Published in final edited form as:

Gastroenterology. 2008 September ; 135(3): 907–916.e2. doi:10.1053/j.gastro.2008.06.034.

Hoxc6 is overexpressed in gastrointestinal carcinoids and interacts with JunD to regulate tumor growth

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Abstract

Background and Aims—The molecular alterations that underlie carcinoid tumor pathogenesis remain poorly defined. The homeobox gene *Hoxc6* was highly upregulated in human gastrointestinal carcinoid tumors, and we sought to define its pathogenic role.

Methods—The functional and physical properties of *Hoxc6* were investigated by establishing carcinoid cells that stably overexpressed *Hoxc6* or were deficient in *Hoxc6*. Cellular proliferation assays, luciferase reporter assays, western blotting, immunoprecipitation, DNA affinity precipitation, and DNA microarray studies were performed

Results—Expression of *Hoxc6* in cultured human BON1 carcinoid cells enhanced their proliferation, and knock-down of *Hoxc6* inhibited their growth. *Hoxc6* activated the oncogenic AP-1 signaling pathway through a physical interaction with JunD. Mutations in the homeodomain of *Hoxc6* blocked this interaction and inhibited proliferation of carcinoid cells. Of note, *Hoxc6* induced the expression of genes that are characteristically upregulated in carcinoid tumors, including neurotensin (NT) and connective tissue growth factor (CTGF).

Conclusions—A novel molecular pathway has been identified that links *Hoxc6* with oncogenic signaling through the AP-1 pathway in carcinoid tumorigenesis.

Keywords

carcinoid; AP-1; homeobox; neuroendocrine tumor

Introduction

Carcinoid tumors are neuroendocrine neoplasms that can arise in multiple organ sites but are most commonly localized to the gastrointestinal tract¹. They are typically slow-growing,

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No conflicts of interest exist.

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well-differentiated tumors that secrete 5-hydroxytryptamine, and excess levels can result in the clinical carcinoid syndrome². Gastrointestinal carcinoids are often classified together with neuroendocrine tumors of the pancreas, as they share many clinical and biological features. However, key insights into their underlying molecular pathogenesis are lacking.

Individuals with the Multiple Endocrine Neoplasia Type 1 syndrome are at high risk for the development of neuroendocrine tumors of the pancreas and pituitary, as well as parathyroid gland hyperplasia and gastric carcinoids. The *MEN1* gene that underlies the syndrome was cloned in 1997³. *MEN1* also plays a role in sporadic pancreatic neuroendocrine tumors and gastrointestinal carcinoids, but only a subset of these harbors *MEN1* mutations⁴. The *MEN1* gene product, menin, has diverse functions⁵, and most recently has been demonstrated to regulate gene transcription through interactions with a histone methyltransferase complex^{6,7}. Some of the targets of menin include the homeobox genes Hoxc6 and Hoxc8⁶ as well as the cell cycle inhibitors p18 and p27^{8,9}.

Homeobox (Hox) genes belong to the homeoprotein family of transcription factors that are developmental regulators of growth, patterning, and differentiation. There are 4 clusters of Hox genes (HOXA-D), and Hox proteins contain a homeodomain that consists of three helices separated by a short loop. Helix III (the C-terminal helix) contacts A/T-rich domains of DNA in the regulatory regions of many genes¹⁰. Because most homeobox proteins have similar DNA binding specificity *in vitro*, the timing of expression as well as the interactions between Hox proteins and other transcriptional regulators are likely to be critical for their functional specificity. Deregulated expression of Hox genes has been reported in many tumors, including lung, breast, ovarian, sarcoma, and leukemia¹⁰. The specific mechanisms by which Hox genes contribute to the tumorigenic phenotype are incompletely described. In some cases, they may function as transcription factors that stimulate the expression of growth factors, such as Hoxb7-mediated upregulation of fibroblast growth factor in melanomas¹¹. Overexpression of Hoxc6 is observed in human prostate cancer, where it functions to inhibit apoptosis^{12,13}.

Because of the link between the Hoxc6 and Hoxc8 gene clusters and the *MEN1* gene⁶, we were curious whether expression of these Hox genes was altered in human neuroendocrine tumors. Previous DNA microarray studies indicated that Hoxc6 mRNA was strongly upregulated in gastrointestinal carcinoid tumors but not in closely related pancreatic neuroendocrine tumors¹⁴. There are 2 Hoxc6 variants that arise from differential promoter usage¹⁵. Hoxc6-variant 1 (Hoxc6-V1) is derived from a 1.8 kb mRNA transcript encoding a 27 kD protein, and Hoxc6-variant 2 (Hoxc6-V2) is derived from a 2.2 kb mRNA transcript that encodes an 18 kD protein. Although both possess the identical 61 amino acid homeodomain, their functional differences are unknown. We now verify that Hoxc6 is overexpressed in human carcinoid tumors and demonstrate that expression of Hoxc6 stimulated the growth of carcinoid cells *in vitro*. Hoxc6 enhanced signaling through the AP-1 pathway, and this was mediated by a novel interaction between Hoxc6 and the JunD component of AP-1. These findings link a developmental regulator to an oncogenic signaling pathway. In addition to providing an important new insight into the pathogenesis of carcinoid tumors, these studies point to a molecular pathway that may be a novel target for therapy in carcinoids.

Materials and methods

Quantitative PCR and RT-PCR

RNA from eleven carcinoid tumors (Table 1), eight pancreatic endocrine tumors, two samples of normal ileum, and six of normal pancreas were isolated using the RNeasy Protect Mini Kit (QIAGEN). Reverse transcription was performed using the SuperScript III

platinum Two-Step qRT-PCR Kit (Invitrogen). The primers for Hoxc6 variant 1 (Hoxc6-V1) were; sense 5'-CCTTCCTTATCCTGCCACCT-3', antisense 5'-GCTGGAAGTGAACACGACAT-3' (NM_004503). The primers for Hoxc6 variant 2 (Hoxc6-V2) were; sense 5'-CTCCTCTCTCCCAGGCTCTT-3', antisense 5'-GCTGCTGTCACTCTCCCTCT-3' (NM_153693). The primers for Hoxc8 were; sense 5'-CTCAGGCTACCAGCAGAACC-3', antisense 5'-TTGGCGGAGGATTTACAGTC-3' (NM_022658). 18S rRNA served as an endogenous control. PCR cycles were: 2 min at 95 °C, followed by 40 cycles with an annealing temperature of 60 °C. A fluorogenic SYBR Green and MJ research detection system were used for real time quantification. Primer sequences for neurotensin (NT), peptide YY (PYY), and connective tissue growth factor (CTGF) are available upon request.

The results were presented as parameter threshold cycle (CT) values. CT was the difference in the CT values derived from the specific gene being assayed and 18S rRNA while ΔCT represented the difference between the paired samples, as calculated by the formula $\Delta CT = CT \text{ of a sample} - CT \text{ of a reference}$. The amount of target, normalized to 18S and relative to a reference, was expressed as $2^{-\Delta CT}$.

Stable cell lines

BON1 cells (a human pancreatic carcinoid cell line) were utilized to generate cells stably overexpressing Hoxc6-Variant1, Hoxc6-Variant 2, or an siRNA to Hoxc6. These stable cells are designated BON-Flag control, BON-Hoxc6-V1, BON-Hoxc6-V2, BON-si Hoxc6, and two independent clones of each construct were analyzed. The pCMV FLAG-tag expression vector (Invitrogen) with the coding region of the human Hoxc6 variant 1 (NM_004503) or variant 2 (NM_153693) inserted between the BamHI and XhoI sites was transfected into BON1 cells. The cDNA of Hoxc6 V1 and V2 were a kind gift of Carlos Moreno (Emory University, Atlanta)¹³. The N191Q mutation in Hoxc6-V1 and N109Q mutation in Hoxc6-V2 (AAC → CAG) in the homeodomain¹⁶ was introduced using the Quik Change IIXL Site-Directed Mutagenesis Kit (Stratagene). The siRNA against Hoxc6 was made using pSR-neo vectors (Oligoengine, Seattle, WA). The oligonucleotides were inserted between the BglII and HindIII sites to produce short hairpin RNA. Two different sequences derived from human Hoxc6 mRNA (5'-GGGAAAUAUACAAAAGAG-3', 5'-GCGAAUGAAUUCGCACAGU-3'), common to both variants, were selected. For control siRNA, we used the sequence 5'-GCGCGCTTTGTAGGATTCG-3' that does not correspond to any known human gene. The stable transfected cells were selected in 200 µg/ml geneticin (GIBCO) for 14 days, and clones were screened for Hoxc6 stable expression by Western Blotting using ANTI-FLAG M2 (Sigma) and for Hoxc6 silencing by q-PCR. The cells were maintained in a 1:1 mixture of F12 and DMEM with 10% fetal bovine serum (Cellgro) supplemented with 1% penicillin/streptomycin (Invitrogen) in 5% CO₂ at 37 °C.

Proliferation assay

The stable cells were plated in 96-well plates (1,500–3,000 cells per well). Proliferation of cells was measured by a colorimetric assay using the CellTiter 96 aqueous one solution cell proliferation assay kit (Promega) on day 1, 3, and 7 after plating.

Cell culture, transfections, and luciferase reporter assay

DLD-1 and 293 cells were obtained from ATCC, and TPC-1 cells were a gift from James Fagin. One day after plating BON cells in a 12 well plate, 0.25 µg of an AP-1 luciferase reporter construct that contains 4 consensus AP-1 binding sites and 5 ng of the pRL-CMV Renilla vector (Promega) were co-transfected using Lipofectamine 2000 (Invitrogen). Luciferase activity was measured with the Dual Luciferase Reporter Assay System (Promega) 48 hours after transfection. In independent experiments, 0.25 µg of a menin

expression vector (gift of Matthew Meyerson) was transiently transfected into BON cells and RNA was harvested for qRT-PCR.

siRNA of AP-1 components

siRNA knockdown of the AP-1 components, cFos, JunD, and cJun, was performed with specific duplex oligos. The sequences were; 5'-GGAACAGUUAUCUCCAGAA-3' for cFos (NM_005252), 5'-GCAUGAUGAAGAAGGACGC-3' for JunD (NM_005354), and 5'-GGCACAGCUAAACAGAAA-3' for cJun (NM_002228). A control siRNA that does not correspond to any known human gene, 5'-GGCGAUCAUAAACGACAAA-3', was also utilized. The cells were transfected with 20 nM siRNA duplexes using Lipofectamine 2000 (Invitrogen). AP-1 reporter constructs were co-transfected and dual luciferase assays were performed 48 hours after transfection.

Western Blotting and Immunoprecipitation

Cells were lysed in chilled lysis buffer (Cell Signaling) supplemented with proteinase inhibitors (PSC, Roche). 15–20 µg protein lysate were resolved on 4–12% NuPAGE Bis-Tris polyacrylamide gels (Invitrogen) and transferred to polyvinylidene difluoride membranes (Millipore). Nuclear extracts were isolated using NE-PER kit (Pierce). The blots were probed with Flag and α -Actin (Sigma) antibodies. Immunoreactive proteins were visualized using Western Lighting Chemiluminescence Reagent Plus (Perkin Elmer).

Nuclear extracts were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Reagents (PIERCE). 300 µg of nuclear proteins were incubated with ANTI-FLAG M2 beads (Sigma) overnight at 4°C. The beads were washed 3 times with RIPA buffer (Boston BioProducts) for 5 min, and were boiled in NuPAGE SDS Sample Buffer (Invitrogen) for 10 min. The samples were run on NuPAGE 4–12% Bis-Tris Gels and detected by Western blotting using antibodies against JunD (sc-74, Santa Cruz) and Flag (Sigma). The expression levels of JunD and Hoxc6 in the samples were confirmed by Western Blotting.

DNA affinity precipitation assay

200 – 400 µg nuclear extracts of BON-Flag control, BON-Hoxc6-V1, BON-mHoxc6-V1, BON-Hoxc6-V2 and BON-mHoxc6-V2 cells were incubated with binding buffer (10mM Tris, 50mM KCl, 1mM DTT pH7.5, 50ng/µl Poly(dI-dC), 10% Glycerol and 1x HALT protease inhibitor cocktail (Pierce)) for 5 min at room temperature. Samples were pre-cleared with Streptavidin-conjugated Agarose beads (Pierce) for 5 min. After centrifugation for 2 min at 2000 rpm, supernatants were incubated with 80 fmol of 5'-biotinylated AP1-probes for 30min. 25 µl of Streptavidin-conjugated Agarose beads were added and samples incubated for 2 hours at 4°C with gentle rotation. The samples were centrifuged at 2000 rpm, beads were washed 3 times with binding buffer (without Poly(dI-dC)) and proteins eluted from the beads by the addition of 4x LDS sample buffer (Invitrogen). Proteins were separated on 4–12% NuPAGE Bis-Tris polyacrylamide gels and transferred to polyvinylidene difluoride membranes. Blots were probed with a Flag antibody.

DNA microarray studies and analysis

RNA isolated from BON-Hoxc6-V1, BON-Hoxc6-V2 and BON-siHoxc6 cells was amplified and labeled with Cy5 (Amersham Biosciences). Control RNA amplified from BON-Flag cells was used as the reference sample and labeled with Cy3. Dye-swapping was also performed by replacing Cy3 with Cy5 labeling of the samples. Competitive hybridization of Cy5- and Cy3-labelled RNA samples was carried out on glass slides spotted with 34,594 oligonucleotide probes. The microarray slides were scanned using the MDS/Axon GenePix system (Molecular Devices) according to manufacturer's instructions. The

studies were performed at the Microarray core facility at the Department of Molecular Biology, Massachusetts General Hospital. Data analysis is detailed in Supplementary Material.

Statistical analysis

Statistical differences were analyzed by the student's t-test, and p-values <0.05 were considered statistically significant.

Results

Upregulation of Hoxc6 in gastrointestinal carcinoids

Previous DNA microarray studies indicated that Hoxc6 and Hoxc8 were upregulated in gastrointestinal carcinoid tumors but not pancreatic neuroendocrine tumors¹⁴. Quantitative RT-PCR was performed to define expression levels of the two Hoxc6 variants in normal pancreas, normal ileum, intestinal carcinoids, and pancreatic neuroendocrine tumors. The expression level in normal pancreas was normalized to 1. For Hoxc6-V1, there was an 11.5 fold increase in expression in normal ileum, 35.6 fold increase in pancreatic neuroendocrine tumors, and a 1244.8 fold increase in intestinal carcinoids. For Hoxc6-V2, the findings were similar. There was a 37.8 fold increase in expression in normal ileum, 27.4 fold increase in pancreatic neuroendocrine tumors, and a 950.5 fold increase in ileal carcinoids (Figure 1).

In carcinoid tumors, there was also an upregulation of Hoxc8, although the level was somewhat lower. Compared to normal pancreas, there was a 26.1 fold increase in pancreatic neuroendocrine tumors, and an 877.4 fold increase in ileal carcinoids. Thus, overexpression of both Hoxc6 variants and Hoxc8 appears to be a distinctive feature of gastrointestinal carcinoid tumors but not pancreatic neuroendocrine tumors.

Interestingly, Hoxc6 and Hoxc8 have been reported as targets of the *MEN1* tumor suppressor gene in murine embryonic fibroblasts⁶. However, overexpression of menin in BON1 carcinoid cells did not induce Hoxc6 or Hoxc8 mRNA levels, as measured by qRT-PCR. Specifically, levels of Hoxc6 were induced 0.97-fold $\pm$ 0.14 and levels of Hoxc8 were induced 1.05 $\pm$ 0.21.

Hoxc6 stimulates cellular proliferation of BON1 cells *in vitro*

Because Hoxc6 was more significantly upregulated than Hoxc8 in carcinoids, we focused attention on this gene. To determine the functional consequences of Hoxc6 expression in carcinoid cells, BON1 stable cell lines were generated in which either Hoxc6-V1 or Hoxc6-V2 was overexpressed. Two independent clones of each construct were analyzed. *In vitro* growth assays revealed that overexpression of variant 1 was associated with a 74% (clone #3, $p < 0.01$) to 92% (clone #17, $p < 0.01$) increase in cell number compared to control transfected cells at 7 days (Figure 2). Similarly, overexpression of variant 2 was associated with a 68% (clone #3, $p < 0.01$) to 109% (clone #2, $p < 0.01$) increase in cell number (Figure 2).

To verify this effect, BON1 cells were also generated that stably expressed an siRNA to endogenous Hoxc6. Hoxc6 expression levels were determined by quantitative RT-PCR in multiple cell lines. In the DLD1 colon cancer cell line and the TPC thyroid cancer cell line, Hoxc6 levels were undetectable. Hoxc6 expression was detected in 293, Bon, and Panc1 cells. Compared to 293 cells, Hoxc6 expression levels were 2.3 fold higher in BON1 cells and 3.3 fold higher in Panc1 pancreatic adenocarcinoma cells. The Hoxc6 siRNA sequence utilized was common to both variants and therefore targeted both Hoxc6 transcripts. The silencing efficiency of two independent siRNA constructs to Hoxc6 was excellent. In BON-

siHoxc6#1 cells, levels of Hoxc6-V1 were suppressed to 16% of baseline and Hoxc6-V2 levels to 18% of baseline. Similarly, in BON-siHoxc6#3 cells, levels of Hoxc6-V1 were suppressed levels to 18% of baseline and levels of Hoxc6-V2 to 14% of baseline. When compared to BON1 cells expressing a control siRNA, there was a 43% decrease ($p<0.01$) in the number of BON-siHoxc6#1 cells and 49% decrease ($p<0.01$) in the number of BON-siHoxc6#3 cells at 7 days (Figure 2). Thus, Hoxc6 can regulate the proliferation of carcinoid cells *in vitro*.

Hoxc6 enhances signaling through the AP-1 pathway

To determine the mechanism through which Hoxc6 may control cellular proliferation, we tested the consequences of Hoxc6 overexpression on the AP-1 signaling pathway that is frequently activated in human malignancies. An earlier report suggested that Hoxb4 can enhance signaling through the AP-1 pathway by upregulating the expression of Jun-B and Fra-1 in Rat-1 cells¹⁷. AP-1 is a heterodimeric transcription factor that is a downstream target of multiple oncogenes, and it can regulate the expression of genes critical for cellular growth¹⁸. A consensus AP-1 luciferase reporter construct was introduced into BON1 cells that stably overexpressed Hoxc6. Compared to control BON1 cells, there was a 2.1 to 3.0 fold ($p<0.01$) increase in AP-1 reporter activity in BON-Hoxc6-V1 cells, and a 2.1 to 2.8 fold ($p<0.01$) increase in BON-Hoxc6-V2 cells (Figure 3). This induction was observed in two independent clones. Similarly, in BON1 cells with Hoxc6 stably knocked-down, there was a 70% reduction ($p<0.01$) in AP-1 reporter activity in BON-siHoxc6#1 cells and a 66% reduction ($p<0.01$) in BON-siHoxc6#3 cells compared to control parental BON1 cells (Figure 3). Thus, Hoxc6 can enhance signaling through the AP-1 pathway.

c-Fos and JunD are the functional components of AP-1 in BON1 cells

AP-1 is a heterodimeric transcription factor comprised of Jun and Fos proteins, and the expression of these various components is cell-specific. In order to determine whether Hoxc6 mediates its effects through one or more particular component, each of these factors was selectively knocked down in BON1 cells stably overexpressing Hoxc6. The silencing efficiency of each AP-1 component is shown in Figure 4a. Knockdown of c-Fos or JunD blocked the induction of AP-1 activity mediated by both Hoxc6 variants (Figure 4b,c). However, knockdown of c-Jun had no effect upon AP-1 reporter activity (Figure 4d). These findings suggested that c-Fos and JunD were the critical components of AP-1 in carcinoid cells, and that Hoxc6 may enhance AP-1 activity through an interaction with c-Fos or JunD, but not c-Jun.

Regulation of JunD expression by Hoxc6

In BON1 cells stably overexpressing Hoxc6, there was a modest increase in JunD mRNA levels. In BON-Hoxc6-V1 cells, there was a 2.23 fold increase ($p<0.05$) in JunD mRNA levels, but only a 1.56 fold increase ($p=0.09$) in BON-Hoxc6-V2 cells. In cells stably expressing Hoxc6 siRNA, there was a 35% decrease ($p=0.07$) in JunD mRNA levels compared to control cells. These findings suggest that Hoxc6 may regulate the expression of JunD, but that the level of induction is modest. Of note, menin mRNA levels were unchanged in BON1 cells stably expressing either variant of Hoxc6.

Hoxc6 physically interacts with JunD through its homeodomain

The effects of Hoxc6 on expression of JunD were relatively modest, and we were curious whether there may be additional mechanisms controlling AP-1 activity by Hoxc6. A physical interaction between the yeast homeodomain protein Hex and c-Jun has been previously described, and Helix III of the Hex homeodomain mediated this interaction¹⁶. Because c-Jun was not involved in the functional activation of AP-1 by Hoxc6, we

determined whether Hoxc6 may instead interact with JunD. Immunoprecipitation of Hoxc6 from BON1 cells stably overexpressing Hoxc6 followed by immunoblotting with a JunD antibody revealed a specific interaction between endogenous JunD and both Hoxc6 variants (Figure 5a). Control studies were performed to determine whether Hoxc6 may bind to c-Jun, and immunoprecipitation of Hoxc6 followed by blotting with a c-Jun antibody failed to identify a specific interaction (Figure 5b).

To test whether this interaction was mediated by Helix III of the homeodomain, a point mutation was introduced in the Hoxc6 construct. There is a conserved asparagine residue in Helix III of Hex that was previously demonstrated to be essential for its interaction with c-Jun¹⁶. This asparagine residue was selectively mutated in both Hoxc6 variants (N191Q in variant 1, designated mHoxc6-V1, and N109Q in variant 2, designated mHoxc6-V2). Immunoprecipitation-western studies revealed that both of these Hoxc6 mutants failed to interact with JunD (Figure 5a).

To verify that Hoxc6 formed a complex with AP-1 that physically bound to DNA, a DNA-precipitation assay was performed. Nuclear extracts from BON1 cells overexpressing either Hoxc6-V1 or Hoxc6-V2 were incubated with a biotinylated AP-1 consensus element probe, and the AP-1 probe was precipitated with streptavidin beads. Precipitated proteins were separated on an acrylamide gel, and immunoblotting for Hoxc6 was performed. In control cells, no Hoxc6 was identified in the precipitated complex. However, an interaction between Hoxc6 and the AP-1 probe was identified in extracts from cells overexpressing either wild-type Hoxc6 variant 1 or variant 2. In contrast, when cells expressed mutant Hoxc6 (mHoxc6-V1 or mHoxc6-V2), no interaction with the AP-1 complex was observed (Figure 6). Thus, Hoxc6 directly binds to the AP-1 complex on DNA, and this interaction is mediated by the homeodomain of Hoxc6.

Mutant Hoxc6 inhibits AP-1 activity and cellular growth

The functional effects of the interaction between Hoxc6 and AP-1 were verified by expressing the mutant Hoxc6 that fails to interact with JunD. In BON1 cells expressing either mHoxc6-V1 or mHoxc6-V2, there was a 57% ($p<0.01$) to 51% ($p<0.01$) reduction of AP-1 reporter activity, respectively, indicating that this Hoxc6 mutant can inhibit AP-1 activity. The growth rate of BON1 cells that stably expressed mHoxc6 was measured. In cells expressing either mHoxc6-V1 or mHoxc6-V2, there was a 55% to 50% (both $p<0.01$) reduction in cell number at 7 days, respectively (Figure 7). Thus, mutant forms of Hoxc6 that cannot interact with JunD can inhibit AP-1 activity as well as cellular growth.

Gene targets of Hoxc6 in BON1 cells

To better define the role of Hoxc6 in carcinoid tumorigenesis, microarray studies were performed with RNA isolated from BON1 cells stably expressing Hoxc6-v1, Hoxc6-v2, siHoxc6, or a control plasmid. Differential gene expression patterns between these cells were identified utilizing a 1.5-fold change in expression levels and an FDR-adjusted $p<0.05$. There were 202 genes that were coordinately regulated by both Hoxc6-v1 and Hoxc6-v2, relative to siHoxc6, and heatmaps of these expression patterns are illustrated in the Supplementary Figure. There are 61 genes overexpressed in both BON-Hoxc6-V1 and BON-Hoxc6-V2 cells, relative to BON-siHoxc6, and the top 15 of these genes overexpressed in BON-Hoxc6-V1 cells that were also overexpressed in BON-Hoxc6-V2 cells were APOA2, ARHGDIB, S100A2, DHRS2, NTS, PYY, NPTX2, RBP1, EGR1, TM4SF3, CTGF, INHBB, BPIL1, C10orf10, and TMEM2. Interestingly, 3 of these genes are characteristically expressed in carcinoid cells. Both peptide YY (PYY) and neurotensin (NTS) are gut hormones that are often overexpressed in neuroendocrine tumors, including carcinoids^{19,20}. Furthermore, connective tissue growth factor (CTGF) has recently been

described as an autocrine growth factor for gastric enterochromaffin-like cells²¹. qRT-PCR utilizing RNA from 3 BON1 clones overexpressing Hoxc6-V1 and 2 BON1 clones overexpressing Hoxc6-V2 confirmed a 1.8 fold induction of NT levels and 2.7 fold induction of CTGF levels. In both cases, expression of mHoxc6-V1 or mHoxc6-V2 resulted in a 30% reduction of NT and CTGF levels. No induction of PYY was seen by QPCR. Of note, both the NT and CTGF genes have key AP-1 regulatory sites in their promoters^{22,23}.

Discussion

Intestinal carcinoid tumors remain a fascinating clinical entity. Unfortunately, the therapeutic options for advanced disease are limited¹⁹. The poor understanding of the molecular genetic alterations that underlie these tumors has hindered the search for new therapies. Defining the key facets of the molecular circuitry of these tumors is an important prerequisite to the rational design of novel targeted approaches. The current studies have identified a new molecular pathway in carcinoid tumorigenesis that involves the Hoxc6 homeobox gene.

HOX genes are critical regulators of cellular proliferation and differentiation during development. Deregulated expression of HOX genes has been observed in many tumor types including leukemia, breast, lung, and prostate cancer. Among these tumors, a pathogenic role for a HOX gene has been demonstrated in leukemias²⁴. However, the mechanisms by which HOX genes contribute to tumorigenesis are poorly defined. Although HOX genes are homeodomain transcription factors that recognize a consensus TAAT motif, their functions in cancer are far more diverse¹⁰. Overexpression of homeobox genes can induce cellular transformation^{25, 26}, and this may be mediated by the upregulation of genes normally targeted during development, such as fibroblast growth factor¹¹. However, other non-transcriptional functions have been defined as well. For example, Hox11 can control the G2 cell-cycle checkpoint through its physical interaction with protein phosphatases PP2A and PP4²⁷.

Our studies have identified a new role for Hoxc6 in carcinoid tumorigenesis. In the developing mouse embryo, Hoxc6 is expressed in the spinal cord, prevertebrae, kidney, and selected compartments of lung, stomach, and intestine²⁸. In human tumors, overexpression of Hoxc6 has thus far been implicated in the pathogenesis of prostate cancer^{12,13}. In the prostate, Hoxc6 appears to regulate the expression of bone morphogenetic protein-7, fibroblast growth factor receptor-2, insulin-like growth factor binding protein-3, and platelet derived growth factor receptor-A, all of which have been implicated in tumorigenesis²⁹. These genes were not identified in our microarray analysis, suggesting that the functions of Hoxc6 are tissue-specific. This is consistent with prior studies that have demonstrated distinctive functions of Hox genes depending upon the tissue in which they were expressed³⁰.

In carcinoid tumors, Hoxc6 appears to function in a novel manner as a potent enhancer of signaling through the AP-1 pathway, and this is mediated through JunD. There are at least 2 distinct mechanisms for this effect. First, Hoxc6 can modestly upregulate JunD. In addition, Hoxc6 can physically interact with JunD to enhance transcriptional activation through AP-1, and this interaction is mediated by the homeodomain of Hoxc6. This contrasts with the related yeast transcription factor Hex. Hex also binds to AP-1 proteins through its homeodomain, but Hex functions to inhibit AP-1 activity. This inhibitory activity is strengthened when a Hex binding site in addition to an AP-1 binding site is present in the promoter¹⁶. Thus, homeobox proteins may be important regulators of AP-1 activity, and Hoxc6 functions as an enhancer of AP-1 activity in carcinoid tumors.

The AP-1 transcription factor regulates a diverse set of cellular processes, and these effects are not only cell-specific but also dependent upon the balance of specific Fos and Jun proteins. In contrast to c-Jun, the precise functions of JunD remain incompletely described¹⁸. Depending upon the particular environment, JunD can exhibit either pro-proliferative or growth inhibitory properties³¹. The tumor suppressor protein menin has been reported to interact with JunD, and it can suppress the growth promoting activity of JunD^{32, 33}. Our studies revealed that Hoxc6 enhances the effects of JunD to stimulate proliferation in carcinoid cells. This was specific to JunD, as no effect was observed when the related factor c-Jun was silenced. Furthermore, there was no physical interaction between Hoxc6 and c-Jun.

The relationship between Hoxc6 and menin in human carcinoid tumors remains uncertain. Studies in murine embryonic tissue have previously demonstrated an induction of Hoxc6 by menin⁶. However, menin mRNA levels in human carcinoid tumors and BON1 cells were low, and overexpression of menin in BON1 cells did not induce Hoxc6 gene expression. These observations may be attributable to the differences in species or in the developmental stages of the tissues studied. Whether menin may independently regulate the physical interaction between Hoxc6 and JunD remains to be determined.

This dramatic and selective induction of Hoxc6 in carcinoid tumors but not pancreatic neuroendocrine tumors, normal pancreas, or normal intestine suggests that there are likely to be tissue-specific mechanisms that regulate the expression of Hoxc6. During development, regulation of Hox gene expression is tightly controlled and highly dependent upon the precise activity of chromatin-remodeling proteins³⁴. The pathways that control aberrant expression of Hox genes in adult tumors are unknown. Our study has focused on carcinoids arising in the intestine, and it would be important to determine whether Hoxc6 overexpression is observed in carcinoids from lung and stomach. In addition, it would be of interest to define when during tumor progression this upregulation first occurs. Our tumors were primarily advanced metastatic lesions, and this reflects the rarity with which small benign carcinoids are recognized pre-operatively.

In addition to its role in tumor pathogenesis, Hoxc6 may also play a role in specifying the neuroendocrine phenotype, as Hoxc6 upregulates multiple neuroendocrine markers including neurotensin. Hoxc6 also upregulates CTGF, which is an important growth factor in gastric carcinoids²¹. This selective upregulation suggests that Hoxc6 may be an important therapeutic target. Potential strategies include inhibition of Hoxc6 directly, inhibition of the interaction between Hoxc6 and JunD, or inhibition of the AP-1 signaling pathway in general. Regardless, these observations have revealed a new pathway that underlies the molecular pathogenesis of carcinoid tumors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Stephen and Caroline Kaufer Fund for Neuroendocrine Tumor Research, Caring for Carcinoid Foundation. EMD was supported by a fellowship from the Deutsche Forschungsgemeinschaft. AN was supported by a fellowship from the Crohn's and Colitis Foundation of America. We would also like to thank Carlos Moreno (Hoxc6 constructs).

Abbreviations

Hoxc6-V1	Hoxc6 variant 1
Hoxc6-V2	Hoxc6 variant 2
PYY	peptide YY
NT	neurotensin
CTGF	connective tissue growth factor
AP-1	activator protein-1
MEN1	multiple endocrine neoplasia 1

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Figure 1. Upregulation of Hoxc6 in gastrointestinal carcinoids

Quantitative PCR for Hoxc6 variants 1 and 2 was performed in six samples of normal pancreas, two of normal ileum, eight malignant pancreatic neuroendocrine tumors (mPET), and eleven malignant carcinoid tumors. The expression level in normal pancreas was normalized to 1.

Figure 2. Hoxc6 stimulates proliferation of BON1 cells in vitro

Proliferation of BON1 cells stably expressing Hoxc6 variant 1 (Hoxc6V1#3, Hoxc6V1#17), Hoxc6 variant 2 (Hoxc6V2#2, Hoxc6V2#3), or control pCMV vector was measured by a colorimetric assay on days 1, 3, and 7 after plating. Also, proliferation of BON1 cells stably expressing siRNA to Hoxc6 (siHoxc6#1, siHoxc6#3) or a control siRNA (pSR vector) was similarly measured. The results on days 1 and 7 are illustrated and expressed as the mean $\pm$ S.D. of duplicate wells performed twice.

p<0.01 vs pCMV vector control

* p<0.01 vs pSR vector control

Figure 3. Hoxc6 enhances signaling through the AP-1 pathway

A consensus AP-1 luciferase reporter construct was introduced into BON1 cells stably expressing Hoxc6 variant 1 (Hoxc6V1#3, Hoxc6V1#17), Hoxc6 variant 2 (Hoxc6V2#2, Hoxc6V2#3), or pCMV control vector. Similarly, the AP-1 luciferase reporter was also introduced into BON1 cells stably expressing siRNA to Hoxc6 (siHoxc6#1, siHoxc6#3) or control siRNA (pSR vector). The results shown are the ratio of AP-1 luciferase activity in these cells relative to those expressing vector control. Results are expressed as the mean $\pm$ S.D. of duplicate wells performed at least two times.

Figure 4a

* $p < 0.05$ vs si control
 ** $p < 0.01$ vs si control

Figure 4b

++ $p < 0.01$ vs si control

Figure 4c

+ p<0.05 vs si control

Figure 4d

Figure 4. c-Fos and JunD are functional components of AP-1 in BON1 cells

a) siRNA knockdown of c-Fos, JunD, or c-Jun was performed with specific duplex oligos. The silencing efficiency was measured by quantitative PCR. The results are shown as relative ratios with control siRNA normalized to 1. The mean $\pm$ S.D. of duplicate studies are shown. AP-1 reporter luciferase activity was measured in BON1 cells stably overexpressing Hoxc6-V1 or Hoxc6-V2 in which c-Fos (b), JunD (c), or c-Jun (d) were knocked down. The results are shown as the relative ratio of AP-1 activity compared to vector-transfected cells. The results are expressed as the mean $\pm$ S.D. of duplicate wells performed two times.

Figure 5a

Figure 5b

Figure 5. Hoxc6 interacts with JunD through its homeodomain

Immunoprecipitation of Hoxc6 (Flag) from BON1 cells stably overexpressing Flag tagged-Hoxc6-V1 or -V2 followed by immunoblotting with a JunD (a) or a c-Jun antibody (b). The mutant constructs have a N191Q mutation in Hoxc6-V1 and N109Q mutation in Hoxc6-V2 homeodomain (AAC → CAG).

Figure 6. Hoxc6 directly binds to the AP-1 complex on DNA

A DNA affinity purification assay was performed using nuclear extracts of BON1 cells overexpressing Flag-HoxC6-V1, Flag-mutant HoxC6-V1 and Flag-control vector (top) or Flag-HoxC6-V2, Flag-mutant HoxC6-V2 and Flag-control vector (bottom). Extracts were incubated with a biotinylated AP-1-probe and immunoprecipitated. Blots were probed with a Flag-antibody. Only wild-type HoxC6 can bind to the AP-1 complex. Mutation of the homeodomain of each variant disrupts binding of HoxC6 to the AP-1 complex.

DNAP=DNA affinity precipitation. Input = nuclear extracts in which DNAP was not performed.

Figure 7. Mutant Hoxc6 inhibits cellular growth

Proliferation of BON1 cells stably expressing mutant Hoxc6 variant 1 or variant 2 was measured by a colorimetric assay on days 1, 3, and 7 after plating. The results are expressed as the mean $\pm$ S.D. of duplicate wells performed two times.

Table 1

Clinical features of neuroendocrine tumor samples

GI Carcinoids						
Sample	Age	Sex	Primary site	Tumor size (cm)	Stage	Carcinoid syndrome
2974-1	72	nk	ileum	0.4	metastatic to liver	yes
11898-1	52	F	ileum	2	metastatic to liver	yes
33762-1	67	M	ileum	1.5	metastatic to liver	yes
53456-1	59	F	colon	nk	metastatic to liver	nk
67494-1	53	F	ileum	2.5	T3N1M0	no
80570-1	70	F	ileum	3.5	metastatic to liver	no
139	55	F	ileum	1.7	metastatic to liver	no
177	51	F	ileum	2	metastatic to liver	yes
183	58	M	small bowel	1.6	metastatic to liver	yes
192	56	F	small bowel	3	metastatic to liver	no
205	57	M	ileum	3.1	metastatic to liver	yes

Pancreatic Neuroendocrine Tumors						
Sample	Age	Sex	Tumor type	Tumor size	WHO classification	Metastases
2	62	M	PTHrPoma	8.5	WDEC	lymph nodes, liver
53	69	F	Nonfunctional	13	WDET - LM	none
56	73	M	Insulinoma	2.5	WDEC	lymph nodes
59	65	M	ACTHoma	6.5	WDEC	lymph nodes, liver
61	58	F	Gastrinoma	12	WDEC	duodenal wall invasion
69	48	F	Gastrinoma	3	WDEC	lymph nodes
83	47	F	Nonfunctional	7	WDEC	lymph nodes
84	59	M	Gastrinoma	nk	WDEC	lymph nodes

nk: not known

WDET: LM: well-differentiated endocrine tumor, low grade malignant

WDEC: well-differrefiliated endocrine carcinoma