《Original Article》

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, and immunoprecipitation 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.

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

Keywords: Hoxc6, carcinoid, AP-1, neuroendocrine tumor

Abbreviations: Hoxc6-V1, Hoxc6 variant 1; Hoxc6-V2, Hoxc6 variant 2; AP-1, activator protein-1; MEN1, multiple endocrine neoplasia 1

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Introduction

Carcinoid tumors are neuroendocrine neoplasms that can arise in multiple organ sites but are most commonly localized to the gastrointestinal tract [1]. They are typically slow-growing, well-differentiated tumors that secrete 5-hydroxytryptamine (serotonin), and excess levels can result in the clinical carcinoid syndrome [2]. 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

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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* tumor suppressor gene that underlies the syndrome was cloned in 1997 [3]. *MEN1* also plays a role in sporadic pancreatic neuroendocrine tumors and gastrointestinal carcinoids, but only a subset of these sporadic tumors harbors *MEN1* mutations [4]. The *MEN1* gene product, menin, has diverse functions [5], 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 Hoxc8 [6] 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 genes [10]. 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 leukemia [10]. 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 melanomas [11]. 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* gene [6], 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 tumors (in press). We now verify these findings 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

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 was analyzed. The pCMV FLAG-tag expression vector (Invitrogen) with the coding region of the human Hoxc6 variant 1 (V1) (NM_004503) or variant 2 (V2) (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 Dr. Carlos Moreno (Emory University, Atlanta) [13]. The N191Q mutation in Hoxc6 V1 and N109Q mutation in Hoxc6 V2 (AAC -> CAG) in the homeodomain [14] 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'-GGGAAAAUUACAAAAAGAG-3', 5'-GCGAAUGAAUUCGCACAGU-3'), common to

both variant 1 and variant 2, 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 (Cellgro) 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.

Luciferase reporter assay

One day after plating the 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.

Western Blotting and Immunoprecipitation

Cells were lysed in chilled lysis buffer (Cell Signaling) supplemented with proteinase inhibitor (PSC, Roche). 15-20 ug 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 β-Actin (Sigma) antibodies. Immunoreactive proteins were visualized using Western Lighting Chemiluminescence Reagent Plus (Perkin Elmer Life Sciences).

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 (Invitrogen) and detected by Western blotting using antibodies against JunD (sc-74, Santa Cruz) and Flag (ANTI-FLAG M2, Sigma). The expression levels of JunD and Hoxc6 (FLAG) in the samples were confirmed by Western Blotting using 10 μg of nuclear protein.

Statistical analysis

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

Results

Hoxc6 stimulates cellular proliferation of BON1 cells *in vitro*

Because Hoxc6 was significantly upregulated in carcinoids, we focused our attention on this gene. To determine the functional consequences of Hoxc6 expression in BON1 carcinoid cells, stable cell lines were generated in which either Hoxc6-V1 or Hoxc6-V2 was overexpressed (Figure 1). 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 generated that stably expressed an siRNA to endogenous Hoxc6. Wild-type BON1 cells express low levels of Hoxc6. The siRNA sequence utilized is common to

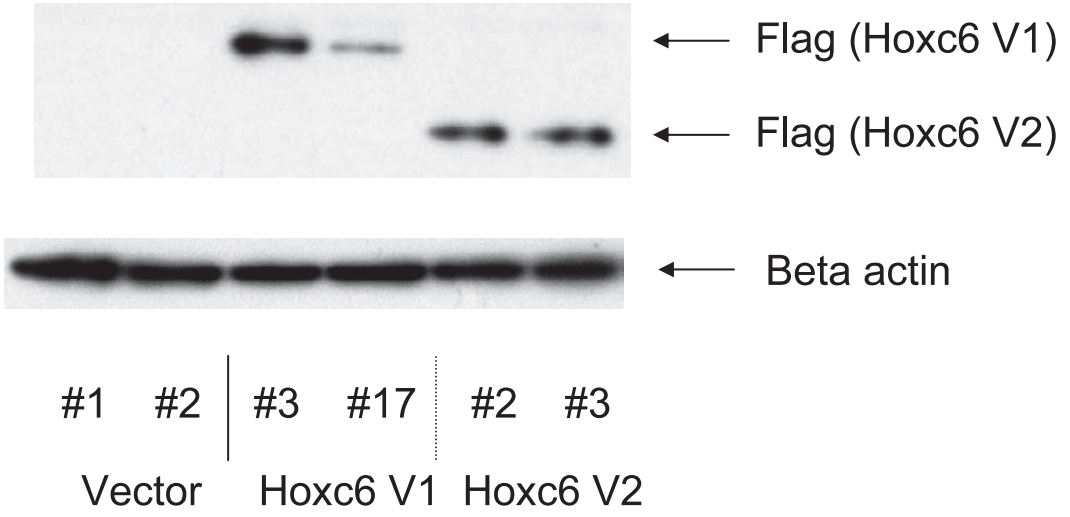

Figure 1 Hoxc6 expression in BON1 carcinoid cells

Stable cell lines were generated in which either Flag tagged Hoxc6 variant 1 or Hoxc6 variant 2 was expressed. Two indepedent clones of each construct were analyzed. Expression was confirmed by western blotting for Flag. Vector = BON1 cells stably expressing an empty Flag vector.

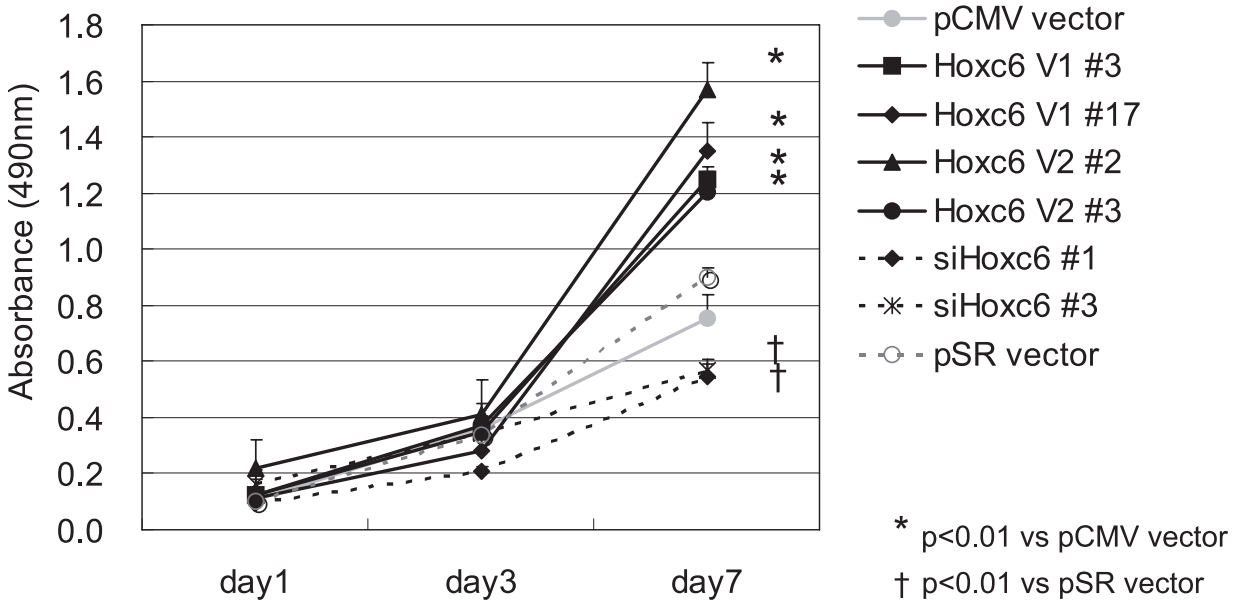

Figure 2 Hoxc6 stimulates proliferation of BON1 cells in vitro

Proliferation of BON1 cells stably expressing Hoxc6 variant 1 (Hoxc6V1#3, Hoxc6V1#17), Hoxc6 variant 2 (Hoxc6V2#2, Hoxc6V2#3), or siRNA to Hoxc6 (siHoxc6#1, siHoxc6#3) was measured by a colorimetric assay on days 1, 3, and 7 after plating.

both variants and therefore targets both Hoxc6 transcripts. The silencing efficiency of two independent siRNA constructs to Hoxc6 is shown in Figure 3. 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 in the number of BON-siHoxc6#1 cells and 49% decrease in the number of BON-siHoxc6#3 cells at 7 days (Figure 2). Thus, Hoxc6 can regulate the proliferation of BON1 carcinoid cells *in vitro*.

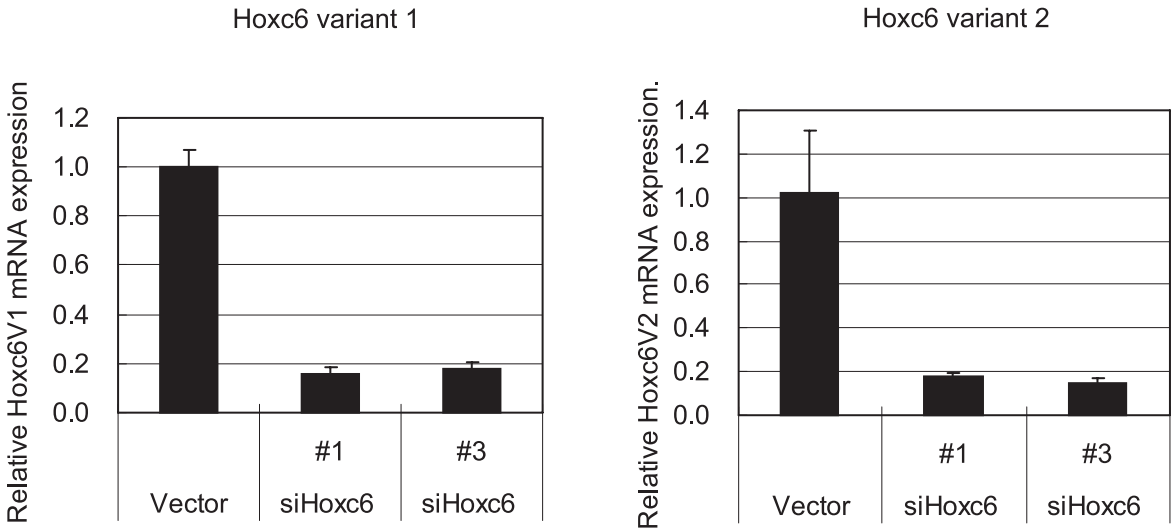

Figure 3 The silencing efficiency of Hoxc6 knock down stable cell lines

Vectors expressing si RNA against Hoxc6 were stably transfected in BON1 cells. The silencing efficiency of Hoxc6 knock down stable cell lines was shown by quantitative PCR for Hoxc6 variant 1 and variant 2. The results are normalized to values from cells expressing a control siRNA.

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 cells [18]. AP-1 is a heterodimeric transcription factor that is a downstream target of multiple oncogenes including Ras, and it can regulate the expression of many genes critical for cellular growth [19]. A consensus AP-1 luciferase reporter construct was introduced into BON1 cells that stably overexpressed either Hoxc6 variant 1 or variant 2. 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 4a). 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 (Figure 4b). Thus, Hoxc6 can enhance signaling through the AP-1 pathway.

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. These findings suggest that Hoxc6 may regulate the expression of JunD, but that the level of induction is modest.

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 interaction [14]. 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

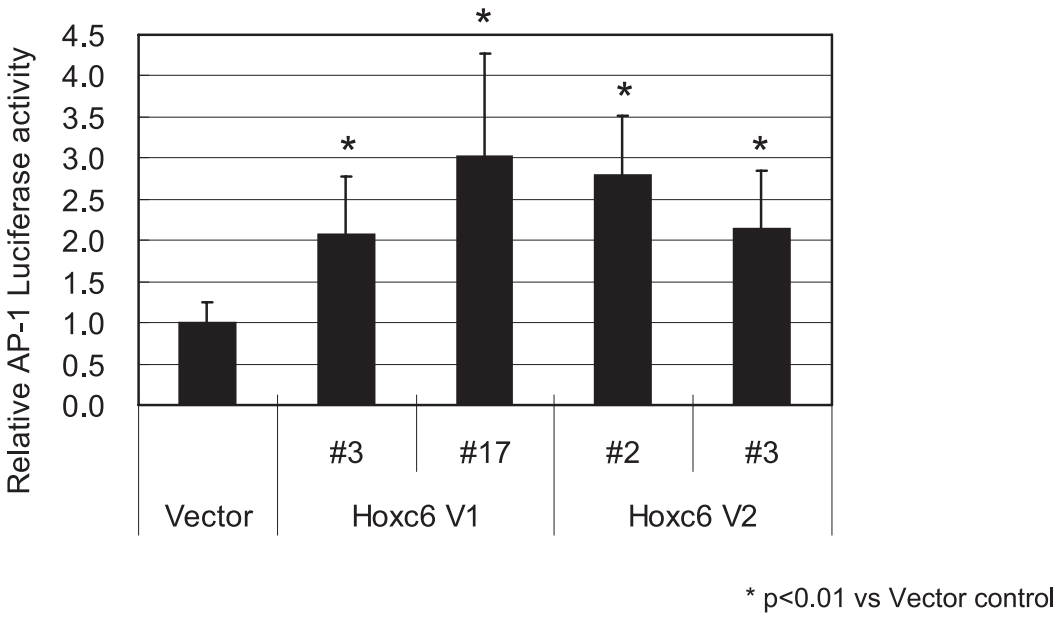

Figure 4a Hoxc6 enhances signaling through the AP-1 pathway

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). The results shown are the ratio of AP-1 luciferase activity in these cells relative to those expressing vector control.

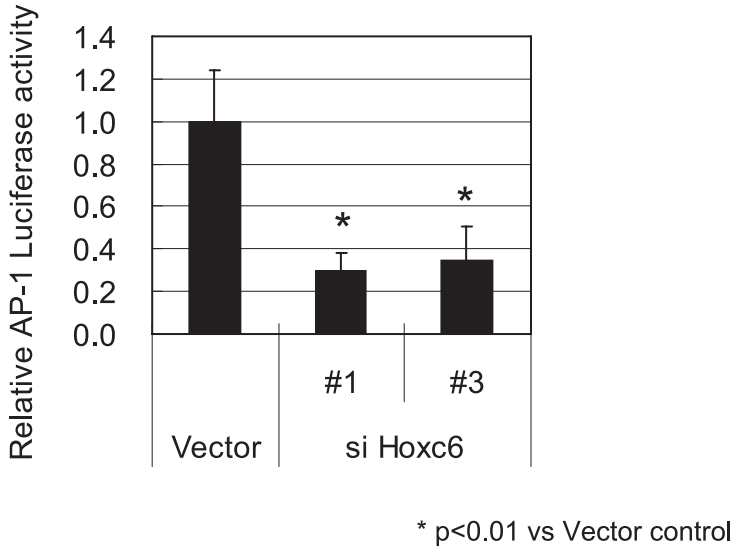

Figure 4b

A consensus AP-1 luciferase reporter construct was introduced into BON1 cells stably expressing siRNA to Hoxc6 (siHoxc6#1, siHoxc6#3). The results shown are the ratio of AP-1 luciferase activity in these cells relative to those expressing vector control.

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 5).

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-Jun [14]. 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).

Figure 5 Hoxc6 interacts with JunD through its homeodomain
Immunoprecipitation of Hoxc6 (Flag) from BON1 cells stably overexpressing Flag tagged-Hoxc6 followed by immunoblotting with a JunD antibody. The mutant constructs have a N191Q mutation in Hoxc6-V1 and N109Q mutation in Hoxc6-V2 homeodomain (AAC -> CAG).

Immunoprecipitation-western studies revealed that both of these Hoxc6 mutants failed to interact with JunD (Figure 5).

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 5). 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 then 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 6). Thus, mutant forms of Hoxc6 that cannot interact with JunD can inhibit AP-1 activity as well as cellular growth.

Discussion

Intestinal carcinoid tumors remain a fascinating clinical entity. Unfortunately, the therapeutic options for advanced disease are very limited [20]. 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 unique tumors is an important prerequisite to the rational design of novel targeted approaches. The current studies have identified a new molecular pathway in carcinoid

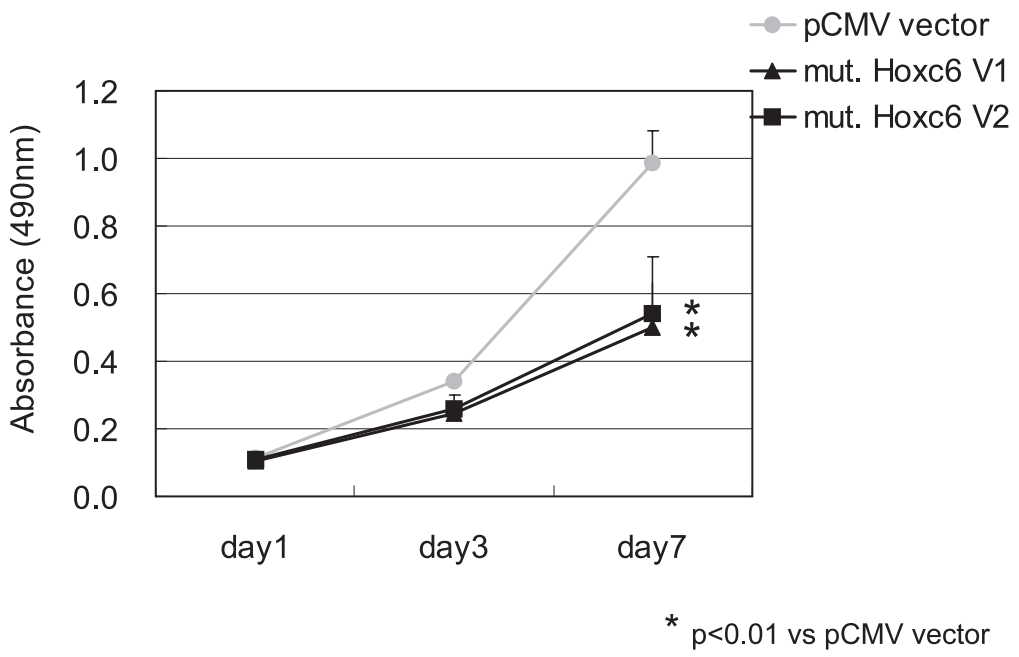

Figure 6 Mutant Hoxc6 inhibits cellular growth

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.

tumorigenesis that involves the Hoxc6 homeobox gene.

HOX genes are critical regulators of cellular proliferation and differentiation during development. A link between deregulated expression of HOX genes and cancer has been observed in many tumor types, but no consistent mechanism of action has yet been defined. Although HOX genes are homeodomain transcription factors that recognize a consensus TAAT motif, their functions in cancer appear to be far more diverse [10]. Overexpression of homeobox genes can induce cellular transformation [25, 26], and some of this may be mediated by the upregulation of genes normally targeted during development, such as fibroblast growth factor [11]. 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 PP4 [27].

Our studies have identified a 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 intestine [28]. In human tumors, overexpression of Hoxc6 has thus far been implicated in the pathogenesis of prostate cancer [12, 13]. In prostate cancer, Hoxc6 appears to regulate neutral endopeptidase (NEP) and insulin-like growth factor binding protein-3 (IGFBP-3), both of which may in turn regulate apoptosis [13].

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 yeast Hex transcription factor. Hex also binds to AP-1 proteins through the homeodomain, but Hex functions to inhibit AP-1 activity. This inhibitory activity is strengthened when a Hex binding site is also present in the promoter [14]. Thus, homeobox proteins may be important regulators of AP-1 activity, and Hoxc6 is 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 described [19]. Depending upon the particular environment, JunD can exhibit both pro-proliferative as well as growth inhibitory properties [29]. The tumor suppressor protein menin has been reported to interact with JunD, and it can suppress the growth promoting activity of JunD [30, 31]. In contrast, Hoxc6 appears to function in an opposing manner to regulate JunD. The relationship between Hoxc6 and menin in carcinoid tumors remains uncertain. Studies in murine embryonic tissue have demonstrated a strong induction of Hoxc6 by menin [6]. However, menin mRNA levels in carcinoid tumors and BON1 cells were very low, and overexpression of menin in BON1 cells did not induce Hoxc6 gene expression (data not shown). Thus, menin does not appear to be an important regulator of Hoxc6 expression in adult neuroendocrine tissues.

The 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 Hoxc6 expression in carcinoids. In tumor pathogenesis, Hoxc6 may also play a role in specifying the neuroendocrine phenotype. This selective upregulation also 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.

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