Signaling and Regulation

p120-Catenin Is Transcriptionally Downregulated by FOXC2 in Non–Small Cell Lung Cancer Cells

Fariborz Mortazavi1,2, Jiabin An¹, Steven Dubinett3, and Matthew Rettig1,2

Abstract

p120-catenin (p120ctn) plays a major role in cell adhesion and motility through the regulation of E-cadherin and interaction with RhoGTPase and Rac1. p120ctn is downregulated in several malignancies including non–small cell lung cancer (NSCLC). Here, we investigated transcriptional regulation of p120ctn in NSCLC. We cloned a 1,400-bp amplicon of chromosome 11 from position -1,082 to +320 relative to the transcription start site into a firefly luciferase reporter vector and prepared serial deletion constructs to pinpoint cis-acting elements involved in the regulation of p120ctn. We transfected NSCLC cell lines and immortalized normal human respiratory epithelial cells with the abovementioned constructs. We found reduced p120ctn promoter activity, protein level, and mRNA message in lung cancer cells compared with noncancerous immortalized lung epithelial cells. Serial deletion analysis of p120ctn promoter identified a region between positions +267 and +282, which mediated the transcriptional repression of p120ctn. This region harbored putative binding sites for FOXC2 and FOXL1 transcription factors. Direct binding of FOXC2 to the p120ctn promoter between positions +267 and +282 was confirmed by electromobility shift assay. RNAi-mediated silencing of FOXC2 in A549, H157, and H358 cells resulted in increasing p120ctn promoter activity as well as mRNA and protein levels. Finally, silencing FOXC2 in these NSCLC cells enhanced E-cadherin level, which was reversed by simultaneous silencing of p120ctn. In summary, our data support the notion that FOXC2 mediates the transcriptional repression of p120ctn in NSCLC. Mol Cancer Res; 8(5); 762–74. ©2010 AACR.

Introduction

The p120-catenin protein (p120ctn) is a regulator of E-cadherin, which promotes cell-cell adhesion at adherens junctions. When localized to the plasma membrane, p120ctn stabilizes E-cadherin and thereby enhances cell-cell interactions. Loss of p120ctn expression results in the destabilization of the E-cadherin complex, a critical step in invasion and metastasis (1-10). Downregulation of p120ctn has been shown to disrupt cell-cell adhesions in several studies. p120ctn knockdown by small interfering RNA (siRNA) expression results in the dose-dependent destabilization of epithelial, placental, neuronal, and vascular endothelial cadherins, and complete loss of cell-cell adhesion (1). Lower expression of p120ctn at the protein and mRNA levels in several malignancies including all subtypes of non–small cell lung cancer (NSCLC) is associated with increased metastatic potential of these tumors. Compared with the normal uninvolved tissue counterpart, reduced expression of p120ctn has been observed in multiple tumor models, including NSCLC (4, 11, 12). Interestingly, it seems that reduction in p120ctn protein is also associated with a decrease in p120ctn mRNA transcript. As such, we reasoned that the dysregulation of p120ctn expression in malignant versus nonmalignant tissue is at least in part attributable to differential p120ctn gene transcription.

The transcriptional regulation of p120ctn expression has not been studied. Here, we investigated the regulation of p120ctn transcription in NSCLC by cloning the p120ctn promoter and generating several deletion mutants to elucidate the cis-acting regulatory elements involved in p120ctn transcriptional regulation. Here, we report the role of FOX2C2 in the downregulation of p120ctn. FOXC2 is a mesenchymal forkhead-related protein that is involved in specifying mesenchymal cell fate during embryogenesis. FOXC2 is also known as a mediator of epithelial-mesenchymal transition. As part of the epithelial-mesenchymal transition process, malignant epithelial cells lose their adhesive epithelial phenotype and acquire characteristics of mesenchymal cells, such as loss of association to underlying epithelial cell sheets and the acquisition of invasiveness and motility.

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Note: Supplementary data for this article are available at Molecular Cancer Research Online (http://mcr.aacrjournals.org/).

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doi: 10.1158/1541-7786.MCR-10-0004

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Materials and Methods

Cell cultures. A549, H157, and Rh2 cells were routinely cultured in RPMI supplemented with antibiotics and 10% heat-inactivated fetal bovine serum (Omega Scientific). Immortalized normal human epithelial cells (BEAS-2B) were cultured in Bronchial Epithelial Cell Basal Medium supplemented with all the additives (Lonza/Clonetics Corp.).

Cloning of p120ctn promoter and preparation of deletion constructs. Using chromosome 11 sequence data available through the Genbank nucleotide database (reference: NM_001331), we identified a 1,400 nucleotide region spanning both the 5’ and 3’ regions of the p120ctn transcriptional start site. Computer-based promoter analysis of this sequence identified numerous putative transcription factor binding sites (i.e., cis-acting elements). Two separate constructs were generated, one spanning from position −1,082 to +320 (numbers relative to transcriptional start site) and another from position −540 to +320. We amplified the aforementioned two segments of p120ctn gene by PCR from human genomic placental DNA by using primers directed at sequences starting at position −1,082 and −540 in relation to the transcription initiation site to position +320 (end of exon 1). The resulting amplicons were 1,400 and 861 bp and were cloned into a firefly luciferase reporter vector (pGL4.16 Luciferase reporter vector, Promega) to generate p120ctnshort-luc and p120ctnlong-luc, respectively.

Primers used for the amplification of p120ctn promoter were as follows:

- **p120ctnshort-forward**: 5′-GCTCTATATCGAATATCTCCAAAAC-3′
- **p120ctnlong-forward**: 5′-GGCATATAATGTTGAGATGACATAGA-3′
- **p120ctnshort and long-reverse**: 5′-GACAAAAATTCGACTTTGCTTATCC-3′

Multiple deletion constructs lacking 5′ or 3′ segments of p120ctnshort were also prepared by PCR, using Taq DNA polymerase (Roche Applied Science). These deletion mutants were subcloned into pGL4.16 vector. All constructs including deletion mutants were verified by sequencing. Initial deletions were made at the 5′ end and further deletions were made at the 3′ end of the p120ctnshort-luc to keep the TATA box within the construct. Primers used for creating deletion constructs were as follows:

- **-221**: 5′-GCAGTAATTTCCCTACAGAGATTTGACAGTC-3′
- **-71**: 5′-AGCTGCACCTTTATCCAAATAG-3′
- **-205**: 5′-AGTGGATGTACGCCCCCTTGAG-3′
- **-95**: 5′-TTCCGTTTTCTTAAGTGATGATG-3′
- **+19**: 5′-AACAACGTACCTG-3′
- **+69**: 5′-CACCTAAAATGGAAATGGAC-3′
- **+127**: 5′-GATCCCCAAAGAGGAGGAGG-3′

Measurement of p120ctn promoter activity by dual luciferase assay. p120ctn promoter constructs were transfected into NSCLC cell lines as well as immortalized normal human respiratory epithelial cells (BEAS-2B) by Lipofectamine 2000 (Invitrogen). Twenty-four hours after transfection, cells were washed with PBS and lysed using a Branson Sonifier in 1× passive lysis buffer (Promega) at room temperature. Reporter gene expression was assessed by using the Dual-Luciferase Reporter Assay System kit (Promega) according to the manufacturer’s instructions in a TD-20/20 Luminometer (Turner Biosystems). We normalized for transient transfection efficiency (i.e., firefly luciferase activity) by cotransfection of a Renilla luciferase expressing control vector (pRL-SV40). The ratio of Firefly to Renilla luciferase activity determined for each sample separately. The luciferase activity of each deletion mutant construct was tested in triplicate groups; the mean and SD of Firefly to Renilla luciferase activity ratio was calculated for each group and 95% confidence interval was used for comparing means among separate groups. Each experiment was done at least twice.

Preparation of A549 cell lines with low levels of FOXC2 or FOXL1. Stable cell lines expressing low levels of FOXC2 or FOXL1 were created by using short hairpin RNA (shRNA) directed against FOXC2 or FOXL1. Lentiviral plasmid constructs expressing shRNA were purchased (Open Biosystems, FOXC2: TRC0000013978; FOXL1: TRC0000013978). Lentiviral particles were prepared and titrated by the University of California at Los Angeles’ vector core facility and A549 cells were transduced 48 hours after transduction. A549 cells were selected with puromycin at 1.0 μg/mL of medium for 2 days. Messenger RNA levels of FOXC2 or FOXL1 were measured by reverse transcription-PCR (RT-PCR) in these cell lines A549/FOXC2low and A549/FOXL1low. As a negative control, another stable A549 cell line was prepared using shRNA empty vector A549/PLKO.1.

SiRNA-mediated silencing of FOXC2 and p120ctn. To examine the effect of FOXC2 on p120ctn regulation and also to assess the role of p120ctn in biological effects that are mediated by FOXC2, we silenced FOXC2 and p120ctn in A549, H157, and H358 cells by siRNA. Initially, A549 cells were transfected with two independent sets of siRNA for both FOXC2 and p120ctn.

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- **FOXC2 siRNA #1**: 5′-CAACGUGGCGGGAUACUUC-3′
- **FOXC2 siRNA #2**: 5′-CUACCUGAGCAGGCGAGAUU-3′
- **p120ctn siRNA #1**: 5′-GCCAUUGAGACCCUGAUUA-3′
- **p120ctn siRNA #2**: 5′-CUAUGAUGACCCUGAUAU-3′

Transfection was done by using Lipofectamine 2000 (Invitrogen). As a control, scrambled nonsilencing siRNA was
We also measured FOXC2 and FOXL1 levels quantitatively by qRT-PCR as explained above using the Taqman probes: FOXC2 assay ID Hs00270951_s1 and FOXL1 assay ID Hs00534264_s1 (Applied Biosystems).

**Electrophoretic mobility shift assay.** A549/FOXC2low, A549/FOXL1low, and A549/PLKO.1 cells were grown in 10-cm Petri dish up to ~70% confluency. Cells were washed with cold PBS and harvested by scraping from the dish. Buffer A (10 mmol/L of pH 7.9 HEPES, 1.5 mmol/L MgCl2, 10 mmol/L KCl, and 1 mmol/L DTT) was added before pulverization with a KONTES Dounce Tissue Grinders on ice. Cells were lysed using 5 to 10 strokes and checked for adequate lysis before proceeding. Subsequently, nuclei were pelleted, lysed with Buffer C [20 mmol/L HEPES (pH 7.9), 0.42 mol/L NaCl, 1.5 mmol/L MgCl2, 0.1 mol/L EDTA, 25% glycerol, and 1 mmol/L DTT], and then passed several times through a 25-gauge needle. Debris was removed by centrifugation at 4°C for 30 minutes. Wild-type and mutant FOX oligonucleotide probes were designed and Oct-1 oligonucleotide probes were purchased from (Santa Cruz Biotechnology). Twenty micrograms of nuclear protein was combined with end-labeled, double-stranded oligonucleotide probe, 1 μg of polyIdC (Amerham Pharmacia Biotech), in a final reaction volume of 20 μL for 20 minutes at room temperature. The DNA protein complex was run on a 4% nondenaturing polyacrylamide gel with 0.4× Tris-borate EDTA running buffer before subsequent autoradiography. Cold competition experiments were done with a 100-fold molar excess of double-stranded, cold wild-type or cold mutant FOX oligonucleotide probes. As a control, electrophoretic mobility shift assays (EMSA) for Oct-1 were done in a similar fashion.

**FOX-forward:** 5′-AGAAATGTATGTACCTGACGGG-3′
**FOX-reverse:** 5′-CCCCGTACGTACATACATTCTT-3′
**FOX-mutant-forward:** 5′-AGAAATGCGTGGCGGTACGGG-3′
**FOX-mutant-reverse:** 5′-CCCCGTACGCGCAGCTTTTCT-3′
**Oct-1-forward:** 5′-TGTCGAAATTGCAACCTAGAA-3′
**Oct-1-reverse:** 5′-TTCTAGTGATTTCATGCAACG-3′
**Oct-1-mutant-forward:** 5′-GCGCGCUUUGUAGGAUUCGdTdT
**Oct-1-mutant-reverse:** 5′-3TTAGTGCGTGGCGATTCAAGC-3′

**Western blots.** NSCLC cell lines were seeded in 10-cm Petri dishes at 5 × 105 cells per dish, which resulted in 30% to 40% confluency 24 hours after plating. Cells were harvested at 24 hours by adding trypsin, were pelleted, and were lysed in 100 μL of lysis buffer (15 mmol/L NaCl, 0.5 mmol/L EDTA, and 10 mmol/L Tris) using a Branson Sonifier. Cell debris was collected by centrifugation at 4°C and protein concentration was measured by the bichinchonic acid method. Protein was resolved by SDS-PAGE and was transferred to a nitrocellulose membrane. The

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**Measurement of p120ctn mRNA level by quantitative RT-PCR.** p120ctn mRNA level in NSCLC cell lines were measured by quantitative RT-PCR (qRT-PCR) with a Taqman probe directed toward all isoforms of p120ctn (assay ID: Hs00931670_m1; Applied Biosystems). In each cell type, total RNA was extracted by using the Trizol method. A cDNA library was prepared by a RT-PCR using SuperScript-II RT kit (Invitrogen) and Oligo (dT)s according to the manufacturer’s instructions. For each cDNA sample, an internal control (β actin) was also measured by Taqman probe. To compare the expression level of p120ctn between samples, we used the Comparative Ct Method (ΔΔCt). Relative expression of p120ctn compared with β actin in each sample was calculated (ΔCt) and relative expression of p120ctn among samples was determined by calculating the difference in (ΔCt) between samples (ΔΔCt). All relative quantitative PCRs were done, recorded, and analyzed by using the ABI 7300Prism Sequence Detection System (Applied Biosystems). All samples were carried out in triplicate (10 ng of total RNA per well) and repeated at least twice. Controls without template or without reverse transcriptase (no-RT) were run in each experiment.

**Measurement of FOXC2 and FOXL1 mRNA level by RT-PCR.** We extracted total RNA from each NSCLC cell line by using the Trizol method and prepared cDNA libraries using SuperScript-II RT kit (Invitrogen) and Oligo (dT)s according to the manufacturer’s instructions. The following primer sets were used to amplify a short segment of FOXC2 and FOXL1 cDNA.

**FOX-forward:** 5′-AGAAATGTATGTACCTGACGGG-3′
**FOX-reverse:** 5′-CCCCGTACGTACATACATTCTT-3′
**FOX-mutant-forward:** 5′-AGAAATGCGTGGCGGTACGGG-3′
**FOX-mutant-reverse:** 5′-CCCCGTACGCGCAGCTTTTCT-3′
**Oct-1-forward:** 5′-TGTCGAAATTGCAACCTAGAA-3′
**Oct-1-reverse:** 5′-TTCTAGTGATTTCATGCAACG-3′
**Oct-1-mutant-forward:** 5′-GCGCGCUUUGUAGGAUUCGdTdT
**Oct-1-mutant-reverse:** 5′-TTTCTAGTGCGTGGCGATTCAAGC-3′

**Western blots.** NSCLC cell lines were seeded in 10-cm Petri dishes at 5 × 105 cells per dish, which resulted in 30% to 40% confluency 24 hours after plating. Cells were harvested at 24 hours by adding trypsin, were pelleted, and were lysed in 100 μL of lysis buffer (15 mmol/L NaCl, 0.5 mmol/L EDTA, and 10 mmol/L Tris) using a Branson Sonifier. Cell debris was collected by centrifugation at 4°C and protein concentration was measured by the bichinchonic acid method. Protein was resolved by SDS-PAGE and was transferred to a nitrocellulose membrane. The

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**Concentrations of siRNA were kept at 100 nmol/L among groups.**

**Overexpression of FOXC2 in A549 cells.** We obtained the plasmids p-Babe human FOXC2 and pBabe-puro (Addgene, Inc., deposited by Robert A. Weinberg), and transiently transfected A549 cells by using Lipofectamin 2000 (Invitrogen). For measuring p120ctn promoter activity, p120ctn promoter constructs (p120ctn plasmids and SV40) were cotransfected along with the abovementioned (Invitrogen). For measuring transiently transfected A549 cells by using Lipofectamin 2000 (dgene, Inc., deposited by Robert A. Weinberg), and transiently transfected A549 cells by using Lipofectamin 2000 (Invitrogen). For measuring p120ctn promoter activity, p120ctn promoter constructs (~221 to +288) and (pRL-SV40) were cotransfected along with the abovementioned plasmids and p120ctn promoter activity was measured 48 hours after transfection.

**Measurement of activated expression of p120ctn among samples was determined by calculating the difference in (ΔCt) between samples (ΔΔCt). All relative quantitative PCRs were done, recorded, and analyzed by using the ABI 7300Prism Sequence Detection System (Applied Biosystems). All samples were carried out in triplicate (10 ng of total RNA per well) and repeated at least twice. Controls without template or without reverse transcriptase (no-RT) were run in each experiment.**

**Measurement of FOXC2 and FOXL1 mRNA level by RT-PCR.** We extracted total RNA from each NSCLC cell line by using the Trizol method and prepared cDNA libraries using SuperScript-II RT kit (Invitrogen) and Oligo (dT)s according to the manufacturer’s instructions. The following primer sets were used to amplify a short segment of FOXC2 and FOXL1 cDNA.

**FOXforward:** 5′-AGAAATGTATGTACCTGACGGG-3′
**FOX-reverse:** 5′-CCCCGTACGTACATACATTCTT-3′
**FOX-mutant-forward:** 5′-AGAAATGCGTGGCGGTACGGG-3′
**FOX-mutant-reverse:** 5′-CCCCGTACGCGCAGCTTTTCT-3′
**Oct-1-forward:** 5′-TGTCGAAATTGCAACCTAGAA-3′
**Oct-1-reverse:** 5′-TTCTAGTGATTTCATGCAACG-3′
**Oct-1-mutant-forward:** 5′-GCGCGCUUUGUAGGAUUCGdTdT
**Oct-1-mutant-reverse:** 5′-TTTCTAGTGCGTGGCGATTCAAGC-3′

**Western blots.** NSCLC cell lines were seeded in 10-cm Petri dishes at 5 × 105 cells per dish, which resulted in 30% to 40% confluency 24 hours after plating. Cells were harvested at 24 hours by adding trypsin, were pelleted, and were lysed in 100 μL of lysis buffer (15 mmol/L NaCl, 0.5 mmol/L EDTA, and 10 mmol/L Tris) using a Branson Sonifier. Cell debris was collected by centrifugation at 4°C and protein concentration was measured by the bichinchonic acid method. Protein was resolved by SDS-PAGE and was transferred to a nitrocellulose membrane. The
membrane was blocked with TBS with 5% nonfat powdered milk.

Membranes were immunoblotted with the following primary antibodies: p120ctn (BD Biosciences), FOXC2 (Santa Cruz), and E-cadherin (BD Biosciences). Horse radish peroxidase–conjugated pertinent secondary antibodies were used for detection of bands by chemiluminescence (enhanced chemiluminescence Western blotting detection reagents, Amershams Biosciences).

**Chromatin immunoprecipitation assay.** We followed the method described by Boyd and Farnham (13) and Y. Shang et al. (14) with some modifications. (a) A549 and H157 cells (2 × 10^7 in a 150-mm dish) were grown to confluence. (b) Cells were cross-linked by adding formaldehyde to a final concentration of 1% (0.68 mL of 37%/25 mL media) directly into the media and mixed on a rocker for 10 minutes at room temperature. (c) The cross-linking was stopped by adding glycine to a final concentration of 125 mmol/L (3.75 mL of 1 mol/L/25 mL media) for 5 minutes at room temperature. (d) The cell monolayers were washed thrice with ice-cold 1× PBS. Cells were then scraped into 1× PBS (5 mL) plus protease inhibitors and collected by centrifugation (700× g for 4 min). (e) The cell pellets were resuspended in cell lysis buffer [5 mmol/L Pipes (KOH; pH 8.0)/85 mmol/L KCl/0.5% NP40] containing the protease inhibitors 1 μg/mL leupeptin, 1 μg/mL aprotinin, and 1 mmol/L phenylmethylsulfonyl fluoride, and incubated for 10 minutes on ice. The efficiency of cell lysis was checked with trypan blue and, if needed, cells were homogenized on ice with a type B homogenizer. (f) Nuclei were pelleted by centrifugation (5,000 rpm for 5 minutes) and resuspended in nuclear lysis buffer [50 mmol/L Tris (pH 8.1)/10 mmol/L EDTA/1% SDS containing the same protease inhibitors as in cell lysis buffer] followed by incubation on ice for 10 minutes. (g) Chromatin was sheared by using a sonifier to an average length of ∼600 bp while keeping samples on ice. For both cell lines, we used a Branson Sonifier 250 with power setting of 5 in 20-second bursts followed by 1 minute of cooling on ice for a total sonication time of 3 minutes per sample. (h) Debris was cleared by centrifugation at maximum speed for 10 minutes at 4°C. (i) The supernatant was transferred to a new tube and diluted 5-fold in a chromatin immunoprecipitation assay (ChIP) dilution buffer [0.01% SDS/1.1% Triton X-100/1,2 mmol/L EDTA, 16.7 mmol/L Tris (pH 8.1)/167 mmol/L NaCl plus protease inhibitors]. (j) To reduce nonspecific background, we precleared the samples with 80 μL of salmon sperm DNA/protein A/G agarose slurry for 30 minutes at 4°C with agitation. (k) We collected the beads by a brief centrifugation and separated the supernatant fraction. (l) Twenty percent of the total supernatant was saved as total input control and processed with the eluted immunoprecipitates. (m) The rest of the supernatant was divided into two fractions: one for an immunoprecipitation with IgG control and the second was incubated with 5 μg of FOXC2 antibody (Santa Cruz) overnight at 4°C with rotation. (n) We collected immune complexes with 60 μL of the salmon sperm DNA/protein A/G agarose slurry for 1 hour at 4°C with rotation. (o) Beads were then washed consecutively for 3 to 5 minutes on a rotating platform with 1 mL of each solution: a low-salt wash buffer [0.1% SDS/1% Triton X-100/2 mmol/L EDTA, 20 mmol/L Tris (pH 8.1)/150 mmol/L NaCl], a high-salt wash buffer [0.1% SDS/1% Triton X-100, 2 mmol/L EDTA, 20 mmol/L Tris (pH 8.1), 500 mmol/L NaCl], a LiCl wash buffer [0.25 mol/L LiCl/1% NP40/1% deoxycholate, 1 mmol/L EDTA/10 mmol/L Tris (pH 8.0)], and twice in 1× TE buffer. (p) Complexes were eluded by adding 250 μL of elution buffer (1% SDS/0.1 mol/L NaHCO₃) to pelleted beads by mixing and shaking by vortexer for at least 15 minutes. Beads and eluted proteins were separated by centrifugation at 14,000 rpm for 3 minutes. This step was repeated and both elutions were combined in the same tube. (q) Formaldehyde cross link reverses were removed by adding 1 μL 10 mg/mL RNase and 5 μL NaCl to a final concentration of 0.3 mol/L to the elutants and incubation in a 65°C for 4 to 5 hours. (r) DNA was precipitated by adding 2.5 volumes of 100% ethanol overnight at −20°C. (s) DNA and debris were collected by centrifugation at high speed and resuspended in 100 μL of water. We added 2 μL of 0.5 mol/L EDTA, 4 μL 1 mol/L Tris (pH 6.5), and 1 μL of 20 mg/mL Proteinase K; incubated samples for 1 to 2 hours at 45°C; and purified DNA by using QiaQuick spin columns. (t) Two microliters of this DNA sample was used in RT-PCR with the following primers.

FOXChIP-forward: 5′-GATCCCGAAAGGAGGAAGAG-3′
FOXChIP-reverse: 5′-CGACTTTGCTTATCCTCC-TTTC-3′

**Results**

**Promoter activity of p120ctn is significantly reduced in NSCLC cell lines compared with immortalized normal human respiratory epithelial cell line BEAS-2B.** Because a reduction in p120ctn protein is associated with reduced p120ctn mRNA levels in NSCLC patient samples (11), we postulated that altered expression of p120ctn was attributable to the transcriptional downregulation of the p120ctn promoter. As such, we sought to compare p120ctn promoter activity in the NSCLC cell lines to that of an immortalized normal respiratory epithelial cell line, BEAS-2B. BEAS-2B cells represent human bronchial epithelial cells infected by the SV40 virus, which results in a nonmutogenic immortalized cell line.

To analyze the p120ctn promoter, we focused on 1,400 nucleotide region spanning both the 5′ and 3′ regions of the p120ctn transcriptional start site, which encompasses numerous predicted transcription factor binding sites (i.e., cis-acting elements). Two separate constructs were generated, one spanning from position −1,082 to +320 (numbers relative to transcriptional start site) and another
from position −540 to +320; both were cloned into a firefly luciferase reporter vector (pGL4.16 Luciferase reporter vector, Promega) to generate p120ctnlong-luc and p120ctnshort-luc, respectively (Fig. 1A). Our two p120ctn promoter constructs were transiently transfected into A549 and BEAS-2B cells and reporter gene activity was measured at 24 hours (Fig. 1B). The p120ctn promoter activity in BEAS-2B cells was ∼3-fold higher than in A549 cells. Both the p120ctnshort-luc and p120ctnlong-luc constructs showed a similar pattern of promoter activity (data only shown for short construct). These findings suggest that differential transcriptional regulation of
p120ctn in malignant versus nonmalignant lung epithelial cells contributes to the metastatic potential of malignant lung epithelium. To examine the generalizability of our results, we compared the p120ctn promoter activity in multiple NSCLC lines to that of BEAS-2B. The same pattern of p120ctn promoter activity is also observed among other NSCLC lines when transfected with p120ctnshort-luc construct (Fig. 1C), indicating that the

![Graph A](image)

**FIGURE 2.** A, analysis of p120ctn promoter by creating serial deletion constructs. These deletion constructs of full-length p120ctn promoter were prepared by deletions from both the 5' and 3' ends of the p120ctnshort-luc construct. Both A549 and BEAS-2B cells were transfected with this mutant constructs, and 24 h later, cells were lysed and subjected to dual Luciferase assay; data only shown for A549 cells. Relative promoter activity is normalized to full-length promoter activity in A549 cells. B, measuring p120ctn promoter activity following deletion of segment (+267 to +282) in several NSCLC cell lines. Error bars, 95% confidence interval. C, detailed analysis of the 3' end of p120ctn promoter between positions +267 and +320 relative to transcription initiation site. Luciferase activity significantly changes between position +267 and +282 both in A549 and BEAS-2B cells; data shown only for A549 cells. Values were normalized to that of A549 cells transfected with 1.0 μg of p120ctnshort-luc.
phenomenon of reduced \(p120\text{ctn}\) promoter activity is observed in multiple NSCLC cell lines of different histologic origin. Because we observed similar results with both the long and short constructs, we focused subsequent promoter experiments on the \(p120\text{ctn}_{\text{short}}\)-luc construct (−540 to +320 relative to the transcription initiation site).

**Promoter activity of \(p120\text{ctn}\) is reflective of \(p120\text{ctn}\) mRNA and protein level in NSCLC and normal human respiratory epithelial cell lines.** We next sought to confirm that a reduction in \(p120\text{ctn}\) promoter activity in NSCLC cell lines correlates with reduced \(p120\text{ctn}\) mRNA transcripts and protein levels. Therefore, as a validation step, we measured the \(p120\text{ctn}\) mRNA and protein expression in NSCLC cell lines and compared it to that of BEAS-2B cells. Indeed, qRT-PCR showed an overall 50% to 60% reduction of \(p120\text{ctn}\) mRNA transcripts in the A549 (squamous cell), Rh2 (squamous cell), H157 (undifferentiated), and H596 (adenosquamous; data not shown) cells, compared with the nonmalignant BEAS-2B cells (Fig. 1D). Next, we examined the protein expression of \(p120\text{ctn}\) in several NSCLC cell lines. For this purpose, we used an antibody directed against the COOH-terminus of \(p120\text{ctn}\), which detects all isoforms of this protein (Figs. 1E and 5). Analogous to our qRT-PCR results, a reduced level of \(p120\text{ctn}\) was observed in NSCLC cell lines A549, Rh2, and H157 compared with BEAS-2B cells.

Thus, our results showing a reduction of mRNA transcript of \(p120\text{ctn}\), which detects all isoforms of this protein (Fig. 1D). Next, we examined the protein expression of \(p120\text{ctn}\) in several NSCLC cell lines. For this purpose, we used an antibody directed against the COOH-terminus of \(p120\text{ctn}\), which detects all isoforms of this protein (Figs. 1E and 5). Analogous to our qRT-PCR results, a reduced level of \(p120\text{ctn}\) was observed in NSCLC cell lines A549, Rh2, and H157 compared with BEAS-2B cells.

**Deletion mutation analysis of the \(p120\text{ctn}_{\text{short}}\)-luc promoter construct.** Given the finding of reduced \(p120\text{ctn}\) promoter activity in NSCLC cells, we next sought to identify the cis- and trans-acting elements that mediate the transcriptional repression of \(p120\text{ctn}\) in malignant lung epithelium. To our knowledge, the biochemical signals that regulate \(p120\text{ctn}\) promoter activity have not been studied to date. To identify the important and relevant sequences in the \(p120\text{ctn}\) promoter region involved in \(p120\text{ctn}\) transcriptional downregulation, we performed serial deletions of our \(p120\text{ctn}_{\text{short}}\)-luc promoter construct. Routinely, these analyses are initiated by generating sequentially larger and larger deletions of the 5′ end of the promoter. Our initial 5′ deletions of the \(p120\text{ctn}_{\text{short}}\)-luc promoter construct did not diminish reporter gene expression [mutant (−221 to +320); Fig. 2A]. However, further 5′ deletions resulted in significant loss of promoter activity equivalent to that of the empty vector control, which was most likely due to loss of the TATA box and core promoter region [mutant (+127 to +320); Fig. 2A]. Consequently, our subsequent deletional promoter analyses were modified so that we maintained the TATA box, the core promoter region, and the transcription initiation site intact. Specifically, we created 3′ deletion mutant constructs spanning from position −221 to positions +267, +239, +69, +19, −9, −20, and −71 relative to transcription initiation site. The schematic view of the deletion mutant constructs and their relative promoter activity compared with full-length promoter in A549 cells are shown in (Fig. 2A).

Our first 3′ deletion yielded perhaps the most interesting result [mutant (−221 to +267); Fig. 2A]. Deletion of this 53-bp segment at the 3′ end of \(p120\text{ctn}_{\text{short}}\)-luc construct (+267 to +320) resulted in a robust increase in luciferase activity, which was observed in both A549 and BEAS-2B cells. This finding was corroborated in H157, Rh2, and H5358 cells, indicating the generalizability of the result (Fig. 2B). These findings strongly suggest the existence of transcriptional repressive elements within the region of +267 to +320 of the \(p120\text{ctn}\) promoter. Further 3′ deletions of \(p120\text{ctn}_{\text{short}}\)-luc construct revealed other key regulatory regions of \(p120\text{ctn}\) promoter. Noticeably, deletion of segment +19 to +69 of the \(p120\text{ctn}_{\text{short}}\)-luc construct resulted in a dramatic decrease in luciferase activity [mutant (−221 to +19); Fig. 2A]. Detailed analysis of this segment and adjacent core promoter region of \(p120\text{ctn}\) promoter is currently under way in our laboratory. In this report, we focus on segment +267 to +320 of the \(p120\text{ctn}\) promoter and its putative suppressive elements as was revealed by dele- tional mutational analysis. Sequential deletions within segment +267 to +320 of \(p120\text{ctn}\) promoter shows that the putative suppressive elements in fact reside between position +267 and +282 relative to transcription initiation site in a short 15 bp stretch (Fig. 2C).

To identify putative cis-acting elements that regulate \(p120\text{ctn}\) promoter, we used a computer-based DNA sequence analysis known as JASPAR, an open access eukaryotic transcription factor binding site database. JASPAR uses matrix-based analysis to predict potential transcription factor binding sites in a given DNA sequence (15). By using position-specific weight matrix models, the computer software assigns a score to each candidate binding sequence. Relative scores are calculated based on the percentage of the maximum possible score for each matrix. Therefore, the higher the relative scores for a given candidate binding site, the higher the similarity of that candidate site to a

| Table 1. Predicted Forkhead-related proteins binding sites in segment +267 to +282 of the \(p120\text{ctn}\) promoter |
|-----------------|-----------------|
| **Predicted binding site** | **Relative score** |
| +267 | +282 |
| agaaaatgtatgactgacggg | FOXC2 0.92 |
| tgtatgta | FOXC2 0.86 |
| aaaagtga | FOXL1 0.80 |
| tgtatgta | FOXL1 0.81 |

[Published OnlineFirst May 11, 2010; DOI: 10.1158/1541-7786.MCR-10-0004](https://mcr.aacrjournals.org/content/8/5/768)
known binding site sequence. We found multiple putative transcription factor binding sites (e.g., CAAT/enhancer binding protein-α, NF-κB, AP-2α, GATA-1, FOXC, and FOXL1) within the immediate region upstream and also downstream of the transcription start site (Fig. 1A). When we subjected the putative repressive sequence +267 to +282 of p120ctn promoter to the JASPAR matrix-based sequence analysis, we identified potential binding sites for FOX family members (Table 1). Interestingly, two separate FOXC (i.e., FOXC1 and FOXC2) and FOXL1 binding sites are predicted within this region of p120ctn promoter by setting the profile score threshold at 80%.

Exploring the expression of FOXC2 and FOXL1 in NSCLC cell lines. FOXC1 and FOXC2 are members of forkhead family of transcription factors, both of which have identical DNA binding domains. The function of FOXC1 is yet to be determined and defects of this gene are associated with ophthalmic and cardiovascular anomalies (16). On the other hand, FOXC2 is involved in determining the fate of mesenchymal tissues and also in the development of lymphatic vessels. Moreover, FOXC2 overexpression has been recently observed in aggressive breast cancer with high metastatic potential (15, 17). With regard to FOXL1, the function of this protein has not been well studied, but evidence supports the involvement of FOXL1 in the hedgehog pathway, which functions during embryogenesis and plays a potential role in oncogenesis (18). Thus, we chose to explore the roles of FOXC2 and FOXL1 in the regulation of p120ctn. To verify whether lung cancer cell lines express FOXC2 and/or FOXL1 transcription factors, we checked the mRNA expression levels of FOXC2 and FOXL1 by RT-PCR in a few NSCLC cell lines (Supplementary Fig. S1). In RT-PCRs, FOXC2 expression was detected in A549 and H157 cells and FOXL1 in A549 cells.

FOXC2 directly binds to segment +267 to +282 of the p120ctn promoter. We next sought evidence

![Figure 3](https://www.aacrjournals.org/mcr/8/5/769/fig3a.png)

**FIGURE 3.** A, EMSA in A549/FOXC2low, A549/FOXL1low, and A549/PLKO.1 cell lines. B, qRT-PCR measuring the degree of FOXC2 and FOXL1 silencing in A549/FOXC2low, A549/FOXL1low, and A549/PLKO.1 cell lines. C, ChIP in A549 and H157 cells; immunoprecipitation with anti-FOXC2 antibody or IgG control, PCR with primers encompassing segment +127 to +309 of the p120ctn promoter. Error bars, 95% confidence interval.
that whether FOXC2 or FOXL1 directly interact with the putative suppressive cis-acting elements between positions +267 and +282 of the p120ctn promoter. To investigate this issue, we examined DNA-protein interactions in EMSA and ChIP assays. First, we prepared A549 cell lines with low levels of FOXC2 or FOXL1 by using shRNA expressed by lentiviral particles. We transduced A549 cells with lentiviral particles expressing shRNA directed against FOXC2 or FOXL1 and selected the transduced cells with puromycin generating A549/FOXC2 low and A549/FOXL1 low cell lines. As a negative control, lentiviral empty vector PLKO.1 was also used to transduce A549 cells generating A549/PLKO.1 cell line. Efficiency of silencing was measured by qRT-PCR (Fig. 3B). Sense and anti-sense oligonucleotides representing the segment between positions +267 to +282 of the p120ctn promoter were annealed and end labeled with γ-32P-ATP to make a FOX DNA probe. Nuclear protein from A549/FOXC2 low, A549/FOXL1 low and A549/PLKO.1 cells were extracted and subjected to EMSA using the FOX DNA probe (Fig. 3A). Unlabeled (cold) as well as a mutant cold DNA probes were used as competitors to establish the specificity of our DNA-protein binding.

We observed a reduced DNA-protein binding between the A549/FOXC2 low nuclear extract and the DNA probe representing the repressive region of the p120ctn promoter (+267 to +288), whereas we did not observe any reduction in the intensity of the EMSA signal from nuclear protein extract of A549/FOXL1 low or A549/PLKO.1 cells. These findings suggest that FOXC2 but not FOXL1 interacts with cis-acting elements within positions +267 to +282 of p120ctn promoter.

In addition to EMSA, we examined the protein-DNA binding between FOXC2 and p120ctn promoter in A549 and H157 cells by ChIP. As described in the experimental procedure section, a 25-μg chromatin sample of each cell line was prepared and incubated with either FOXC2 antibody or control IgG. Following immunoprecipitation and appropriate washing steps, harvested DNA was used in a
PCR to amplify a short 182-bp segment of the \( p120^{ctn} \) promoter from position +127 to +309 encompassing segment +267 to +282 (Fig. 3C). We observed that FOXC2 antibody successfully immunoprecipitates a segment of \( p120^{ctn} \) promoter, which contains segment +267 to +282, thereby confirming the direct binding of FOXC2 to endogenous \( p120^{ctn} \) promoter in these two NSCLC cell lines.

Silencing FOXC2 results in increased \( p120^{ctn} \) promoter activity, mRNA, and protein levels. Deletion mutagenesis experiments earlier suggested a repressive role for cis-acting elements residing between positions +267 to +282 of \( p120^{ctn} \) promoter with putative transcription factors being predicted to be FOXC2. Therefore, we predicted that silencing FOXC2 would result in increasing \( p120^{ctn} \) promoter activity, mRNA copy number, and protein levels. To verify this notion, we used a siRNA technique to silence FOXC2. Two independent siRNA sets were used to silence FOXC2 in A549 cells, both of which showed successful silencing of this gene (Supplementary Fig. S1). We transfected A549; H157 and H358 cells with siRNA directed against FOXC2. As a negative control, these cells were transfected with nonsilencing scrambled siRNA. Efficiency of silencing was measured by qRT-PCR (Fig. 4C). Next, we subjected these NSCLC cells with low levels of FOXC2 to a dual luciferase assay using the \( p120^{ctn} \) short-luc promoter construct (−540 to +320; Fig. 4A). All three NSCLC cell lines show a robust increase of ~2-fold in their \( p120^{ctn} \) promoter activity compared with their counterpart cells transfected with nonsilencing siRNA.

In concert with previous results, we expected to observe an increase in \( p120^{ctn} \) mRNA and protein levels by silencing FOXC2. Therefore, following silencing of FOXC2 by siRNA in A549; H157 and H358 cells, we
measured the mRNA levels of p120ctn by qRT-PCR with a Taqman probe (Fig. 4B). Silencing FOXC2 results in an ∼2-fold increase in p120ctn mRNA levels in these cell lines. Western blot analysis also shows an increase in p120ctn protein level in all the examined NSCLC cell lines (Fig. 6B). These results strongly suggest the role of FOXC2 in transcriptional regulation of p120ctn.

Silencing FOXC2 results in increased level of E-cadherin. This effect is mainly mediated through p120ctn regulation. Expression level of FOXC2 has an inverse correlation with E-cadherin level in epithelial cells. Forced expression of FOXC2 in Madin-Darby canine kidney cells resulted in the downregulation of E-cadherin (17). On the other hand, the p120ctn expression level in epithelial cells has a direct correlation with E-cadherin level. Silencing p120ctn in NSCLC cells resulted in the downregulation of E-cadherin (19). In concert with these specifics, we have identified a similar expression pattern (i.e., direct p120ctn correlation and reverse FOXC2 correlation with regard to E-cadherin level) in a panel of NSCLC cell lines (Fig. 5). Because we have shown earlier that FOXC2 directly regulates p120ctn, we sought to examine whether the alteration in E-cadherin level caused by changes in FOXC2 is mediated through the regulation of p120ctn. For this purpose, we simultaneously silenced FOXC2 and p120ctn in A549; H157 and H358 cells. Following silencing FOXC2, E-cadherin level increases in all of these NSCLC cells. This effect of FOXC2 on E-cadherin is reversed by silencing p120ctn at the same time (Fig. 6A). This finding strongly points to the notion that the downregulation of E-cadherin level caused by FOXC2 is mainly mediated through the regulation of p120ctn. Moreover, if FOXC2 was controlling E-cadherin level mainly through other pathways, simultaneous silencing of p120ctn would have little effect on FOXC2 mediated E-cadherin changes.

Overexpression of FOXC2 in A549 cells results in the repression of p120ctn promoter activity and protein level. Our data are suggesting a role for FOXC2 in the negative regulation of p120ctn promoter; therefore, it is expected that overexpression of this transcription factor causes reduced p120ctn promoter activity and p120ctn expression. In fact, in transient transfection experiments, we observed a significant repression of p120ctn promoter activity as well as decreased p120ctn protein level in A549 cells transfected with FOXC2-expressing vector compared with empty vector (Fig. 7).

Discussion

Metastasis is one of the main characteristics of malignant tumors. Tumor recurrence in distant sites is seen in 50% to 60% of patients even in early-stage NSCLC who were treated by surgical resection at presentation. This notion indicates that metastasis usually occurs very early in the process of tumorigenesis. Therefore, identifying mechanisms orchestrating invasion and metastasis is an important step in containing this deadly disease.

p120ctn as part of adherens junction participates in cell-cell adhesion and is affected in several different ways in human tumors. First, in some tumors, loss of expression of p120ctn results in reduced stabilization and expression of the whole cadherin-catenin complex. Downregulation of p120ctn expression, which occurs in 60% to 80% of NSCLC cases, is associated with more advanced tumor-node-metastasis stage, higher grade, and reduced overall survival of these patients. Recently, Liu et al. (11) showed a decreased p120ctn isoforms 1 and 3 in 50 patients with NSCLC compared with uninvolved lung tissue. In another study, Wang et al. (4) investigated the expression of p120ctn in 143 patients with NSCLC. Immunohistochemistry and Western blot analyses showed decreased expression of p120ctn in ∼80% of subjects. This low expression level was associated with poor differentiation, high tumor-node-metastasis stage, and lymph node metastasis. Patients with tumors expressing low p120ctn also had shorter survivals with a median survival of 20 versus 70 months. Second, loss of E-cadherin during tumor progression causes the mislocalization of p120ctn to the cytoplasm and the nucleus, which disables the adherens junctions, thereby reducing cell adhesion. Lastly, posttranslational modification of p120ctn (most likely through phosphorylation) possibly by src may enhance the adhesive properties of malignant cells (20).
p120ctn also participates in cell motility, although its role in cell movement is rather complicated as reviewed by Anastasiadis (20). For example, when E-cadherin expression is lost, p120ctn can localize to the cytoplasm where it interacts with RhoA and Rac1, thereby promoting cell motility. However, although the effects of p120ctn on cell motility have not been fully elucidated, the evidence to date suggests that p120ctn may function differently in this regard in epithelial versus mesenchymal tissues.

In NSCLC cell lines, ablation of p120ctn resulted in the enhancement of invasion and metastasis as was show by Liu et al. (19). In this study, the downregulation of p120ctn by siRNA in NSCLC cell lines resulted in reduced levels of E-cadherin and β-catenin proteins. Furthermore, p120ctn silencing inactivated RhoA and increased Rac1 activity, promoting the invasiveness of lung cancer cells. Moreover, as mentioned above, lower p120ctn levels in NSCLC tumors compared with adjacent uninvolved lung tissue was reported in two separate studies (4, 11). Therefore, accumulating evidence points to the fact that reduced expression of p120ctn both in vitro and in vivo results in the destabilization of cell-cell adhesions and contributes to the metastatic process. Importantly, reduced mRNA expression of this gene in several tumors types indicates that reduced p120ctn in the process of tumorigenesis is most likely due to transcriptional repression (21).

Our data revealed a lower promoter activity of p120ctn in NSCLC cells compared with immortalized normal respiratory cell line. This finding suggested an altered transcriptional regulation of p120ctn in malignant respiratory epithelium. Through deletion mutational analysis of the p120ctn promoter, here, we identified the region +267 to +288 of p120ctn promoter to harbor repressive cis-acting elements. We also identified FOXC2 (members of forkhead family of transcription factors) as a negative regulator of p120ctn. Through a series of biochemical experiments, we established that FOXC2 suppresses p120ctn transcription and showed that FOXC2 directly binds to this region of p120ctn promoter. We also showed that the biological effects of FOXC2 (manifested as E-cadherin alteration) are mainly mediated through the regulation of p120ctn.

The metastatic promoting effects of FOXC2 are probably mediated through the transcriptional regulation of several downstream genes. These targets genes of FOXC2 are gradually being identified. Hayashi et al. (22) recently reported the role of FOXC2 in the regulation of integrin β3. Here, our data identifies p120ctn as another target of FOXC2.

In summary, these findings provide evidence for a role of the members of the FOX family of transcription factors in the regulation of invasiveness and cell-cell adhesion of NSCLC cells that is mediated mainly through p120ctn regulation. Further investigations in this field will help us identify primary events resulting in increased tendency of malignant tumors to invade the local microenvironment and subsequently metastasize.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Grant Support

Grant from the Tower Cancer Research Foundation.

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Received 01/05/2010; revised 03/09/2010; accepted 03/24/2010; published OnlineFirst 05/11/2010.

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Molecular Cancer Research

p120-Catenin Is Transcriptionally Downregulated by FOXC2 in Non–Small Cell Lung Cancer Cells

Fariborz Mortazavi, Jiabin An, Steven Dubinett, et al.

Mol Cancer Res 2010;8:762-774. Published OnlineFirst May 11, 2010.

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doi:10.1158/1541-7786.MCR-10-0004

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