Activin Upregulation by NF-κB 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 (CIC), 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-κB activity is required to upregulate INHBA/Activin, a morphogen in the TGFβ 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 are 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. Cancer Res; 75(2): 426–35. © 2014 AACR.

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, squamous cell carcinomas (SCC), and large cell carcinomas (LCC). The 5-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, multistep 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 reprogramming event known as the epithelial-to-mesenchymal transition (EMT; refs. 2, 3). Mesenchymal cells acquire resistance to apoptotic cues, upregulate genes that promote self-renewal, and display properties of cancer-initiating cells (CIC; ref. 2).

TGFβ is one of the best-studied inducers of EMT (4, 5). TGFβ, Nodal, and activins are all members of the TGFβ superfamily (6). Although TGFβ signaling drives EMT, efficient transition depends on the activation of other costimulatory pathways. TNF, a proinflammatory cytokine, activates NF-κB to synergize with TGFβ in inducing EMT (7–9).

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

NF-κB transcription is commonly deregulated in NSCLC and correlates with metastasis and poor clinical outcome (18). NF-κB and IκB signaling are known to regulate self-renewal of CICs. Mice expressing a nonfunctional IκBα protein suppressed mammary tumor development by impairing self-renewal of CICs (19). Moreover, expression of a nondegradable form of IκBα inhibited NF-κB activation and delayed mammary tumor development by reducing the CIC population (20). Both the canonical and noncanonical NF-κB 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-κB activity is required for expression of Twist1/Twist, SNAI2/Slug, and ZEB2/Sip1 to drive EMT and CIC phenotypes in NSCLC (9).

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

Materials and Methods

Cell culture and reagents

NSCLC adenocarcinoma (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 TGFβ-1 (Invitrogen, 2 ng/mL; ref. 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) IκBα (A549.I) or vector control (A549.V) were created by retroviral delivery of pBABE-Puro (vector) or pBABE-Flag-SR-IκBα (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; Supplementary Table S1). Following puromycin (1.5 μg/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 (Supplementary Fig. S4A and S4B). 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 μg/mL), and type I DNase (Sigma; 20 μg/mL) for 30 minutes. Following tissue digestion, cells were pelleted, washed, and resuspended in RPMI/10% FBS/penicillin/streptomycin 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 TGFβ were harvested and filtered through a 0.22-μm pore. Supernatants from 2D and 3D cultures untreated or treated with TNF and TGFβ for 96 hours were harvested and filtered (0.22 μm). An ELISA kit (R&D) was used to measure activin levels in the supernatants (100 μL aliquots) as per manufacturer’s protocol.

Transwell assays and lung metastasis

Spheroid cultures were disaggregated by incubation in 0.05% trypsin for 10 minutes with intermittent gentle trituration. Transwell assays were performed to measure migration and invasion as previously described (9). For lung metastasis, 4- to 5-week-old female outbred Crl:NU/Nu nude mice (Jackson Laboratories) were injected subcutaneously (1 × 10⁶ cells/site) with cells from disaggregated A549.C or A549.A spheroid cultures left untreated or treated with cytokines. After 40 days, animals were euthanized following approved procedures by the University of Virginia (UVA; Charlottesville, VA) ACUC. Lung surface metastatic foci were counted using a dissecting scope 40 days following subcutaneous injection.

Immunohistochemistry of activin

Zinc formalin-fixed paraffin-embedded tissue blocks for adenocarcinoma and SCC were retrieved from the archives of the UVA Pathology Department and prepared as previously described (24). Alternatively, LCC tissue microarrays (TMA) were purchased (LC1211a, US Biomax). The avidin–biotin immunoperoxidase technique was performed as previously described (24) and slides were counterstained with hematoxylin. Immunohistochemistry (IHC) was performed using activin antibody HPA020031 (Sigma-Aldrich). Immunostains were examined in an objective semiquantitative fashion with a score assigned to staining intensity (1, weak; 2, moderate; 3, strong) and a quartile score for percentage 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).

Statistical analysis

All data are presented as mean ± SD of three independent biologic experiments. When two experimental groups were compared, values were log₂ transformed, and a one-tailed Student t test in Microsoft Excel was performed. Differences were
Results

Mesenchymal A549 cells produce soluble factors capable of inducing EMT

Recently published data from our laboratory demonstrate that TNF and TGFβ-stimulated NSCLC spheroid (3D) cultures effectively undergo EMT over a 4-day period (9). Because the half-life of recombinant cytokines in growth media is short (25), we postulated that A549 cells produce autocrine factors to maintain the mesenchymal state. To test this hypothesis, we harvested 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 downregulated E-cadherin while upregulating N-cadherin and vimentin (Fig. 1A). This effect was not due to residual TNF or TGFβ 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 TGFβ, because CM from spheroid cultures treated with either alone did not induce EMT in 3D cultures, as assayed by downregulation of E-cadherin or increased expression of N-cadherin and vimentin (Fig. 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 TGFβ, showed massive and reproducible changes in gene expression upon stimulation. This 1,351 gene list was then analyzed for overlap with a library of 1,636 genes encoding secreted/extracellular proteins (Secreted Protein Database, SPD; ref. 26). Microarray analysis identified 4,064 differentially regulated genes specific to the 3D-treated versus untreated cultures. Of these, 1,351 genes showed increased expression upon stimulation. This 1,351 gene list was then analyzed for overlap with a library of 1,636 genes encoding secreted/extracellular proteins (Secreted Protein Database, SPD; ref. 26). As illustrated in the Venn diagram in Fig. 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 1).

Interestingly, this list included Inhibin βA (INHBA). Inhibin β subunits dimerize with each other to form activins or with the α subunit to make Inhibins (28). Under physiologic conditions, activin A (inhibin βA homodimer) functions as a pluripotent factor for human embryonic stem cells (hESC; refs. 29, 30) and in endoderm and mesoderm formation (31–33). However, in melanoma and pancreatic cancer, Nodal/Activin A signaling has been linked to more aggressive phenotypes and self-renewal of CICs (34, 35).

Table 1. Most upregulated genes encoding secreted ligands following cytokine stimulation in 3D cultures

<table>
<thead>
<tr>
<th>Rank</th>
<th>Symbol</th>
<th>GenBank</th>
<th>Fold change (3D − 3D − )</th>
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<tr>
<td>1</td>
<td>GAL</td>
<td>NM_05973.3</td>
<td>220</td>
</tr>
<tr>
<td>2</td>
<td>CSF2</td>
<td>NM_000758.3</td>
<td>201</td>
</tr>
<tr>
<td>3</td>
<td>INHBA</td>
<td>NM_00292.2</td>
<td>16</td>
</tr>
<tr>
<td>4</td>
<td>PDGFB</td>
<td>NM_002608.2</td>
<td>111</td>
</tr>
<tr>
<td>5</td>
<td>CCL5</td>
<td>NM_002985.2</td>
<td>100</td>
</tr>
<tr>
<td>6</td>
<td>IL1</td>
<td>NM_000641.3</td>
<td>980</td>
</tr>
<tr>
<td>7</td>
<td>BMP6</td>
<td>NM_001718.4</td>
<td>580</td>
</tr>
<tr>
<td>8</td>
<td>PTHLH</td>
<td>NM_002082.2</td>
<td>410</td>
</tr>
<tr>
<td>9</td>
<td>EDIL3</td>
<td>NM_005713.3</td>
<td>400</td>
</tr>
<tr>
<td>10</td>
<td>CRLF1</td>
<td>NM_004750.4</td>
<td>150</td>
</tr>
<tr>
<td>11</td>
<td>CYR61</td>
<td>NM_001554.4</td>
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<td>CSF3</td>
<td>NM_000759.3</td>
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<td>IL8</td>
<td>NM_000584.3</td>
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<td>14</td>
<td>PDGFA</td>
<td>NM_002607.5</td>
<td>430</td>
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<tr>
<td>15</td>
<td>CCL2</td>
<td>NM_002982.3</td>
<td>430</td>
</tr>
</tbody>
</table>

NOTE: Mesenchymal NSCLC significantly upregulated INHBA/Activin transcripts. Microarray and bioinformatic analysis identified 128 genes that belong to the SPD and that are significantly upregulated in 3D cultures following TNF and TGFβ 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.

Table 2. Differential expression of TGFβ superfamilies members following cytokine stimulation in 3D cultures

<table>
<thead>
<tr>
<th>Symbol</th>
<th>GenBank</th>
<th>Fold change (2D − 2D − )</th>
<th>Fold change (3D − 3D − )</th>
</tr>
</thead>
<tbody>
<tr>
<td>INHBA</td>
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</tr>
<tr>
<td>INHBB</td>
<td>NM_002193.2</td>
<td>0.6</td>
<td>0.35</td>
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<tr>
<td>INHBC</td>
<td>NM_005538.2</td>
<td>1.0</td>
<td>1.00</td>
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<tr>
<td>INHBE</td>
<td>NM_031479.3</td>
<td>5.2</td>
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<td>INHA</td>
<td>NM_002191.3</td>
<td>1.2</td>
<td>0.80</td>
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<td>TGFBI</td>
<td>NM_000660.4</td>
<td>1.5</td>
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<td>TGFBI</td>
<td>NM_005239.2</td>
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<td>1.00</td>
</tr>
<tr>
<td>NODAL</td>
<td>NM_018055.4</td>
<td>1.0</td>
<td>1.00</td>
</tr>
</tbody>
</table>

NOTE: The differential expression of select TGFβ superfamilies members following cytokine treatment in both 2D and 3D cultures was examined.

*p < 0.005.

*p < 0.05.

*p < 0.01.
Importantly, in our microarray data, other members of the TGFβ superfamily, including NODAL, the TGFβs, and other INHIBINS, are not transcriptionally upregulated to the same extent as INHBA/Activin A in cytokine-treated cultures (Table 2). These results were confirmed by qRT-PCR (Supplementary Fig. S1B). The unique upregulation of INHBA among Inhibin α/β subunits following EMT suggests that these βA subunits predominantly 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 naive 3D cultures.

Upregulation of activin in cells derived from primary lung tumors

To confirm microarray results shown in Tables 1 and 2, qRT-PCR was performed on A549 3D cultures following exposure to TNF, TGFβ, or both cytokines. Although TGFβ alone modestly increased activin mRNA levels, treatment with both TNF and TGFβ significantly upregulated activin transcripts (Fig. 2A). Importantly, A549 and H1299 cells upregulate activin levels specifically in 3D cultures (Fig. 2B). In A549 cells, activin transcription was stimulated within 24 hours of cytokine treatment and steadily increased over a 4-day period, indicating that cells are capable of maintaining activin expression (Supplementary Fig. S2A). 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 (H522), SCC (H226), and LCC lines (Supplementary Fig. S2B). Importantly, the upregulation of activin mRNA levels observed in Fig. 2A and B corresponded with elevated protein levels in the media, as determined by ELISA (Fig. 2C).

Next, CM from cytokine-treated 3D A549 cultures were incubated for 2 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 Fig. 1A, N3D cultures

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Upregulation of activin in NSCLC lines and primary human lung tumors. A, A549 3D cultures were left untreated (−) or treated (+) with TNF, TGFβ, or both TNF and TGFβ before qRT-PCR. B and C, A549 and H1299 cultures (2D and 3D) were left unstimulated (No add) or treated with cytokines (TNF/TGF) before 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 4 hours. N3D cultures were incubated with this pretreated CM before 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 before immunoblot analysis. F, spheroid cultures were generated from A549 cells or from primary lung tumor cells isolated from human adenocarcinoma or SCC and were left untreated or stimulated with TNF and TGFβ before ELISA. qRT-PCR and ELISA results are mean ± SD. *, P < 0.05; **, P ≤ 0.01; ***, P ≤ 0.005; N = 3.
incubated with supernatants from cytokine-treated spheres effectively downregulated E-cadherin and upregulated N-cadherin, compared with N3D cultures incubated with CM from unstimulated spheroid cultures (Fig. 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 TGFβ and TNF (Fig. 2E). Experiments in Fig. 2D and E 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 adenocarcinoma and SCC tumors were disaggregated. Spheroid cultures were generated and either left untreated or stimulated with TNF and TGFβ. 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 (Fig. 2F). Moreover, activin levels further increased in primary NSCLC cultures following exposure to TNF and TGFβ. These data indicate that spheroid cultures from both established and primary NSCLC cells express activin, an event that is potentiated following cytokine treatment.

**NF-kB upregulates activin to promote expression of EMT markers**

Since we and others have recently shown that NF-kB is required for EMT and development of CICs in NSCLC (9, 21, 36), we wanted to determine whether activin is transcriptionally regulated by NF-kB. Before addressing this question, we examined the individual and combined contribution of TNF, TGFβ, and activin on NF-kB and SMAD signaling pathways. Although TGFβ, and activin alone resulted in strong constitutive SMAD2 phosphorylation, maximal RelA phosphorylation was only observed following combined treatment with TNF and TGFβ (Fig. 3A).

Next, A549 stable cell lines were developed that ectopically express the vector control (A549.V) or the dominant negative super-repressor IκBα (SR-IκBα, A549.I). A549.I cells fail to induce NF-kB transcription because expression of the SR-IκBα prevents nuclear translocation in response to physiologic stimuli (9). A549.I cells were unable to fully upregulate activin expression following TNF and TGFβ treatment, compared with controls A549.V (Fig. 3B). In contrast, the A549.I line demonstrated full...
transcriptional induction of bone morphogenic protein 2 (BMP2), another TGFβ superfamily member that is not regulated by NF-κB. As predicted from the qRT-PCR data, A549.I cells secrete less activin into the media than the A549.V line (Fig. 3C). Moreover, when compared with control cells, the A549.I line was unable to produce autocrine factors capable of inducing EMT in N3D cultures (Fig. 3D). Data shown in Fig. 3B–D indicate that the loss of NF-κB 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.

Because results shown in Fig. 3D could be due to a failure of A549.I cells to undergo EMT in the absence of NF-κB activity, we examined whether CM induced EMT through TGFβ/activin/Nodal signaling pathways. To address this question, TGFβ/Activin/Nodal signaling was blocked in cultures using SB431542 or ALK5i. SB431542 blocks activin type I receptor (ALK4), TGFβ type I receptor (ALK5), and Nodal type I receptor (ALK7; ref. 37), whereas the ALK5i inhibitor selectively blocks the TGFβ type I receptor (ALK5). CM were isolated and incubated with N3D cultures in either the absence or presence of SB431542 or ALK5i. Although ALK5i-treated cultures showed a slight reduction in RelA and SMAD2 phosphorylation, compared with vehicle control-treated cultures, SB431542 completely abolished signaling (Fig. 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

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 ± SD, *P ≤ 0.05; ***, P ≤ 0.005; N = 3.
transcription factors SNAIL/Snail, SNAI2/Slug, TWIST1/Twist, and ZEB2/Sip1, and mesenchymal markers CDH2/N-cadherin and VLM/Vimentin, compared with vehicle control-treated CM (Fig. 3F and Supplementary Fig. S3). Although SB431542 blocks signaling in response to activin, Nodal, and TGFβ ligands, only activin is upregulated in A549 3D cultures in response to TNF and TGFβ (Table 2 and Supplementary Fig. S1B). 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 nontargeting 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 (Supplementary Fig. S4A and S4B). 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 with A549.C (Fig. 4A and B). 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, whereas supernatants from A549.A were less efficient at modulating these markers (Fig. 4C). Importantly, A549.A cells exhibit statistically significantly lower levels of EMT master-switch transcription factors SNAIL1, SNAI2, and ZEB2, compared with control cells (Fig. 4D). However, the knockdown of activin had no effect on TWIST1 expression. Activin was required to sustain expression of MYCN, SOX2, KLF4, and HMGA2 (Fig. 4E), supporting the role of activin as a maintenance factor required for the expression of genes known to induce self-renewal in CICs (9, 38–41).

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; Fig. 5A). Moreover, activin expression was required for cell migration and invasion of cytokine-treated spheroid A549 cultures, as assayed in Transwell assays (Fig. 5B and Supplementary Fig. S5). The knockdown of activin resulted in elevated cell migration in unstimulated cultures, through an unknown mechanism that may be linked to the fact that low levels of activin A are required to form Inhibin (28).

Recently, we published that induction of EMT in A549 cells promotes the development of CICs that are highly metastatic under limiting cell dilution (42, 43) without the need for subcutaneous 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 before 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 subcutaneously 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 with control cells (Fig. 5C). Collectively, our results indicate that TNF and TGFβ-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

Because our data thus far indicate 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 and 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 (Fig. 6A and B). Next, IHC was performed on primary NSCLC tumors. Activin protein expression was elevated in 13 of 17 (76%) adenocarcinomas and 18 of 23 (78%) SCCs, while 13 of 36 (36%) LCCs showed positive IHC staining (Fig. 6C). The IHC staining intensities for activin levels in LCC were 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 adenocarcinomas were examined (44), we find that activin expression is elevated in primary tumors adenocarcinoma, 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 (39, 40). 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 (45). Thus, targeting CICs has been proposed as an approach to treat epithelial malignancies (46, 47).

Despite this current knowledge, therapies for eradicating CICs have been limited because of our poor understanding of how these cells maintain their mesenchymal phenotype throughout the metastatic process.

Work presented in this study indicates that TNF- and TGFβ-stimulated NSCLC spheroid cultures upregulate and secret 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 adenocarcinoma, SCC, and LCC show a significant upregulation of activin expression, compared with nonmalignant lung (Fig. 6C). Importantly, our findings are in agreement with the study by Seder and colleagues, which originally indentified activin overexpression in primary lung adenocarcinoma (44). Inhibition of activin signaling using RNAi or pharmacologic 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 (34), 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 (34). Primary pancreatic spheroid cultures cultured in basic fibroblast growth factor (bFGF) and 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 TGFβ family member driving...
self-renewal phenotypes in pancreatic CICs (34). This is in contrast with our findings, indicating that activin, not Nodal, drives EMT, expression of self-renewing factors, and metastatic potential of NSCLC-CICs. In addition, in esophageal carcinoma, activin, not Nodal, contributes to invasive, aggressive phenotypes (48). 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 because 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 TGFβ) dictates whether the CICs upregulate autocrine signaling of either Nodal or activin. The ability of TNF and TGFβ to upregulate activin is supported by our observation that cytokine treatment results in constitutive NF-κB activity (9), which is required to upregulate activin secretion (Fig. 3B and C). These effects are also compounded by the fact that under some cellular contexts, both TGFβ and activin have also been shown to promote proliferation (44).

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 gemcitibine effectively reduced tumor growth in pancreatic carcinoma (34). However, targeting the CIC population alone may not be an effective strategy. Lonardo and colleagues found that tumor-associated stroma not only produces inflammatory mediators that support CIC propagation, but may also provide resistance to the chemosensitizing effect of SB431542 and gemcitibine. 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 (29). In a similar manner, our work supports the role of TNF as a costimulatory signal required to potentiate EMF in response to recombinant activin (Fig. 2E). In light of the recent work by Kendellen and colleagues (21), it will be important to determine whether inflammatory cytokines, such as IL-1β and IL-6, work in concert with activin. This is especially important because TNF was unable to potentiate the ability of activin to signal through NF-κB or SMAD-dependent pathways (Fig. 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.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Acknowledgments
The authors thank Dr. Lisa Shock for discussion on the manuscript, Dr. Pat Pramonojoo for IHC analysis, and the Biorepository and Tissue Research Facility at UVA.

Grant Support
This work was supported by T32 Cancer Research Training in Molecular Biology Grant (CA009109; J.J. Wamsley and S.H. Clift), T32 Training in Cellular and Molecular Biology Training Grant (GM008136; D.F. Allison), T32 Cancer Research Training in Molecular Biology Grant (CA009109), T32 Biophysics Training Grant (GM080186; S.A. Hoang), NIH grants R01CA132580 and R01CA104397, and DB Philanthropy Funds (M.W. Mayo), and NIH R01CA136705 (D.R. Jones).

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Received September 24, 2013; revised July 9, 2014; accepted September 12, 2014; published OnlineFirst November 28, 2014.

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