INHBA Overexpression Promotes Cell Proliferation and May Be Epigenetically Regulated in Esophageal Adenocarcinoma

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Introduction: The expression, mechanisms of regulation, and functional impact of activin (*INHBA*) in esophageal adenocarcinoma (EAC) have not been fully defined.

Methods: *INHBA* expression was examined in 46 esophageal samples (nine Barrett's metaplasia (BM); seven BM/low-grade dysplasia; eight low-grade dysplasia; seven high-grade dysplasia; 15 EAC) using oligonucleotide microarrays and real-time reverse transcription-polymerase chain reaction (RT-PCR) and in 90 tissue samples (79 EAC; 8 dysplastic; 3 BM) using immunohistochemistry (IHC). The proliferation of EAC cell lines FLO and OE-33 was examined after treatment with exogenous activin. The proliferation of OE-33 was also examined after treatment with the activin inhibitor follistatin and *INHBA*-targeting siRNA. OE-33 and FLO cells were treated with 5-aza-2′deoxycytidine (5-AZA) and trichostatin A to investigate the role of epigenetic regulation in *INHBA* expression.

Results: Primary EACs expressed 5.7-times more *INHBA* mRNA than BM samples on oligonucleotide microarray. Transcript overexpression in EAC relative to BM was confirmed on real-time RT-PCR. IHC suggested higher *INHBA* protein expression in EAC (69.6%) than in the dysplastic (37.5%) and BM samples (33.3%). FLO and OE-33 treated with activin demonstrated increased proliferation, and OE-33 cells treated with follistatin and *INHBA*-targeting siRNA demonstrated reduced proliferation, relative to untreated controls. Treatment of FLO cells with trichostatin A and 5-AZA up-regulated *INHBA* mRNA and protein production by real time RT-PCR and IHC.

Conclusions: *INHBA* is overexpressed in EAC relative to dysplastic and BM tissue. *INHBA* overexpression may promote cell proliferation and may be affected by promoter demethylation and histone acetylation in EAC cell lines.

Key Words: Esophageal adenocarcinoma, *INHBA*, Activin, Epigenetic regulation.

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The incidence of esophageal adenocarcinoma (EAC) has risen dramatically over the past 30 years, however, the overall 5-year survival has remained low at approximately 20%.[1] Research dedicated toward the identification of novel biomarkers will be useful in the early identification and treatment of this lethal disease. Recently, gene expression profiling has allowed researchers to correlate tumor expression patterns with clinical outcomes.[2–4] Such high-throughput technology has led to a better understanding of the molecular pathways involved in neoplastic progression, and will be of paramount importance in the fight against EAC.

Activin, the disulfide-linked homodimer of inhibin βA (*INHBA*), is a ligand in the transforming growth factor-beta (TGF-β) superfamily.[5,6] The inhibin βA can also bind the βB and α isoforms to form activin AB and inhibin, respectively. Activin and inhibin are known to act in the hypothalamic-pituitary-gonadal axis and have been implicated in multiple biologic processes, including neoplastic progression.[7–13]

Chronic exposure to activin has been shown to promote tumorigenicity, growth, invasion, and resistance to apoptosis in esophageal squamous cell carcinoma.[11] In addition, activin overexpression in esophageal squamous cell carcinomas has been associated with advanced stage and worse prognosis.[12] Inhibin α–knock-out mice express high levels of activin and are prone to develop gonadal stromal tumors, supporting activin's role in tumorigenesis.[13] Furthermore, colon, prostate, pancreatic, and ovarian cancers have been reported to overexpress activin,[14–17] and women with endometrial or cervical cancer have increased serum activin concentrations.[18] However, the diverse biologic function of activin is demonstrated by its growth inhibitory effect on prostate cancer cells, vascular endothelial cells, mammary epithelial cells, plasmacytomas, and hepatocytes.[19–24] In addition, activin induces endodermal differentiation in human embryonic stem cells.[25]

The role of *INHBA* and activin in EAC has not been fully defined. In the current study, we demonstrate that *INHBA* is overexpressed in EAC relative to both dysplastic and Barrett's metaplasia (BM) tissue. Our data also suggest that EAC cell proliferation may be partially dependent on the presence of activin. Finally, we demonstrate that expression of *INHBA* in EAC cells is affected by a combination of promoter demethylation and histone acetylation.

PATIENTS AND METHODS

Patients and Tissues

Forty-six esophageal tissue samples were obtained from consented patients who underwent esophagectomy for adenocarcinoma at the University of Michigan Medical Center (Ann Arbor, MI). Approval for this study was obtained from the local Institutional Review Board. Tissues were transported on ice in Dulbecco modified Eagle medium (DMEM; Life Technologies, Inc., Carlsbad, CA), frozen in liquid nitrogen, and stored at −80°C. Esophageal tissues demonstrating at least 70% cellularity were identified on hematoxylin and eosin-stained frozen sections. Two pathologists (T.J.G. and J.K.G.) classified the tissues as BM, BM/low-grade dysplasia (LGD), LGD, high-grade dysplasia (HGD), or EAC. Macrodissection was used to obtain samples 2 to 3 mm^3 in size for mRNA and protein isolation. No patients received neoadjuvant chemotherapy or radiotherapy.

Cell Lines

The cell lines used in this study, FLO and OE-33, were derived from EAC, and have been previously described.[26] Both cell lines were grown in DMEM (Invitrogen Corp., Carlsbad, CA) with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin/fungizone (Life Technologies, Inc.) at 37°C in 5% CO_2. Cells were treated when at 70% confluence.

Oligonucleotide Microarray

The RNA was isolated, cRNA synthesized, and oligonucleotide microarrays analyzed as previously described.[27] Results from this data set have been previously published.[28]

Immunohistochemistry of Tissue Microarray (TMA)

Immunohistochemical staining was performed on the DAKO Autostainer (DAKO, Carpinteria, CA) using DAKO LSAB+ and diaminobenzadine as the chromogen, as previously described.[27] De-paraffinized sections of the tissue microarray at 5-μ thickness were labeled with inhibin βA antibody (R&D Systems, Cat # MAB3381, Minneapolis, MN, mouse, 1:20) after microwave antigen retrieval in 10 mM sodium citrate buffer, pH 6. Negative (no primary antibody) and positive (placenta) controls were stained with each immunohistochemical assay. The intensity and extent of immunoreactivity was scored using a three-tier (negative (−), moderate (1+), intense (2+)) grading scheme. All tissue microarrays were viewed with an Olympus BX40 microscope (Olympus, Center Valley PA). Images were acquired using a Spot Insight model 3.2.0 camera (Diagnostic Instruments, Inc., Sterling Heights, MI) and Spot version 4.0.9 (Diagnostic Instruments, Inc.) software.

Immunohistochemistry of Cells Lines

After plating on poly-L-lysine-coated glass slides, cells were air-dried and fixed in −20°C acetone for 10 minutes. Endogenous peroxidases were inactivated using 0.5% hydrogen peroxide. Cells were stained with the Vectastain ABC Kit (Vector Laboratories, Burlingame, CA, Cat # PK-6102, mouse IgG) and Peroxidase Substrate Kit (Vector, Cat # SK-4100) according to the manufacturer's instructions. Inhibin βA antibody (R&D Systems, Cat # MAB3381, 1:20) was used as the primary antibody. Negative controls (no primary antibody) were stained with each slide.

Western Blot Analysis

Western blot analysis was performed as previously described with slight variations.[26] Briefly, 15 μl of FBS or calf serum (CS) was loaded onto a 10% sodium dodecyl sulfate polyacrylamide gel and electrophoresed. Inhibin βA antibody (R&D Systems, Cat # MAB3381, 2 μg/ml) and a secondary antibody (GE Healthcare UK Limited, Chalfont St. Giles, UK, Cat # NA931V, goat antimouse, 1:10,000) were used for protein detection.

Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted with the RNeasy Mini Kit (Qiagen, Valencia, CA, Cat # 74104) and cDNA was made using the Superscript II Kit (Invitrogen, Cat # 18064). Real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed on the Rotor-Gene 6000 (Corbett Life Science, Sydney, Australia) with the Platinum SYBR Green Kit (Invitrogen, Cat # 11733-038). The *INHBA* oligo primers (Invitrogen) for real-time RT-PCR included forward INHBArt2f (5′-AAG TCG GGG AGA ACG GGT ATG TGG-3′) and reverse INHBArt2r (5′-TCT TCC TGG CTG TTC CTG ACT CG-3′). Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) expression was used to standardize the *INHBA* results using *GAPDH* primers (Invitrogen). Optimal annealing temperatures were determined, and melt curves were analyzed to ensure RT-PCR results.

WST-1 Cell Proliferation Assays

Cell viability and proliferation were assessed using Cell Proliferation Reagent WST-1 (Roche Applied Science, Indianapolis, IN, Cat # 11644807001). The experiments were performed in 96-well plates at two different cell densities (FLO: 6.0×10^3 and 8.0×10^3 cells/well; OE-33: 1.5×10^3 and 2.5×10^3 cells/well). Absorbance was measured on a Biotek EL-312e microplate reader (Biotek Instruments, Winooski, VT) at 96 hours.

Treatment of Cell Lines with Activin and Follistatin

Twenty-four hours after plating, FLO and OE-33 cells were treated with 1, 10, and 100 ng/ml activin (Peprotech, Inc., Cat # 120-14, Rocky Hill, NJ) in DMEM with 10% FBS or 10% CS. In addition, OE-33 cells were treated with 1, 10, and 100 ng/ml follistatin (Peprotech, Inc., Cat # 120-13) in DMEM with 10% FBS. All activin and follistatin treatment experiments were performed in quadruplicate.

Transfection of OE-33 Cells with *INHBA*-Targeting Small-Interfering RNA (siRNA)

Transient transfection of OE-33 cells with *INHBA*-targeting siRNA was performed as previously described.[28] Briefly, OE-33 cells were plated at a density of 2.0×10^3

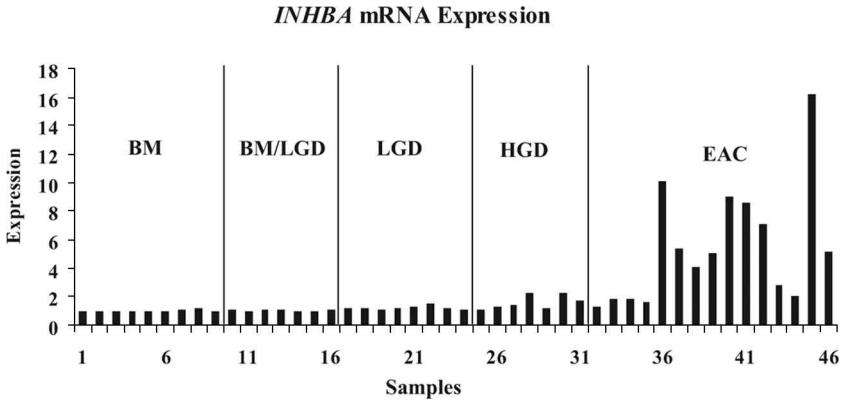

FIGURE 1. Oligonucleotide microarray analysis of 46 esophageal specimens demonstrated at least two-fold expression in 11 of 15 (73.3%) and four-fold expression in 9 of 15 (60%) of esophageal adenocarcinoma (EAC) relative to Barrett's metaplasia (BM). x axis represents tumor identifier; y axis represents transcript expression relative to BM. BM, Barrett's metaplasia; LGD, low-grade dyslasia; HGD, high-grade dyslasia; EAC, esophageal adenocarcinoma.

cells/well in DMEM with 10% FBS in a 96-well format for determination of the optimal siRNA transfection conditions. Twenty-four hours after plating, cells were treated with *INHBA*-targeting SMART pool siRNA (Dharmacon, Lafayette, CO) in the presence of 0.1 μl Lipofectamine RNAiMAX (Invitrogen) in 9.9 μl Opti-MEM I (Invitrogen). *INHBA* knockdown efficiency was assessed by real-time RT-PCR. An optimal working concentration of 10 nM siRNA was used to perform cell proliferation assays. Forty-eight hours after initial transfection, half of the plates were retransfected with 10 nM *INHBA*-targeting siRNA. Transfected cells were incubated for a total of 96 hours at 37°C before gene silencing analysis. Cells treated with transfection reagent were used as a control. All siRNA experiments were performed in quadruplicate.

Treatment of FLO and OE-33 Cells with Trichostatin A (TSA) and 5-aza-2′ Deoxycytidine (5-AZA)

FLO and OE-33 cells were serum starved for 24 hours before treatment to synchronize their cell cycles. Cell lines were then treated with trichostatin A (TSA) at a concentration of 300 nM and/or 5-aza-2′ deoxycytidine (5-AZA) at a concentration of 5 μM in DMEM with 10% FBS. Cells receiving combination treatment were simultaneously exposed to 300 nM TSA and 5 μM 5-AZA. The TSA and 5-AZA was dissolved in dimethyl sulfoxide (DMSO; Sigma Aldrich, Cat # D4540, St Louis, MO) and control cells were treated with equivalent amounts of DMSO. Cells treated with TSA were harvested at 12 or 24 hours and those treated with 5-AZA were harvested at 48, 72, or 96 hours. Combination plates were treated with 5-AZA for 48, 72, or 96 hours and TSA for 24 hours. Cell pellets for mRNA analysis were frozen in liquid nitrogen and stored at −80°C until mRNA was extracted for real-time RT-PCR. All TSA and 5-AZA experiments were performed in duplicate. Cells for immunohistochemistry (IHC) analysis were immediately suspended in PBS and plated on poly-L-lysine coated slides using a Shandon Cytospin 3 machine (Thermo Fisher Scientific, Waltham, MA).

Correlation between *INHBA* and Histone Deacetylase, Histone Acetyltransferase, and DNA Methyltransferase Expression

To further examine the potential role of histone acetylation and promoter methylation in the regulation of *INHBA* expression, we examined the oligonucleotide microarray expression of histone deacetylases (HDAC), histone acetyltransferases (HAT), and DNA methyltransferases (DNMT) in the 15 EAC samples. The HDAC, HAT, and DNMT expression values were then correlated with *INHBA* expression.

Statistical Analysis

The Fisher's exact test was used to compare IHC staining between BM, dysplastic, and EAC samples. The *t* test was used to assess the significance of changes in proliferation after treatment. Spearman analysis was used to correlate *INHBA* gene expression with that of the HDACs, HATs, and DNMTs. A p value <0.05 was deemed significant. All statistical analyses were performed using R version 2.7.0 (Vienna, Austria).

RESULTS

INHBA mRNA is Overexpressed in EAC

Oligonucleotide microarrays were used to measure the level of *INHBA* expression in 46 esophageal tissue samples (9 BM; 7 BM/LGD; 8 LGD; 7 HGD; 15 EAC). The EAC expressed two-fold more *INHBA* mRNA than the mean of the BM in 11 of 15 (73.3%) samples and at least four-fold more expression in 9 of 15 (60%) samples (Figure 1). On average, the EAC expressed 5.7-times more *INHBA* mRNA than the BM tissues. The HGD tissue samples demonstrated two-fold expression relative to BM in 2 of 7 (28%) cases. Real-time RT-PCR was used to confirm that *INHBA* was overexpressed in the 15 EAC samples relative to BM (Figure 2). One LGD tissue sample examined in the oligonucleotide microarray was unavailable; therefore, real-time RT-PCR was performed on 45 esophageal tissue samples.

Inhibin βA Protein Overexpression Suggested on Esophageal Tissue Microarray

Immunohistochemical staining of a TMA containing BM, dysplastic tissue, and EAC suggested greater inhibin βA

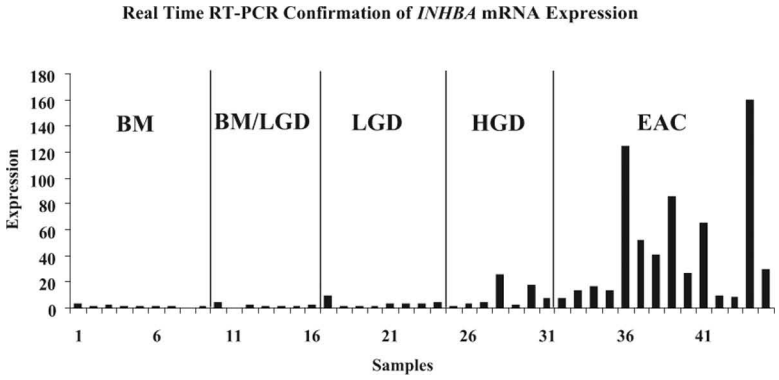

FIGURE 2. Real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis of 45 esophageal specimens used in the oligonucleotide microarray (Figure 1) confirming overexpression of *INHBA* in esophageal adenocarcinoma (EAC) relative to Barrett's metaplasia (BM). x axis represents tumor identifier; y axis represents transcript expression relative to BM. BM, Barrett's metaplasia; LGD, low-grade dyslasia; HGD, high-grade dyslasia; EAC, esophageal adenocarcinoma.

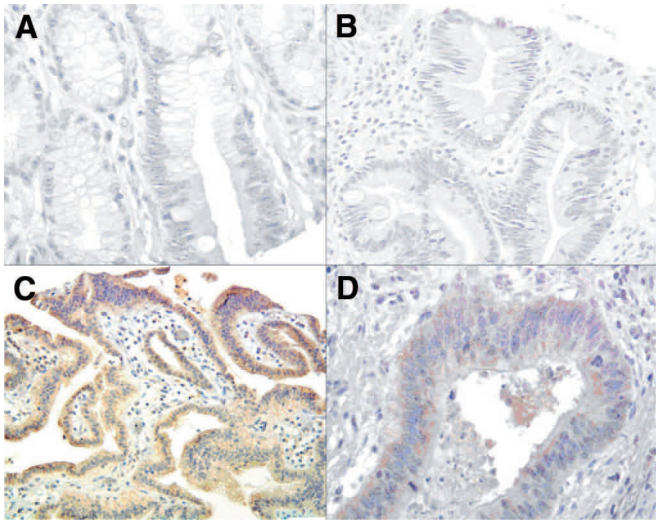

FIGURE 3. Representative sections of tissue microarray. *A*, and (*B*) Inhibin βA protein staining was not detected in most Barrett's metaplasia, but was seen in (*C*) and (*D*) 69.6% of esophageal adenocarcinomas by immunohistochemistry. *A*, and (*D*) ×40 magnification; *B*, ×20 magnification; *C*, ×10 magnification.

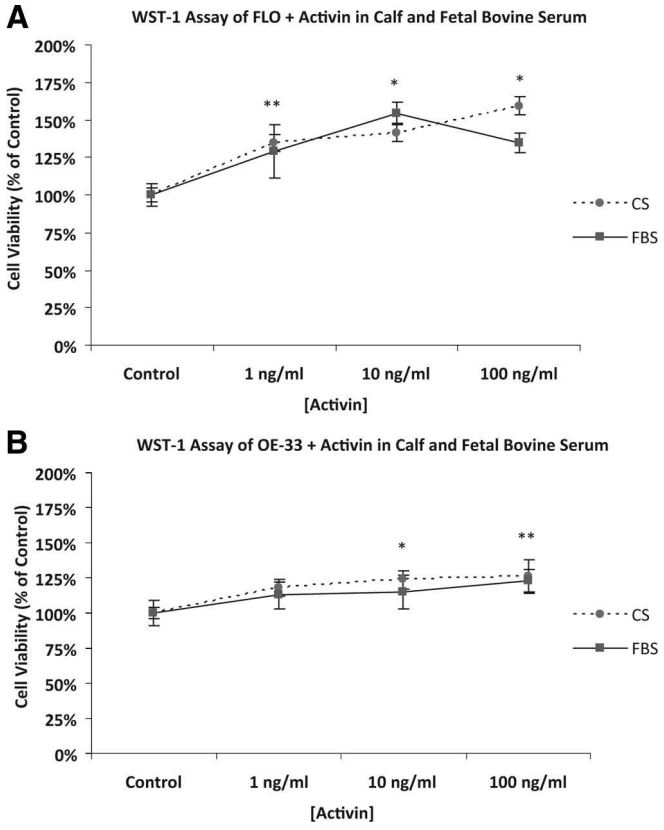

FIGURE 4. *A*, FLO and (*B*) OE-33 cells treated with activin demonstrated increased proliferation at 96 hours as assessed using WST-1 assays. The effect of activin treatment was greater in calf serum (CS) than in fetal bovine serum (FBS). All experiments repeated in quadruplicate. *$p < 0.05$.

expression in EAC than BM and dysplastic samples. All inhibin βA staining was cytoplasmic. Although not statistically significant, immunoreactivity was found in 69.6% (55 of 79) of EAC (Figures 3*C*, *D*), although only 37.5% (3 of 8; $p = 0.11$) of dysplastic samples and 33.3% (1 of 3; $p = 0.23$) of BM (Figures 3*A*, *B*) demonstrated INHBA immunoreactivity.

Activin Induces Proliferation in FLO and OE-33 Cells

EAC cell lines FLO and OE-33 treated with activin at concentrations ranging from 1 to 100 ng/ml demonstrated increased proliferation relative to untreated controls at 96 hours. The effect of activin treatment was greater in the presence of CS than FBS in both cell lines. FLO cells demonstrated the greatest growth proliferative response after treatment with 100 ng/ml activin in CS (159%) and with 10 ng/ml activin in FBS (154%) (Figure 4*A*). Treatment with 100 ng/ml activin induced the greatest OE-33 growth proliferative response in both CS and FBS, at 126 and 123% of controls, respectively (Figure 4*B*). The growth proliferative response was inversely related to the baseline *INHBA* transcript expression as assessed by real time RT-PCR. FLO cells demonstrated low *INHBA* transcript levels (Ct = 27.2, *GAPDH* Ct = 11.6), although OE-33 cells had moderate *INHBA* transcript expression (Ct = 24.5, *GAPDH* Ct = 11.8). At baseline, neither FLO nor OE-33 cells demonstrated inhibin βA immunoreactivity on Western blot or IHC.

Inhibin βA is More Abundant in Fetal Bovine Serum than Calf Serum

We hypothesized that the blunted proliferative response seen in the presence of FBS upon activin treatment was due to a higher inhibin βA concentration in FBS than CS. Inhibin βA protein was seen in all four replicate FBS lanes on Western blot, but none of the four CS lanes, suggesting a higher concentration of activin in FBS than CS (data not shown). This finding was repeated and confirmed with different batches of FBS and CS.

Inhibin βA Inhibitor Follistatin Reduces Proliferation in OE-33 Cells

EAC cell line OE-33 treated with 1 to 100 ng/ml follistatin demonstrated a dose-dependent reduction in proliferation relative to untreated controls, with a maximum of 23% reduction in proliferation (Figure 5*A*).

Transfection of OE-33 Cells with *INHBA*-Targeting siRNA Affects *INHBA* Expression and Cell Proliferation

An *INHBA*-silencing efficiency of 70% relative to mock-controls was achieved by transfecting OE-33 cells with *INHBA*-targeting siRNA at a concentration of 10 nM (data not shown), as assessed using real-time RT-PCR. This concentration of *INHBA*-targeting siRNA produced a 5% reduction in proliferation at 96 hours. When cells were retransfected 48 hours after the initial transfection, a 10% reduction in proliferation was observed at 96 hours (Figure 5*B*).

Epigenetically Mechanisms May Affect *INHBA* Expression in EAC Cell Lines

We treated FLO and OE-33 cells with 5-AZA and TSA to determine if epigenetic regulation affected *INHBA* expression. FLO, a cell line with low baseline *INHBA* expression, demonstrated a 51.7-fold (range, 38.3–78.8-fold) increase in mRNA production relative to the mean of the controls with 12 hours of TSA exposure (Figure 6*A*). When cells were treated with a combination of TSA for 24 hours and 5-AZA for 96 hours, a 46.1-fold (range, 20.5–60.5-fold) increase in *INHBA* mRNA expression was observed. OE-33 cells, which demonstrated higher baseline *INHBA* expression, exhibited an attenuated *INHBA* up-regulation with TSA and 5-AZA treatment (Figure 6*B*). Consistent with our real-time RT-PCR results, IHC of FLO cells treated with TSA for 12 hours demonstrated up-regulated inhibin βA protein expression (Figure 7*B*) relative to the untreated control (Figure 7*A*). This protein up-regulation was not seen in the OE-33 cells treated with TSA and 5-AZA (data not shown).

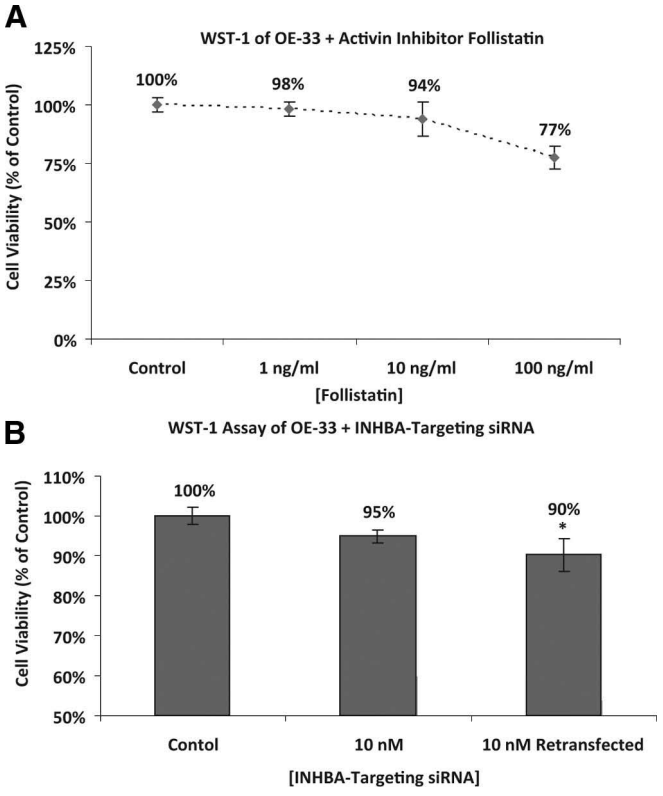

FIGURE 5. OE-33 cells treated with (*A*) activin inhibitor follistatin and (*B*) 10 nM *INHBA*-targeting siRNA demonstrated reduced proliferation at 96 hours relative to mock controls using WST-1 assays. Repeat transfection after 48 hours resulted in further growth-inhibition relative to controls. All experiments repeated in quadruplicate.

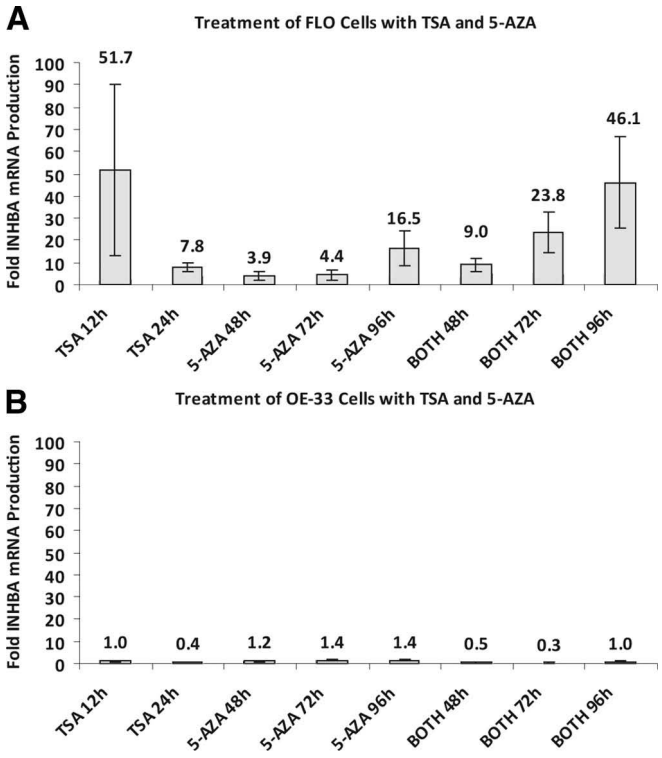

FIGURE 6. *INHBA* mRNA production in (*A*) FLO was up-regulated upon treatment with histone deacetylase inhibitor trichostatin A (TSA) and demethylating agent 5-aza-2′deoxycytidine (5-AZA). *B,* A significantly blunted response was seen upon exposure of OE-33 to TSA and 5-AZA.

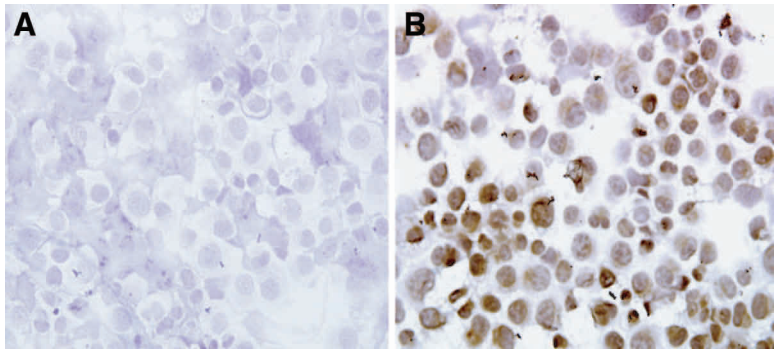

FIGURE 7. Immunohistochemistry of *A*, untreated FLO cells did not demonstrate inhibin βA protein expression. *B*, Treatment of FLO cells for 12 hours with trichostatin A (TSA) significantly up-regulated inhibin βA protein expression (×40 magnification).

Correlation between *INHBA and HAT1* Expression

We compared the oligonucleotide microarray expression of *INHBA* to the HDACs, HATs, and DNMTs in the 15 EAC samples using Spearman correlation analysis. No significant correlations were found between *INHBA* and the HDACs or DNMTs. However, *HAT1* was found to have a strong positive correlation ($\rho = 0.442$) with *INHBA* expression (see Appendix). This correlation is consistent with our cell line findings. However, due to the small sample size, this correlation failed to reach statistical significance ($p = 0.087$).

DISCUSSION

We have demonstrated that *INHBA* mRNA and protein are overexpressed in primary EAC relative to Barrett's and dysplastic tissue. In addition, we have shown that the addition of exogenous activin leads to EAC cell proliferation. Furthermore, partial EAC activin-dependency is suggested by the decrease in proliferation seen with follistatin and *INHBA*-targeting siRNA treatment in OE-33.

Upon treatment with high concentrations of activin in the presence of FBS, FLO cells demonstrated an attenuated proliferative response. This effect was not seen when the cells are treated in CS, which unlike FBS, contains no activin by Western blot. These findings suggest that at high doses, activin might have growth inhibitory effects on some EAC cell lines. The complex actions of activin are characteristic of the TGF-β superfamily, which are known to exert both tumor suppressive and oncogenic effects. In the current model, tumor suppressor activities dominate in normal tissues, but changes in cellular response may promote oncogenesis in tumors.[29–31] Activin seems to exert a similar dose-dependent dual effect on FLO cells, which express small amounts of activin at baseline. The moderate *INHBA*-expressing cell line, OE-33, demonstrated a blunted proliferative response with activin treatment. However, the growth-proliferative effects of activin are seen in a dose-dependent manner, suggesting that OE-33 cells tolerate higher concentrations of activin without growth inhibition.

Although the exact concentrations of specific growth factors are not available for most commercially obtained serums, multiple studies have demonstrated that the concentration of activin A rises in maternal serum throughout pregnancy and is present at high levels in amniotic fluid and fetal serum.[32–38] In addition, studies have suggested that deletion of *INHBA* in mice results in severe craniofacial abnormalities, including cleft palate and lack of lower incisors. Furthermore, lack of type IIA activin receptors in fetal mice results in impaired reproductive function.[39,40] Such evidence implicates the role of activin A in fetal development, and is consistent with our western blots demonstrating detectable levels of the inhibin βA in fetal bovine serum, but not in CS.

Follistatin was first identified as an inhibitor of pituitary FSH secretion[41,42] and was subsequently found to serve as an activin-binding compound.[43] Follistatin is known to bind activin with high affinity (50–500 pM), thereby regulating its function.[44] When we treated OE-33 cells with follistatin, a moderate decrease in proliferation was seen, which was consistent with activin inhibition. A slightly smaller, but statistically significant, growth inhibitory effect was also seen upon treatment of OE-33 with *INHBA*-targeting siRNA. Although follistatin will bind other members of the TGF-β superfamily with very low affinity, and siRNA transfection has the potential to exert off-target effects, our data consistently demonstrated a reduction in cell proliferation with differing methods of activin inhibition. The moderate growth reduction seen in OE-33 cells with activin inhibition suggests agents targeting *INHBA* may be therapeutically useful, potentially in combination with other agents.

The growth-proliferative effects of activin in two EAC cell lines prompted us to explore mechanisms for its overexpression. Previous reports have established that epigenetic mechanisms can affect gene expression in normal and neoplastic tissues.[45–47] Our cell line data and microarray correlation analysis suggest that the overexpression seen in a subset of primary tumors might be due to histone acetylation and/or promoter demethylation. The greatest increase in *INHBA* mRNA expression occurred in the low-*INHBA* expressing cell line, FLO, after 12 hours of TSA exposure. Treatment of FLO with TSA beyond 12 hours, alone or in combination with 5-AZA, led to a smaller increase in *INHBA* expression. As TSA and 5-AZA are globally acting agents, prolonged exposure may affect other genes that result in the down-regulation of *INHBA* transcription. The blunted *INHBA* up-regulation seen in OE-33 cells relative to FLO cells may be a reflection of the higher baseline expression of OE-33, thus resulting in a smaller fold-increase. Alternatively, OE-33 and FLO may have differing mechanisms of regulating *INHBA* expression.

INHBA may represent a novel target in the treatment of EAC. Clearly, *INHBA* overexpression is not the sole mechanism responsible for the unregulated growth of EAC, as some EACs do not express high levels of *INHBA* and inhibition of *INHBA* does not completely arrest EAC growth. However, our data show that activin exposure does stimulate proliferation and that EAC cells are partially dependent on activin exposure. Thus, the use of activin-antagonists, such as follistatin, monoclonal antibodies, and small molecule inhibitors holds therapeutic potential, especially in combination with other agents. Since *INHBA* is widely expressed and has a variety of physiologic functions, the feasibility of systemic therapy needs further examination before in vivo application. Our findings also suggest that that histone acetylation and promoter demethylation may play a role in the *INHBA* overexpression observed in a subset of EAC. This study provides data to support the continued investigation of *INHBA* in the progression of EAC.

ACKNOWLEDGMENTS

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APPENDIX. Expression of *INHBA* and *HAT1* in EAC

Probe ID	Gene	EAC1	EAC2	EAC3	EAC4	EAC5	EAC6	EAC7	EAC8	EAC9	EAC10	EAC11	EAC12	EAC13	EAC14	EAC15
210511_s_at	*INHBA*	1.33	1.82	1.79	1.58	10.07	5.36	4.07	5.00	8.98	8.61	7.10	2.78	2.08	16.21	5.14
203138_at	*HAT1*	0.65	0.69	1.12	0.69	0.97	1.01	0.77	1.03	0.87	0.60	0.99	1.09	1.77	2.07	0.78

EAC, esophageal adenocarcinoma.

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