β-Arrestin-1 Mediates Nicotine-Induced Metastasis through E2F1 Target Genes That Modulate Epithelial–Mesenchymal Transition

Smitha Pillai1, Jose Trevino2, Bhupendra Rawal3, Sandeep Singh4, Michelle Kovacs1, Xueli Li5, Michael Schell5, Eric Haura5, Gerold Bepler7, and Srikumar Chellappan1

Abstract

Cigarette smoking is a major risk factor in the development of non–small cell lung cancer (NSCLC), which accounts for 80% of all lung cancers. Nicotine, the major addictive component of tobacco smoke, can induce proliferation, invasion, and epithelial-to-mesenchymal transition (EMT) in NSCLC cell lines and promote metastasis of NSCLC in mice. Here, we demonstrate that the scaffolding protein β-arrestin-1 is necessary for nicotine-mediated induction of mesenchymal genes vimentin and fibronectin as well as EMT regulators ZEB1 and ZEB2. Nicotine induced changes in cell morphology and ablate tight junctions consistent with EMT; β-arrestin-1, but not β-arrestin-2, was required for these changes. β-Arrestin-1 promoted the expression of the mesenchymal genes, as well as ZEB1 and ZEB2, through the mediation of the E2F1 transcription factor; this required Src kinase activity. Stimulation of multiple NSCLC cell lines with nicotine led to enhanced recruitment of β-arrestin-1 and E2F1 on vimentin, fibronectin, and ZEB1 and ZEB2 promoters. Furthermore, there was significantly more β-arrestin-1 and E2F1 associated with these promoters in human NSCLC tumors, and β-arrestin-1 levels correlated with vimentin and fibronectin levels in human NSCLC samples. A549-luciferase cells lacking β-arrestin-1 showed a significantly reduced capacity for tumor growth and metastasis when orthotopically implanted into the lungs of SCID-beige mice. Taken together, these studies reveal a novel role for β-arrestin-1 in the growth and metastasis of NSCLC.

Introduction

Cigarette smoking is highly correlated with the development of non–small cell lung cancer (NSCLC), which accounts for 80% of all lung cancers (1). Nicotine, the addictive component of tobacco smoke, has been found to induce cell proliferation, angiogenesis, and epithelial-to-mesenchymal transition (EMT) through nicotinic acetylcholine receptors (nAChR; refs. 2, 3). In addition, nicotine confers resistance to apoptosis induced by chemotherapeutic agents (4). Although nicotine is not known to initiate tumors, it can promote the growth and metastasis of solid tumors in vivo, suggesting that nicotine might promote the progression of tumors already initiated by tobacco-specific nitrosamines (5, 6).

The arrestin family consists of four members, including visual arrestins (arrestin1 and 4), which are expressed exclusively in the retina and ubiquitously expressed nonvisual arrestins (β-arrestin-1 and 2 or ARRB1 and ARRB2). Although it is established that β-arrestins promote internalization and desensitization of GPCRs (G protein-coupled receptors), they also regulate signaling by Notch, endothelin A receptor, frizzled, smoothened, and T-cell receptors (7–11). Our earlier studies demonstrated that nAChR signaling induces nuclear translocation of β-arrestin-1 (arrestin, β1 or ARRB1) in a Src-dependent manner, where it recruited the histone acetyltransferase p300 to E2F1-regulated proliferative promoters, facilitating their transcription (12).

Nicotine can induce changes in gene expression consistent with EMT, a signature of more advanced and less differentiated cancers. The EMT program is a highly conserved developmental event that promotes epithelial cell dissociation and migration to appropriate sites during embryogenesis (13); it also facilitates the metastasis of tumors (14). During EMT, epithelial markers such as E-cadherin (CDH1) and catenins are downregulated with a concomitant upregulation of mesenchymal markers, including vimentin (VIM) and fibronectin (FN1; ref. 15). TGFβ is known to induce EMT robustly in many cell types (16). Here, we demonstrate that fibronectin and vimentin promoters are E2F1 regulated and the expression of these genes is induced by nicotine in a β-arrestin-1–dependent manner. There was a direct correlation between the levels of these genes and β-arrestin-1 in human NSCLC samples. In addition, β-arrestin-1 regulated the expression of EMT-promoting transcription factors such as ZEB1 and ZEB2. β-Arrestin-1–depleted cells could not form tumors or metastasize in SCID mice in response to nicotine stimulation. Our results suggest that β-arrestin-1 plays a key and specific role in nicotine-induced EMT and metastasis.

Materials and Methods

Cell lines and reagents

NSCLC cell lines A549, H1650, H358, H1975, Calu6, H23, and PC9 were obtained from ATCC and were used within 6
 Construct and transfections
Fibronectin (pFN 1.2 kb) and vimentin (VimPro-1.5 kb) promoter luciferase constructs were kindly provided by Dr. Jesse Roman (Emory University, Atlanta, GA; ref. 17) and Dr. C. Gilles (Liege University, Belgium; ref. 18). The A549 and H1650 cells were transfected using Fugene HD reagent (Roche; Supplementary Fig. S1A and S1B). Selective knockdown of β-arrestin-1; cells transfected with empty vector were used as control (shcontrol). These cells were rendered quiescent by serum starvation and stimulated with 1 μmol/L nicotine or TGFβ for 24 hours in the presence of 0.2 μmol/L dasatinib or 0.5 μmol/L PP2.

Detailed experimental procedures for orthotopic lung tumor model, migration assay, 3D culture, immunofluorescence, Western blot analyses, chromatin immunoprecipitation (ChIP) assays, and transfections are provided in the Supplementary Methods section.

siRNA transfections and real-time PCR
For siRNA transfections, 50, 75, or 100 pmol of siRNAs (Santa Cruz Biotechnology) with Oligofectamine were added to cells. For real-time PCR, total RNA was isolated using RNeasy miniprep kit (QIAGEN) following the manufacturer’s protocol, followed by first-strand cDNA synthesis using iScript cDNA Synthesis Kit (Bio-Rad). Data were analyzed by the ΔΔCt method, where gene of interest was normalized to 18s rRNA, then compared with the nontargeting siRNA control sample. Error bars represent the SD of three independent experiments. Details of siRNAs and sequences of primers used for RT-PCR are given in Supplemental Methods.

Wound-healing assay
A549 cells were grown in a 6-well plate (Falcon Becton Dickinson) transfected with siRNAs. These cells were starved in serum-free media for 24 hours and then washed with 1× Dulbecco’s PBS (MediaTech). The cells were scratched with a sterilized 200 μL pipette tip in three separate places in each well and medium containing 1 μmol/L nicotine or starvation media were added to the wells. After 24 hours, the wounds were observed and images were taken in ×20 magnification using Zeiss inverted phase contrast microscope.

Invasion assays
Boyden Chamber assays were used to assess the invasive ability of cells as described previously (2). The upper surface of the 6.5 mm filters (Corning) was coated with collagen (100 μg/filter) and Matrigel (BD Biosciences; 50 μg/filter). Twenty thousand cells were plated in the upper chamber with 0.1% BSA (Sigma). Media containing 20% FBS was placed in the lower well as chemoattractant. The cells that invaded through the filters were quantified by counting three fields under ×20 objective magnifications.

Statistical methods and analysis
A log transformation was performed to make mRNA expression values of four genes [β-arrestin-1, vimentin, fibronectin, and 18SrRNA] approximately normal. Pearson rank correlation (r) was used to assess correlation among these three genes. An exact normal scores test was used to assess the association between mRNA expression values of these genes and pathologic stage. The reduced monotonic regression model (19) was used to assess the association between mRNA expression values of these genes and smoking pack years. An optimal cut point for disease-free survival, defined as the time for surgical resection to disease recurrence or death, was tested for using the maximal χ² test. All statistical analyses were performed using SAS (Version 9.2; SAS Institute).

Results
β-arrestin-1 is necessary for nicotine-induced disruption of tight junctions to facilitate EMT and invasion
We previously observed that nAChR stimulation results in changed reminiscent of EMT (2, 20). To investigate the role of β-arrestin-1 in this process, A549 cells that lack β-arrestin-1 were generated by stably expressing β-arrestin-1–specific shRNA (shβ-arrestin1); cells transfected with empty vector were used as control (shcontrol). These cells were rendered quiescent by serum starvation and stimulated with 1 μmol/L nicotine, which is equivalent to the levels observed in the blood stream of heavy smokers. Immunofluorescence experiments revealed membranous staining of tight junction protein zona occludens 1 (ZO-1) in quiescent cells; nicotine treatment reduced ZO-1 levels in shcontrol cells, but not in shβ-arrestin1 cells (Fig. 1A, top; Supplementary Fig. S1E). Selective knockdown of β-arrestin-1 by shRNA and siRNAs is shown in Supplementary Fig. S1A–S1D. Similar results were observed when β-arrestin-1 was depleted by siRNA in H1650 cells (Fig. 1A, bottom; Supplementary Fig. S1F and S1G), suggesting that β-arrestin-1 is necessary for reducing tight junctions upon nAChR activation. In addition to ZO-1, the levels of E-cadherin, an epithelial marker, were significantly decreased upon nicotine stimulation only in shcontrol cells but not in shβ-arrestin1 cells (Supplementary Fig. S2A and S2B). Shcontrol cells and shβ-arrestin-1 cells were grown as 3D cultures on collagen in the presence or absence of nicotine; shcontrol cells treated with nicotine acquired a more elongated, migratory morphology, but shβ-arrestin1 cells did not show such changes (Fig. 1B).

Shβ-arrestin1 cells were also impaired in their ability to migrate (Supplementary Fig. S2C) or invade in response to nicotine stimulation (Fig. 1C). Depletion of β-arrestin-1 impaired nicotine-mediated invasion of a variety of NSCLC cell lines (Fig. 1D), indicating a definite role for β-arrestin-1 in the induction of mesenchymal features in response to nAChR signaling.
β-Arrestin-1 is necessary for nicotine-induced disruption of tight junctions that correlate with EMT and invasion.

A, nicotine downregulated the membrane localization of the tight junction protein ZO-1 (green) in shcontrol cells but not in shβ-arrestin1 cells. Nuclei were counterstained with DAPI (blue). Scale bar, 50 μm. SS, serum-starved cells. B, shcontrol cells grown in presence of nicotine acquired more elongated, migratory morphology in 3D cultures, whereas shβ-arrestin1 cells did not. Scale bar, 20 μm. C and D, depletion of β-arrestin-1 abrogates nicotine-induced invasive capacity of A549 and H1650 cells (C) and H1975, H23, and H358 cells (D) as seen by Boyden chamber assays. (*) P < 0.05.

β-Arrestin-1 is required for the expression of vimentin and fibronectin in response to nAChR signaling

We examined whether β-arrestin-1 regulates the expression of the mesenchymal genes fibronectin and vimentin upon nAChR stimulation. Western blotting showed that nicotine treatment induced the expression of fibronectin and vimentin in shcontrol cells, but not in shβ-arrestin1 cells (Fig. 2A); this induction occurred at the transcriptional level (Fig. 2B). In contrast, depletion of β-arrestin-1 did not alter TGFβ-mediated vimentin or fibronectin expression, suggesting that β-arrestin-1 plays a role in mediating EMT downstream of nAChRs. Transient transfection assays were then conducted using luciferase reporter constructs driven by human vimentin and fibronectin promoters. Shcontrol and shβ-arrestin1 cells were transfected with the reporters, rendered quiescent by serum starvation, and stimulated with nicotine or TGFβ. Both promoters were
induced by nicotine in shcontrol cells but not in shβ-arrestin1 cells while TGFβ could induce them in cells (Fig. 2D). Similar pattern of induction of fibronectin and vimentin in shcontrol A549 cells, but not in shβ-arrestin1 null cells. C, RT-PCR showing the levels of fibronectin and vimentin upon nicotine and TGFβ stimulation after transfecting a different siRNA targeting β-arrestin-1. D, transient transfection assays in shcontrol and shβ-arrestin1 (#1 and #2 are two clones) cells using vimentin and fibronectin promoter luciferase constructs, demonstrating the role of β-arrestin-1 in nicotine-induced promoter induction. E, Western blot analysis showing the levels of vimentin and fibronectin after siRNA transfection in H358. F and G, depletion of Src and α7nAChR abrogated nicotine-induced vimentin and fibronectin expression as seen by Western blot analysis (F) and RT-PCR (G). H, treatment of cells with a TGFβ inhibitor prevented the TGFβ-mediated, but not nicotine-mediated, vimentin or fibronectin. I, levels of E-cadherin, β-catenin, and ZO-1 in the membrane fractions of serum-starved and nicotine-treated shcontrol or shβ-arrestin1 cells. Levels of Na⁺/K⁺-ATPase, a marker for membrane fraction, were not downregulated upon nicotine stimulation. (C, P < 0.05).

Figure 2. β-arrestin-1 is necessary for nicotine-induced upregulation of fibronectin and vimentin expression. A and B, Western blot analyses (A) or RT-PCR (B) showing the induction of vimentin and fibronectin in shcontrol A549 cells, but not in shβ-arrestin1 null cells. C, RT-PCR showing the levels of fibronectin and vimentin upon nicotine and TGFβ stimulation after transfecting a different siRNA targeting β-arrestin-1. D, transient transfection assays in shcontrol and shβ-arrestin1 (#1 and #2 are two clones) cells using vimentin and fibronectin promoter luciferase constructs, demonstrating the role of β-arrestin-1 in nicotine-induced promoter induction. E, Western blot analysis showing the levels of vimentin and fibronectin after siRNA transfection in H358. F and G, depletion of Src and α7nAChR abrogated nicotine-induced vimentin and fibronectin expression as seen by Western blot analysis (F) and RT-PCR (G). H, treatment of cells with a TGFβ inhibitor prevented the TGFβ-mediated, but not nicotine-mediated, vimentin or fibronectin. I, levels of E-cadherin, β-catenin, and ZO-1 in the membrane fractions of serum-starved and nicotine-treated shcontrol or shβ-arrestin1 cells. Levels of Na⁺/K⁺-ATPase, a marker for membrane fraction, were not downregulated upon nicotine stimulation. (C, P < 0.05).
Because nicotine induces cell proliferation by α7nAChR (CHRNA7)-mediated activation of Src, experiments were conducted to assess whether they contribute to the induction of these mesenchymal promoters. Western blot analysis (Fig. 2F) and RT-PCR (Fig. 2G) showed that depletion of Src and Rb on these promoters. Nicotine stimulation led to an increase in the association of E2F1 and β-arrestin-1 with a concomitant dissociation of Rb from these promoters (Fig. 3A and Supplementary Fig. S6). ChIP assays followed by real-time PCR also showed the increased association of E2F1, β-arrestin-1, p300, and β-arrestin1 cells. As seen in Fig. 2f, nicotine treatment decreased the levels of E-cadherin, β-catenin, and ZO-1 in shcontrol cells, but not in shβ-arrestin1 cells.

**Vimentin and fibronectin promoters are E2F1 and Rb responsive**

Recent studies have indicated that E2Fs function in a wide range of biologic processes in addition to cell-cycle progression (23–26). Because β-arrestin-1 interacts with E2F1 (12), attempts were made to assess whether E2F transcription factors played a role in the induction of fibronectin and vimentin during EMT. An analysis of the promoter regions (1 kb) upstream of transcription start site using MatInspector program (Genomatix) revealed the presence of six potential E2F-binding sites on the vimentin promoter (Supplementary Fig. S4A) and two sites on the fibronectin promoter (Supplementary Table S1).

ChIP assays conducted on A549 cells detected the binding of E2F1 and Rb to vimentin and fibronectin promoters (Supplementary Fig. S4B); further, transient transfection experiments showed that E2F1 could induce using vimentin-Luc and fibronectin-Luc reporters, while Rb suppressed this induction (Supplementary Fig. S3C). Similar results were obtained in additional NSCLC cell lines (Supplementary Fig. S4D and S4E). Although E2Fs 2–5 could also induce these promoters, E2F1 had the maximal effect (Supplementary Fig. S3F). Supporting these results, transfection of 75 pmols of an E2F1 siRNA significantly reduced the levels of endogenous vimentin and fibronectin, (Supplementary Fig. S4G and S4H).

Transient transfections were conducted using deletion mutants of vimentin promoter to fine map the promoter region required for E2F-mediated induction (Supplementary Fig. S5A); it was found that the full-length promoter (1.5kb; six E2F-binding sites) and the deletion mutant vim-387+24 (Vim-411; three sites) could be transcriptionally induced by E2F1, but the shortest mutant vim-245+24 (Vim-269) was not E2F1 responsive (Supplementary Fig. S5B), suggesting that the three proximal sites were sufficient for E2F-mediated transcriptional induction. Similar experiments on the deletion mutants of fibronectin promoter revealed that only a proximal binding site was necessary for induction by E2F1 (Supplementary Fig. S5C and S5D); this was confirmed by mutating this site (Supplementary Fig. S5E). These experiments demonstrate a direct role for E2F1 in regulating these mesenchymal promoters.

**nAChR stimulation leads to recruitment of E2F1 and β-arrestin-1 on fibronectin and vimentin promoters**

Because nAChR stimulation induced the nuclear translocation of a subset of β-arrestin-1 (12), ChIP assays were conducted to determine whether it could associate with vimentin and fibronectin promoters. A549 cells were serum starved and stimulated with nicotine; quiescent cells had robust amount of Rb on these promoters. Nicotine stimulation led to an increase in the association of E2F1 and β-arrestin-1 with a concomitant dissociation of Rb from these promoters (Fig. 3A and Supplementary Fig. S6). ChIP assays followed by real-time PCR also showed the increased association of E2F1, β-arrestin-1, p300, and β-arrestin1 cells. As seen in Fig. 2f, nicotine treatment decreased the levels of E-cadherin, β-catenin, and ZO-1 in shcontrol cells, but not in shβ-arrestin1 cells. Furthermore, Rb was present on vimentin and fibronectin promoters in shβ-arrestin1 cells even after nicotine stimulation (Fig. 3B). An unrelated promoter, c-Fos, was used as the negative control and there was only minimal association of E2F1, Rb, β-arrestin-1, p300, and β-arrestin1 cells. As seen in Fig. 2f, nicotine treatment decreased the levels of E-cadherin, β-catenin, and ZO-1 in shcontrol cells, but not in shβ-arrestin1 cells.

**Nuclear function of β-arrestin-1 is necessary for induction of EMT**

To examine whether nicotine- or TGFβ-induced expression of these mesenchymal markers is dependent on E2F1, we transfected A549 cells with control siRNA or two different E2F1 siRNAs, and stimulated with nicotine or TGFβ. Western blotting (Fig. 3C and D) and RT-PCR showed that (Fig. 3E) E2F1 was necessary for nicotine-mediated, but not TGFβ-mediated, induction of vimentin and fibronectin. To assess whether β-arrestin-1 is required for the transcriptional induction of these promoters by E2F1, shcontrol and shβ-arrestin1 cells were transfected with vimentin or fibronectin promoter constructs along with E2F1. Interestingly, fibronectin and vimentin promoters were induced by E2F1 in shcontrol cells but not in shβ-arrestin1 cells (Fig. 3F and G), suggesting that β-arrestin-1 was necessary for the E2F1-mediated transcription of these genes. Experiments on control siRNA or E2F1 siRNA-transfected cells also showed that E2F1 was required for the induction of vimentin and fibronectin promoters upon nicotine stimulation, but not TGFβ stimulation (Fig. 3H and I). Collectively these results indicate that E2F1 and β-arrestin-1 are required for the nAChR-mediated, but not TGFβ-mediated, expression of fibronectin and vimentin.

**Nuclear function of β-arrestin-1 is necessary for induction of EMT**

To examine whether nuclear translocation of β-arrestin-1 regulates the expression of mesenchymal genes, we performed transient transfection assays using the β-arrestin-1–mutant Q394L, in which glutamine 394 has been mutated to leucine to create a nuclear export signal (27, 28). The mutant Q394L did not enhance E2F1-mediated transcription from fibronectin and vimentin promoters, suggesting that nuclear translocation of β-arrestin-1 is required for the transcription of these genes (Fig. 4A).
To further explore the requirement of the nuclear function of β-arrestin-1 in inducing EMT, shβ-arrestin1 cells were transfected with pcDNA3, WT-β-arrestin1-RFP, or Q394L-β-arrestin-1 constructs. Shcontrol cells transfected with pcDNA3 were used as the control. As shown in Fig. 4B, nicotine-induced expression of fibronectin and vimentin in Shβ-arrestin1 cells was rescued by the WT-β-arrestin-1-RFP construct but not the Q394L-β-arrestin-1 construct. Furthermore, WT-β-arrestin-1, but not Q394L-β-arrestin-1 could rescue the nicotine-induced migration and invasion (Fig. 4C and D) of shβ-arrestin1 cells, supporting the contention that β-arrestin-1 facilitates the transcriptional induction of mesenchymal genes upon nicotine stimulation. The level of β-arrestin-1 after rescue was examined by RT-PCR and Western blot analyses (Supplementary Fig. S8A–S8C).

We next examined whether nuclear functions of β-arrestin-1 require Src activity. A549 and H1650 cells were transfected with vimentin and fibronectin promoters along with a dominant negative or constitutively active Src expression vector. Luciferase assays revealed that dominant negative Src inhibited transactivation of these promoters (Fig. 4E) by E2F1. In addition, Src inhibitors dasatinib and PP2 could strongly inhibit E2F1 and β-arrestin-1–mediated transcription of fibronectin and vimentin reporters (Fig. 4F), suggesting that Src was necessary for the nuclear functions of β-arrestin-1. In addition, we performed ChIP assays on A549 cells treated with nicotine in the presence or absence of dasatinib and PP2. Nicotine treatment enhanced recruitment of E2F1, β-arrestin-1, and p300 on these promoters with concomitant release of Rb; these effects were abrogated in
cells treated with the two Src inhibitors (Fig. 4G and H). Though less specific than dasatinib, treatment with both the agents led to a near complete dissociation of Rb, E2F1, and β-arrestin-1, and p300 with a concomitant release of Rb; there were no detectable levels of amplification from an irrelevant antibody control IgG.

E2F1–β-arrestin-1 interaction is necessary for the expression of mesenchymal genes

Previous studies from our laboratory had shown that amino acids 1-163 of β-arrestin-1 were required for its binding to E2F1 (Supplementary data in ref. 12). We conducted IP–Western blot assays to determine whether delivery of the 1-163 fragment of β-arrestin-1 disrupted the binding of endogenous E2F1 to β-arrestin-1. A549 cells were transfected with either the control vector (pcDNA3) or two levels of pcDNA3-β-arrestin-1 1-163 construct (4 μg or 8 μg). IP-Western blots showed that the binding of endogenous E2F1 and β-arrestin-1 was disrupted by β-arrestin-1 1-163 construct in a dose-

Figure 4.
Rescue of β-arrestin-1 function by wild-type β-arrestin-1 construct. A, transient transfection assays demonstrating that Q394L β-arrestin-1 mutant does not induce vimentin or fibronectin promoters. Of note, 3 μg of wild-type or mutant β-arrestin-1 along with 0.5 μg of promoter construct and 0.5 μg of E2F1 was used in transfections. B, RT-PCR showing the rescue of nicotine-induced expression of vimentin and fibronectin in shβ-arrestin cells transfected with wild-type rat β-arrestin-1 construct (βarr-RFP); Q394L-βarr-Q394L could not rescue the nicotine response (*, P < 0.05). C, transfection of WT-β-arrestin-1, but not Q394L-β-arrestin-1, could facilitate nicotine-induced migration of shβ-arrestin cells in wound-healing assays. D, similar results were observed on invasion (**, P < 0.05). E and F, dominant-negative Src inhibits the transcription from vimentin and fibronectin promoters in A549 and H1650 cells (E); dasatinib and PP2 exerted similar effects, as seen in transient transfections (F). G and H, ChIP assays followed by qRT-PCR on A549 cells show that nicotine treatment enhanced the recruitment of E2F1, β-arrestin-1, and p300 with a concomitant release of Rb; these changes were abrogated by Src inhibitors. There was no detectable level of amplification from an irrelevant antibody control IgG.
levels of overexpressed E2F1 and WT- 
-dependent manner (Fig. 5A). Similar results were obtained when we overexpressed E2F1 and WT- 
-prevented the E2F-1–mediated induction of vimentin and fibronectin. A549 cells were transfected with the promoter luciferase constructs along with E2F1 and 
-alone or with different levels of 
-1 1-163; cotransfection of 
-abrogated promoter induction by E2F1 (Fig. 5C), suggesting that binding of 
-arrestin-1 1-163 fragment (Fig. 5B).

We next examined whether disrupting the binding of 
-arrestin-1 prevented the E2F-1–mediated induction of vimentin and fibronectin. A549 cells were transfected with the promoter luciferase constructs along with E2F1 and 
-alone or with different levels of 
-1 1-163; cotransfection of 
-arrestin-1 1-163 fragment (Fig. 5B).

We performed functional assays to explore whether binding of 
-arrestin-1 to E2F1 is required for nicotine-induced EMT. A549 cells were transfected with pCDNA3-E2F1 and 
-arrestin-1 fragment 1-163. A549 cells were transfected with pCDNA3-E2F1 and 
-arrestin-1 along with 
-arrestin-1 1-163 fragment 1-163 abrogates the induction of vimentin and fibronectin promoters by E2F1, suggesting that binding 
-arrestin-1 in 
-arrestin-1 in nicotine-mediated induction of vimentin and fibronectin.

We hypothesized that 
-arrestin-1 associates with these promoters probably through E2F1. It was found that 
-arrestin-1 was recruited on the promoters of many genes that regulate EMT such as ZEB2 as well as other regulatory pathways (Supplementary Table S2; GEO accession number GSE40689). Because ZEB1 and ZEB2 are upregulated by nAChR stimulation and 
-arrestin-1 mediates the induction of EMT-promoting transcription factors.

Attempts were made to analyze the global association of 
-arrestin-1 with promoters upon nicotine stimulation by ChIP sequencing. It was found that 
-arrestin-1 is recruited on the promoters of many genes that regulate EMT such as ZEB2 as well as other regulatory pathways (Supplementary Table S2; GEO accession number GSE40689). Because ZEB1 and ZEB2 are upregulated by nAChR stimulation and 
-arrestin-1 mediates the induction of EMT-promoting transcription factors.

Role of 
-arrestin-1 in lung cancer metastasis

Given the above results, we examined whether 
-arrestin-1 was necessary for nicotine-mediated growth and metastasis of tumors in mice. Sh 
-arrestin-1 cells showed markedly reduced ability to form tumors compared with control cells when implanted subcutaneously into athymic nude mice (Fig. 6A). The potential role of 
-arrestin-1 in nicotine-mediated induction of metastasis...
was next examined. A549 cells stably expressing luciferase gene (A549-luc) along with β-arrestin-1-specific shRNA (shβarr1-luc) or A549-luc cells stably transfected with an empty vector as control (shcontrol-luc) were orthotopically implanted into the left lung of SCID-Beige mice (12/group). Mice were randomized into two groups and administered nicotine (n = 6) or vehicle (n = 6) every other day by intraperitoneal injection for 7 weeks and tumor growth monitored weekly using the IVIS-Caliper 200

Figure 6. Role of β-arrestin-1 in nicotine induced metastasis. A, shβ-arrestin-1 cells showed significantly lower tumor growth in a subcutaneous xenograft model. B, tumor growth of orthotopically implanted cells, as seen by weekly bioluminescence imaging for 7 weeks. Mice implanted with shβarr1-luc had significantly smaller tumors and nicotine did not increase tumor growth or metastasis; mice implanted with shcontrol-luc cells displayed metastases to brain, adrenal glands, and liver. C, luminescence from lung after ex vivo imaging. D, luminescence from liver, brain, adrenal, and liver after imaging in vivo. E, representative images of mice from each group in the orthotopic lung experiment. F, lung tissues from mice implanted with β-arrestin-1–depleted cells show reduced levels of vimentin and fibronectin, as seen by IHC. Scale bar, 200 μm. G, Western blot analyses showing the levels of β-arrestin-1 in shcontrol-luc and shβarr1-luc cells.
system. Mice implanted with shcontrol-luc cells that received nicotine had significantly larger tumors compared with those who received vehicle (Fig. 6B and E); shcontrol-luc tumors displayed metastases to brain, adrenal glands, and liver (Fig. 6D) and nicotine treatment further enhanced metastases. Mice implanted with shβarr1-luc had significantly smaller tumors and nicotine treatment did not increase tumor growth or metastasis; ex vivo imaging of organs at the termination of the experiment confirmed these results (Fig. 6C).

Vimentin and fibronectin expression was significantly higher in tumors from nicotine-treated mice compared with vehicle-treated mice implanted with shcontrol cells; tumors from mice implanted with shβ-arrestin-1 cells displayed minimal staining for vimentin and fibronectin (Fig. 6F). The levels of β-arrestin-1 depletion in the stable β-arrestin-1 knockdown cells are shown in Fig. 6G. Taken together, these results confirm that β-arrestin-1 is indispensable for the growth and metastasis of lung tumors, especially in the context of nicotine exposure.

β-Arrestin-1 expression levels correlate with fibronectin and vimentin levels in lung tumor tissues from patients

We next examined whether the expression of β-arrestin-1 in human lung tumor samples correlates with levels of vimentin and fibronectin by conducting qRT-PCR on 116 patient samples. As shown in Fig. 7A and B and Supplementary Table S3, β-arrestin-1 expression showed a very strong positive correlation with vimentin (r = 0.59, P < 0.0001) and fibronectin (r = 0.52; P < 0.0001), which are known to be overexpressed in more aggressive tumors (29, 30). No significant association was observed between these gene expression patterns and pathologic stages or smoking history (pack years) and disease-free survival (data not shown), suggesting that β-arrestin-1 might be contributing to the growth and metastasis of lung cancer in both smokers and nonsmokers.

ChIP assays were conducted to examine whether β-arrestin-1 is recruited to the promoters of vimentin, fibronectin, ZEB1, and ZEB2 in human lung tumors. There was enhanced association of E2F1 and β-arrestin-1 and elevated levels of acetylated histone H3 on vimentin, fibronectin, ZEB1, and ZEB2 promoters in tumors compared with normal lung tissues in three sets of tumor samples (Fig. 7C–E). Taken together, these results indicate that β-arrestin-1 might have contributed to the growth and progression of NSCLCs by regulating the expression of E2F-regulated mesenchymal genes and EMT transcription factors.

Discussion

β-arrestins function as scaffold proteins that recruit a broad spectrum of signaling molecules to membrane-bound receptors (11, 31). In addition to their established roles in promoting internalization and desensitization of GPCRs, recent studies have implicated involvement of β-arrestins in Notch, frizzled, Wnt/β-catenin, nAChR signaling and regulation of gene expression by facilitating histone acetylation (32, 33). Accumulating evidences indicate a functional role for β-arrestin-1 as a mediator of cellular

Figure 7.

β-Arrestin-1 expression correlates with vimentin and fibronectin expression. A and B, scatter plots showing the correlation of β-arrestin-1 expression with the expression of vimentin and fibronectin from lung tumors of 116 patients. C–E, ChIP assay performed on NSCLC tumors showed enhanced association of E2F1 and β-arrestin-1 on vimentin, fibronectin, ZEB1, and ZEB2 promoters compared with normal tissues.
migration, invasion, and metastatic progression of colorectal, ovarian, and breast cancer (34–39). In this study, we demonstrate that β-arrestin-1, but not β-arrestin-2, plays a major role in nicotine-induced EMT and metastasis, thus contributing to invasive properties of nicotine. The elevated levels of β-arrestin-1, vimentin, and FN in tumors from both smokers and nonsmokers demonstrate that the molecules contribute to the growth and progression of NSCLCs; at the same time, the induction of these genes could be an important mechanism by which nicotine exerts its tumor-promoting functions. Interestingly, β-arrestin-1 was necessary for the induction of mesenchymal promoters upon nAChR stimulation, it did not play a role in TGFβ-mediated induction of these genes. The induction of these genes by β-arrestin-1 and E2F1 in response to nAChR signaling required Src activity; at the same time, TGFβ induces these mesenchymal genes through SMAD proteins, suggesting a unique role for β-arrestin-1 in mediating signals downstream of nAChRs (40, 41).

In addition, the present study demonstrates the critical role of E2F1 in regulating genes involved in EMT, including ZEB1 and ZEB2. ZEB1 suppresses the expression of basement membrane components, cell polarity factors, and epithelial genes, including E-cadherin (42–48), promoting tumor invasion and metastasis (45, 46). ZEB2 collaborates with the TGFβ signaling pathway by interacting with SMAD factors, and induces tumor cell invasion (47). A recent analysis of gene expression database of NSCLC cell lines identified a mesenchymal gene pattern (low E-cadherin, high vimentin) significantly associated with ZEB1 and ZEB2 expression but not with snail, slug, twist1, or twist2 (48). However, correlations between these genes with proliferative signaling cascades have not been elucidated. Our studies show that motogenic signaling through the nAChRs can also activate components of invasive and metastatic phenotype of cancer.

The Rb–E2F transcriptional regulatory pathway plays a major role in cell-cycle regulation, but its role in other aspects of tumor progression, invasion, and metastasis is relatively less explored. E2F1 has been shown to induce VEGF receptors and MMPs, indicating the importance of the Rb–E2F pathway in promoting tumor angiogenesis and metastasis (23, 24). Recent studies demonstrated that Rb depletion results in disruption of cell–cell adhesion and downregulation of E-cadherin (49). In addition, other EMT-related transcription factors, including slug and ZEB1, are also induced by Rb depletion, suggesting that inhibition of EMT is a novel tumor-suppressor function of Rb (50). Our finding that the Rb–E2F pathway is involved in the regulation of mesenchymal proteins and EMT-related transcription factors provides a molecular mechanism by which Rb and E2F1 facilitate tumor progression. This also raises the possibility that signaling events that inactivate Rb and promote cell proliferation might also promote EMT and metastasis upon the acquisition of additional mutational events or signaling cues, suggesting an interrelated circuitry of signaling and transcriptional events that promote the initiation as well as progression of cancers.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

Conception and design: S.P. Chellappan, S. Pillai, J.G. Trevino, G. Bepler Development of methodology: S. Pillai, J.G. Trevino, S. Singh, G. Bepler Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.G. Trevino, M.J. Schell, E.B. Haura, G. Bepler Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S.P. Chellappan, S. Pillai, J.G. Trevino, B. Rawal, M.J. Schell, E.B. Haura Writing, review, and/or revision of the manuscript: S.P. Chellappan, S. Pillai, J.G. Trevino, M.J. Schell, E.B. Haura, G. Bepler Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S.P. Chellappan, J.G. Trevino, X. Li, G. Bepler Other (performed animal experiments): M. Kovacs, S.P. Chellappan

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β-Arrestin-1 Mediates Nicotine-Induced Metastasis through E2F1 Target Genes That Modulate Epithelial–Mesenchymal Transition

Smitha Pillai, Jose Trevino, Bhupendra Rawal, et al.

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