renewal phenotypes in pancreatic CICs (33). This is in contrast to our findings indicating that Activin, not Nodal, drives EMT, expression of self-renewing factors, and metastatic potential of NSCLC CICs. Additionally, in esophageal carcinoma, Activin, not Nodal, contributes to invasive, aggressive phenotypes (47). It is not clear why different epithelial CICs demonstrate a distinct requirement for

autocrine production of either Nodal or Activin. Although the difference could be due to tissue specificity, this is unlikely since the endoderm is responsible for the development of the lungs, esophagus and pancreas. A more plausible explanation is that exposure of carcinoma spheroid cultures to either growth factors (bFGF and EGF) or cytokines (TNF and TGF β) dictates whether the CICs upregulate autocrine signaling of either Nodal or Activin. The ability of TNF and TGF β to upregulate Activin is supported by our observation that cytokine treatment results in constitutive NF- κ B activity (9), which is required to upregulate Activin secretion (Figures 3B and 3C). These effects are also compounded by the fact that under some cellular contexts both TGF β and Activin have also been shown to promote proliferation (46).

Inhibiting Nodal/Activin signaling offers a potentially important therapeutic strategy to target epithelial CICs when used in combination with chemotherapy. Although the use of the ALK4/5/7 inhibitor SB431542 as a single therapy does not prevent tumor development *in vivo*, combined treatment with both SB431542 and gemcitabine effectively reduced tumor growth in pancreatic carcinoma (33). However, targeting the CIC population alone may not be an effective strategy: Lonardo and colleagues found that tumor-associated stroma not only produce Nodal/Activin that can act in a paracrine-dependent manner to support CICs, but stroma may also provide resistance to the chemosensitizing effect of SB431542 and gemcitabine. Thus, successful chemotherapeutic targeting of CICs will most likely involve the use of multiple agents that abolish Nodal/Activin signaling within the tumor microenvironment.

The inability of ALK4/5/7 inhibitor SB431542 to chemosensitize may also have to do with the built-in redundancy of growth factors and inflammatory mediators that support CIC propagation. Although Activin and Nodal are clearly important for maintaining CIC self-renewal, these factors do not act alone. For example, Activin and Nodal have been shown to maintain pluripotency of hESCs when cultured in the presence of bFGF (28). In a similar manner, our work supports the role of TNF as a costimulatory signal required to potentiate EMT in response to recombinant Activin (Figure 2E). In light of the recent work by Kendellen and colleagues (21), it will be important to determine if inflammatory cytokines, such as interleukin (IL)-1 β and IL-6, work in concert with Activin. This is especially important since TNF was unable to potentiate the ability of Activin to signal through NF- κ B or SMAD-dependent pathways (Figure 3A). Work currently underway in the laboratory will examine how different protein ligands promote CICs development through the upregulation of Activin and will examine how these pathways are impacted by the tumor microenvironment. Such studies will firmly establish the importance of Activin signaling in CIC development and metastasis of NSCLC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Mesenchymal A549 cells produce paracrine factors capable of inducing EMT in N3D cultures

(A) N3D cultures were incubated with CM harvested from 2D or 3D cultures left untreated (–) or stimulated (+) with TNF and TGFβ prior to immunoblot analysis. (B) N3D cultures were incubated with CM harvested from 3D cultures left untreated or treated with TNF, TGFβ, or both TNF and TGFβ prior to immunoblot analysis. (C) Venn diagram indicating the 128 gene overlap (orange) between the 1636 genes from the SPD (red), and the 1351 unique genes that are upregulated in 3D treated cultures (yellow, 5% FDR threshold was applied).

Figure 2. Upregulation of Activin in NSCLC lines and primary human lung tumor cells
(A) A549 3D cultures were left untreated (–) or treated (+) with TNF, TGFβ, or both TNF and TGFβ, prior to QRT-PCR. **(B and C)** A549 and H1299 cultures (2D and 3D) were left unstimulated (No Add) or treated with cytokines (TNF/TGF) prior to QRT-PCR and ELISA analysis. **(D)** CM from 3D cultures either left untreated (–) or stimulated (+) with TNF and TGFβ were incubated with either IgG control antibody or with Activin neutralizing antibody for four hours. N3D cultures were incubated with this pre-treated CM prior to immunoblot analysis. **(E)** Spheroid A549 or H1299 cultures were either left untreated (–) or stimulated (+) with recombinant Activin (100 ng/mL), TNF, TGFβ, or a combination of cytokines prior to immunoblot analysis. **(F)** Spheroid cultures were generated from A549 cells or from primary lung tumor cells isolated from human ADC or SCC and were left untreated or stimulated with TNF and TGFβ prior to ELISA. QRT-PCR and ELISA results are mean ± SD, **p* 0.05, ***p* 0.01, ****p* 0.005, N=3.

Figure 3. NF-κB upregulates *Activin* to promote EMT

(A) Spheroid A549 cultures were either left untreated (–) or stimulated (+) with recombinant Activin, TNF, TGFβ, or a combination of cytokines prior to immunoblot analysis for phospho-specific RelA and SMAD2. (B and C) Spheroid A549 cultures expressing the non-degradable mutant IκBα (A549.I) or vector control (A549.V) were left unstimulated or treated with TNF and TGFβ prior to QRT-PCR and ELISA. (D) N3D cultures were incubated with CM from either cytokine-treated or unstimulated 3D A549.V or A549.I cultures prior to immunoblot analysis. (E) Spheroid A549 cultures were pre-treated for two hours with DMSO, SB431542 (5 μM), or ALK5i (50 nM) prior to incubation with CM and immunoblot analysis performed 96 hours later. (F) N3D cultures were pre-treated for two hours with DMSO or SB431542 prior to incubation with CM from control (No Add) or cytokine-treated (TNF/TGF) 3D cultures. Gene expression was determined by QRT-PCR. QRT-PCR and ELISA results are mean ± SD, **p* 0.05, ***p* 0.01, ****p* 0.005, N=3.

Figure 4. Activin expression is required to maintain EMT in NSCLC

(**A and B**) Knockdown of Activin in A549 (A549.A) cells was sustained in cytokine-treated 3D cultures, compared to A549.C, as determined by QRT-PCR and ELISA. (**C**) N3D cultures were incubated with CM harvested from untreated and cytokine-stimulated 3D A549.C and A549.A cells prior to immunoblot analysis. (**D and E**) A549.C and A549.A spheroid cultures were left untreated or stimulated with cytokines prior to QRT-PCR. QRT-PCR and ELISA results are calculated mean $\pm$ SD, * p 0.05, ** p 0.01, *** p 0.005, N=3.

Figure 5. Invasive and metastatic potential of A549 cells is dependent on Activin expression

(A) A549.C and A549.A spheroid cultures were left untreated or stimulated with cytokines prior to QRT-PCR. (B) Untreated or cytokine-treated A549.C and A549.A 3D cultures were dissociated and subjected to migration and invasion assays. (C) Spheroid A549.C and A549.A cultures, either untreated or treated with cytokines, were disaggregated and injected SC into nude mice (1×10^6 cells/animal). Forty days later, the lungs were removed, and the number of surface metastases was enumerated. Metastasis data are mean $\pm$ SD of five mice per condition, N=2. QRT-PCR, ELISA, and transwell assay data are mean $\pm$ SD, * p 0.05, ** p 0.01, *** p 0.005, N=3.

Figure 6. Activin expression is elevated and correlates with advance stage NSCLC
(A and B) Oncomine results for human NSCLC expression of *INHBA/Activin* (48). **(A)** *Activin* mRNA expression is elevated in ADC and SCC in the Cancer Genome Atlas (TCGA, <https://tcga-data.nci.nih.gov/tcga/tcgaHome2.jsp>) lung. **(B)** *Activin* overexpression in SCC correlates with advance M1+ stage, compared to M0 ($P=0.017$). **(C)** Representative images of human tissue microarray staining results for Activin expression in benign lung and malignant lung as well as the summary of the overall Activin score index. For A and B: dots represent maximum and minimum values, whiskers show 90th and 10th percentiles, boxes show 75th and 25th percentiles, and line shows median. The P -value in B was calculated using Oncomine software Student's t -test.

Table 1
Mesenchymal NSCLC significantly upregulate *Activin* transcripts

(A) Microarray and bioinformatic analysis identified 128 genes that belong to the Secreted Protein Database and that are significantly upregulated in 3D cultures following TNF and TGF β treatment. Of these, the 15 most upregulated genes that encode for ligands are shown. The FDR-adjusted *p*-values for the fold induction were 0.05. FC=fold change. (B) The differential expression of select TGF β superfamily members following cytokine treatment in both 2D and 3D cultures was examined.

TABLE 1A: Most Upregulated Genes Encoding Secreted Ligands Following Cytokine Stimulation in Three-Dimensional Cultures

Rank	Symbol	GenBank	FC (3D ⁻ →3D ⁺)
1	<i>GAL</i>	NM_015973.3	220
2	<i>CSF2</i>	NM_000758.3	201
3	<i>INHBA</i>	NM_002192.2	160
4	<i>PDGFB</i>	NM_002608.2	111
5	<i>CCL5</i>	NM_002985.2	100
6	<i>IL11</i>	NM_000641.3	98
7	<i>BMP6</i>	NM_001718.4	58
8	<i>PTH1H</i>	NM_002820.2	41
9	<i>EDIL3</i>	NM_005711.3	40
10	<i>CRLF1</i>	NM_004750.4	15
11	<i>CYR61</i>	NM_001554.4	15
12	<i>CSF3</i>	NM_000759.3	14
13	<i>IL8</i>	NM_000584.3	5
14	<i>PDGFA</i>	NM_002607.5	4
15	<i>CCL2</i>	NM_002982.3	4

TABLE 1B: Differential Expression of TGF β Superfamily Members Following Cytokine Stimulation in Three-Dimensional Cultures

Symbol	GenBank	FC (2D ⁻ →2D ⁺)	FC (3D ⁻ →3D ⁺)
<i>INHBA</i>	NM_002192.2	1.0	159.8***
<i>INHBB</i>	NM_002193.2	0.6	0.1**
<i>INHBC</i>	NM_005538.2	1.0	1.0
<i>INHBE</i>	NM_031479.3	5.2	1.6
<i>INHA</i>	NM_002191.3	1.2	0.8
<i>TGFB1</i>	NM_000660.4	1.5	2.9*
<i>TGFB2</i>	NM_001135599.2	1.2	1.0
<i>TGFB3</i>	NM_003239.2	1.0	1.0
<i>NODAL</i>	NM_018055.4	1.0	1.0

P-values are as indicated:

* *p* 0.05,

** *p* 0.01,

*** *p* 0.005. FC=fold change.