

GATA6 Promotes Colon Cancer Cell Invasion by Regulating Urokinase Plasminogen Activator Gene Expression^{1,2}

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Abstract

GATA6 is a zinc finger transcription factor expressed in the colorectal epithelium. We have examined the expression of GATA6 in colon cancers and investigated the mechanisms by which GATA6 regulates colon cancer cell invasion. GATA6 was overexpressed in colorectal polyps and primary and metastatic tumors. GATA6 was strongly expressed in both the nuclear and cytoplasmic compartments of the colon cancer cells. GATA6 expression was upregulated in invasive HT29 and KM12L4 cells compared with the parental HT29 and KM12 cells and positively correlated with urokinase-type plasminogen activator (uPA) gene expression. Small interfering RNA (siRNA) knockdown of GATA6 resulted in reduced uPA gene expression and cell invasion. GATA6 bound to the uPA gene regulatory sequences *in vivo* and activated uPA promoter activity *in vitro*. uPA promoter deletion analysis indicated that the promoter proximal Sp1 sites were required for GATA6 activation of the uPA promoter. Accordingly, GATA6 physically associated with Sp1 and siRNA knockdown of Sp1 decreased GATA6 activation of the uPA promoter activity suggesting that Sp1 recruits GATA6 to the uPA promoter and mediates GATA6 induced activation of the uPA promoter activity. On the basis of our results, we conclude that GATA6 is an important regulator of uPA gene expression, and the dysregulated expression of GATA6 contributes to colorectal tumorigenesis and tumor invasion.

Neoplasia (2010) 12, 856–865

Introduction

The GATA factors belong to an evolutionarily conserved family of C2-type zinc finger proteins. There are six members of the vertebrate GATA family. GATA1/2/3 are mainly expressed in the hematopoietic lineages and GATA4/5/6 are expressed in endodermally derived tissues, such as the gut, liver and lungs, and mesodermally derived tissues, such as the heart [1]. In the adult small intestine, GATA4/5/6 are expressed in a partially overlapping pattern along the crypt-villus axis [2,3]. GATA4/5/6 regulate various differentiation marker genes expressed in gastrointestinal tissue by binding to the WGATAR sequences within the regulatory regions of these genes and interacting with other ubiquitous and tissue-enriched transcriptional regulators [2,4–7]. In addition to differentiation, GATA4/5/6 have been associated with cell survival, cell proliferation, and neoplastic transformation of various cell types [8–15]. Among gastrointestinal cancers, *GATA4* is amplified in ~10% of esophageal adenocarcinomas and Barrett metaplasia [16]. The transition from normal esophageal epithelium to Barrett metaplasia to adenocarcinoma is associated with up-regulation of GATA6 [17]. Further, the *GATA6* gene is amplified in pancreaticobiliary cancers [12,18].

Colorectal cancer is the third most common cancer in the United States. Although the localized primary colorectal tumors diagnosed early can be effectively treated, the metastatic tumors that have spread to different body parts are difficult to treat. The initial step in metastasis is local invasion. This process involves changes in the adherence and migratory properties of the primary cancer cells and extensive degradation of the extracellular matrix surrounding the primary tumor cells [19]. The urokinase-type plasminogen activator (uPA) system, which includes the serine protease (uPA), its receptor (uPAR), and the endogenous inhibitors, plasminogen activator

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¹This work was supported by a Veterans Affairs merit review grant to D.H.B.

²This article refers to supplementary materials, which are designated by Tables W1 and W2 and are available online at www.neoplasia.com.

Received 27 January 2010; Revised 9 July 2010; Accepted 9 July 2010

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DOI 10.1593/neo.10224

inhibitors 1 and 2, plays an important role in invasion and subsequent metastasis of various tumors including colorectal tumors [20,21]. uPA is expressed ubiquitously and is regulated both at the transcriptional and posttranscriptional levels [22,23]. At the level of transcription, uPA is regulated mainly by an enhancer located 2 kb upstream from the transcription initiation site [24]. The activity of this enhancer requires the cooperativity among the AP-1/PEA3 composite element, a downstream AP1 site and regulatory factors that bind to *cis*-acting sequences in between these two elements [25,26]. Transcriptional activity of the uPA gene is highly dependent on the integrity of a GC/GA-rich region located in the proximal promoter [22]. This region binds Sp1 proteins [27]. In addition to directly activating uPA gene expression, Sp1 also interacts with and recruits other transcription factors to the uPA promoter [28,29]. Apart from these ubiquitously expressed factors, several tissue-enriched factors, such as GATA6 and RUNX/AML, target different regulatory elements of the *uPA* gene, suggesting that the distinct promoter elements may be used in different cell types to drive uPA gene expression [30,31].

Among the GATA family members, GATA5 and GATA6 are expressed in the adult colon [2,32]. Recent studies have demonstrated that GATA5 is inactivated by promoter methylation and that it negatively regulates colorectal cancer cell proliferation and invasion [33]. In contrast, a strong expression of GATA6 in the proliferative crypt compartment of the intestine has suggested that GATA6 may be associated with cellular proliferation. In support of this, GATA6 is strongly expressed in colon cancer-derived cell lines and upregulated in colon cancers [34]. However, a recent study using a limited number of specimens showed the up-regulation of GATA6 in benign lesions and subsequent down-regulation in malignant lesions [35]. Because of these contradictory reports, it is not clear whether GATA6 expression becomes dysregulated during the initial stages of development of colon cancer such as dysplasia and polyposis or during subsequent stages of malignant progression and metastasis. Understanding the temporal aspects of GATA6 dysregulation and the mechanisms by which GATA6 dysregulation contributes to colon carcinogenesis is important for early diagnosis, determination of the course of treatment, and prognosis of colon cancer. In this study, we show that the expression levels and the localization of GATA6 protein is altered beginning from the earliest stages of dysplasia continuing through the late metastatic lesions, suggesting an important role for GATA6 in the initiation and progression of colon cancer. While GATA6 was primarily nuclear in normal colonic epithelium, GATA6 was strongly expressed in both the nuclear and cytoplasmic compartments of the colon cancer cells. We have identified uPA as a GATA6 target gene in colon cancer cells. GATA6 knockdown resulted in reduced uPA gene expression, colon cancer cell migration, and invasion. We further show that GATA6 activates the uPA promoter and physically interacts with Sp1 and Sp1, and the promoter proximal Sp1 binding sites are required for GATA6-mediated activation of the uPA gene expression.

Materials and Methods

Immunohistochemistry

Surgical samples containing primary tumors, adjacent normal tissues, and metastatic tumors were collected in 10% buffered formalin according to institutional review board-approved protocols and used in the preparation of the customized tissue array. Deparaffinized sections were boiled in TUF target unmasking solution (PanPath, Amsterdam, the Netherlands), blocked in 5% horse serum, and incubated with

1:100 dilution of goat GATA6 antibody (R&D Systems, Minneapolis, MN) or rabbit uPA antibody (American Diagnostica, Greenwich, CT) overnight. Sections were processed using the VectaStain Elite kit (Vector Laboratories, Burlingame, CA) and developed using 3,3'-diaminobenzidine. Images were captured using Olympus BX51 microscope (Center Valley, PA) equipped with CCD camera, and the images were processed using Adobe Photoshop (San Jose, CA).

Recombinant DNA

Full-length uPA promoter-luciferase reporter was constructed by cloning the 2.5-kb *Sma*I fragment containing the human uPA 5' regulatory regions upstream of luciferase reporter in the pGL2 basic vector. Promoter deletion constructs containing -2020, -1500, -510, -86, and -30 bp of uPA promoter were constructed by cloning polymerase chain reaction (PCR)-generated fragments in to pGL2 basic vector. Deletion mutagenesis primer sequences are shown in Table W1. HA epitope-tagged GATA6 expression vector was constructed by cloning the PCR-generated rat GATA6 cDNA between the *Xba*I and *Bam*HI sites of the CMV promoter-enhancer-based pCGN vector.

Cell Culture and Transfections

HCT116 and HT29 cells were cultured in McCoy medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine. KM12 and KM12L4 cells were maintained in minimum essential medium supplemented with 10% fetal bovine serum, L-glutamine, sodium pyruvate, nonessential amino acids, and vitamin solution (Invitrogen, Carlsbad, CA). HCT116 cells plated in 24-well plates were transfected in triplicates with 0.2 µg of the uPA luciferase reporters along with 0.4 µg of GATA6 expression vector or pCGN empty vector. At 36 hours after transfections, cells were harvested and analyzed for luciferase activity. For small interfering RNA (siRNA) transfection experiments, 25, 50, and 100 nM of GATA6 siRNA or a nonspecific control siRNA was transfected. To assess the effect of GATA6 knockdown on uPA promoter-luciferase activity, the 2.5-kb uPA promoter-luciferase reporter was cotransfected with 100 nM of GATA6 or control siRNA. For Sp1 knockdown experiments, Sp1 siRNA pool (Dharmacon/Thermo Fisher Scientific, Lafayette, CO) was transfected into HCT116 using Dharnafect 1. Forty-eight hours later, cells were transfected with the -520 uPA-luciferase construct with GATA6 or pCGN empty vector.

Protein Extract Preparation and Western Blot Analysis

About 25 µg of protein extracts prepared from colon cancer cell lines (HCT116, KM12, KM12L4, HT29, and invasive HT29) or surgically removed tumors and adjacent normal tissues were resolved on sodium dodecyl sulfate-10% polyacrylamide gels, transferred to polyvinylidene fluoride membranes. The membranes were blocked in tris-buffered saline-tween 20 containing 5% nonfat milk for 1 hour at room temperature and reacted with GATA6 and uPA antibodies overnight at 4°C. The blots were probed with appropriate HRP-conjugated secondary antibodies and developed using enhanced chemiluminescence.

Immunoprecipitation

Subconfluent HCT116 cells in 10-cm plates were transfected with 2 µg of HA epitope-tagged GATA6 and 4 µg of FLAG epitope-tagged Sp1, either individually or together. pCDNA3 empty vector was used to balance the total amount of transfected DNA to 6 µg. Cells were lysed 48 hours after transfection in lysis buffer (20 mM Tris-HCl pH 7.5,

100 mM NaCl, 0.5% Nonidet P-40 containing EDTA-free HALT protease inhibitor cocktail; Pierce Biotechnology, Rockford, IL), and 500 µg of cell lysates were immunoprecipitated with 15 µl of either mouse HA or mouse FLAG antibody conjugated to red affinity gel (Sigma, St Louis, MO) for 4 hours at 4°C. Immunoprecipitations (IPs) were washed three times in IP buffer and analyzed by Western blot analysis with rabbit HA or FLAG antibodies.

Chromatin Immunoprecipitation Assays

Chromatin immunoprecipitation (ChIP) assays were performed using the ChIP assay kit (Upstate/Millipore, Billerica, MA). GATA6- and uPA-expressing colon cancer cell line, HCT 116, was used for ChIP experiments. For ChIP assay, 2.5 µg of goat GATA6 antibody (R&D Systems) or control nonimmune goat antibodies was used. The ChIP products were analyzed by semiquantitative PCR using primers spanning the 5' flanking regulatory regions of uPA that contained potential GATA binding sites. The primer sequences are shown in Table W2.

In Vitro Invasion Assays

HCT116 cells plated in the Boyden chambers were transfected with 100 nM GATA6 siRNA (top strand 5'-CCAGGAAACGAAAACCUAA-3') or control siRNA (catalog no. 4611; Ambion/Applied Biosystems, Austin, TX) for 8 hours after which the medium was changed with a fresh medium. At 48 hours after transfection, noninvading cells within the chamber were removed with a sterile cotton swab, and the cells that invaded the matrix and crossed over the membrane were quantified colorimetrically using the invasion assay kit (Chemicon/Millipore, Billerica, MA). More invasive HT29 cells were isolated based on the differential ability of the nonclonal HT29 cells to invade and successfully cross the cell matrix in Boyden chambers. Subcloning of more invasive HT29 cells is explained in the Supplementary Information section.

Statistical Analysis

Results were expressed as mean ± SD and analyzed for significance with one-tailed Student's *t* test.

Results

GATA6 Is Upregulated in Benign and Malignant Human Colon Cancers

Recent studies have demonstrated that GATA6 is overexpressed in a variety of cancers including colon cancers [12,18,34]. However, precisely when during colorectal tumorigenesis GATA6 becomes overexpressed and the potential mechanisms involved in GATA6 overexpression have not been addressed. In addition, it is not known how GATA6 up-regulation contributes to the pathogenesis and progression of colorectal cancers. We have examined GATA6 protein expression in 38 human polyps and a larger customized human colorectal cancer tissue array consisting of 250 primary tumors, 208 specimens of normal adjacent mucosa, and 89 metastatic tumors using immunohistochemistry. In Western blot analysis of HCT116 cells transfected with GATA4, GATA5, and GATA6, the goat GATA6 antibody reacted specifically with GATA6 but not GATA4 and GATA5 confirming the antibody specificity (data not shown). Specificity of the immunostaining was confirmed by substituting the GATA6 antibody with nonimmune goat antibody (Figure 1, *A* and *B*). On the basis of the relative intensity of staining, the immunostaining was graded on

a scale of +1 to +3, with +1 being the staining intensity detected in normal colon, +2 being strong, and +3 being the strongest staining. In normal colon, GATA6 was expressed within the nuclei of crypt epithelial cells and, to a lesser extent, within the differentiated epithelial cells at the top of the crypt (Figure 1, *I* and *J*, *deep brown to black nuclear stain indicated by white arrowheads in J*). Compared with the normal crypts, GATA6 expression was increased in crypts showing the earliest stages of dysplasia (Figure 1, *C*, *D*, *E*, and *F*, *normal and dysplastic regions indicated by white and red arrowheads*, respectively). GATA6 was overexpressed in both tubular- and villous-type adenomas. GATA6 staining in 14% of adenomas was comparable to that of normal colon, whereas majority of adenomas (86%) showed strong (+2) staining. These data demonstrate that up-regulation of GATA6 seems to be a very early event during colorectal tumorigenesis. Further up-regulation of GATA6 was noted in carcinomas (Figure 1, *K*, *L*, *M*, and *N*). Most carcinomas (65%) showed strong staining (+2). Close to 25% of carcinomas stained strongest (+3), and only 10% showed staining comparable to normal colon. Interestingly, while GATA6 expression was localized in the nuclei of normal mucosa and adenomas, adenocarcinomas showed strong nuclear and cytoplasmic GATA6 staining (Figure 1, *K*, *L*, *M*, and *N*).

GATA6 Is Overexpressed in Metastatic Colorectal Cancers

Previously, it has been demonstrated that GATA6 and the related GATA4 are targeted by small GTPase RhoA, a well-known regulator of cytoskeletal remodeling and cell motility, and are implicated in epithelial cell migration [36,37]. Therefore, we hypothesized that the upregulated expression of GATA6 could be related to the invasive phenotype of colon cancer cells. To investigate this hypothesis, we examined GATA6 protein expression in metastatic colon cancers. Strong nuclear and cytoplasmic GATA6 expression was detected in colon cancer metastasis in lymph nodes, liver, and omentum (Figure 1, *O*, *P*, *Q*, *R*, *S*, and *T*). Hence, 42% of the metastatic carcinomas stained strongly (+2) and 58% of the metastatic tumors stained the strongest (+3). Although there seemed to be an increase in GATA6 expression from adenoma to primary carcinoma to metastatic lesions, comparison of GATA6 expression with the patient outcome indicated that GATA6 expression is not an independent prognostic marker of poor prognosis (data not shown).

uPA Expression Is Upregulated in GATA6-Overexpressing Colon Cancers

uPA is strongly upregulated in various malignancies including colorectal cancers and serves as a prognostic marker. Previous studies have shown that uPA up-regulation occurs in benign lesions (polyps) well before transition to an invasive malignancy and subsequent metastasis [38]. Because GATA6 is also upregulated starting from the earliest stages of dysplasia, we hypothesized that the uPA expression may be related to the dysregulated expression of GATA6 in polyps and colorectal cancers. Immunostaining of successive sections of polyps demonstrated precise overlapping of GATA6 and uPA staining in the colonic epithelia, suggesting that GATA6 overexpression may be related to uPA expression during polyposis (Figure 1, *E*, *F*, *G*, and *H*). To examine whether GATA6 and uPA expressions are correlated in adenocarcinomas, protein extracts prepared from three pairs of surgically resected human colon cancers and the neighboring apparently normal mucosa were probed with uPA and GATA6 antibodies. On normalization to protein loading, both uPA and GATA6 levels were strongly upregulated in tumor mucosa compared with the normal

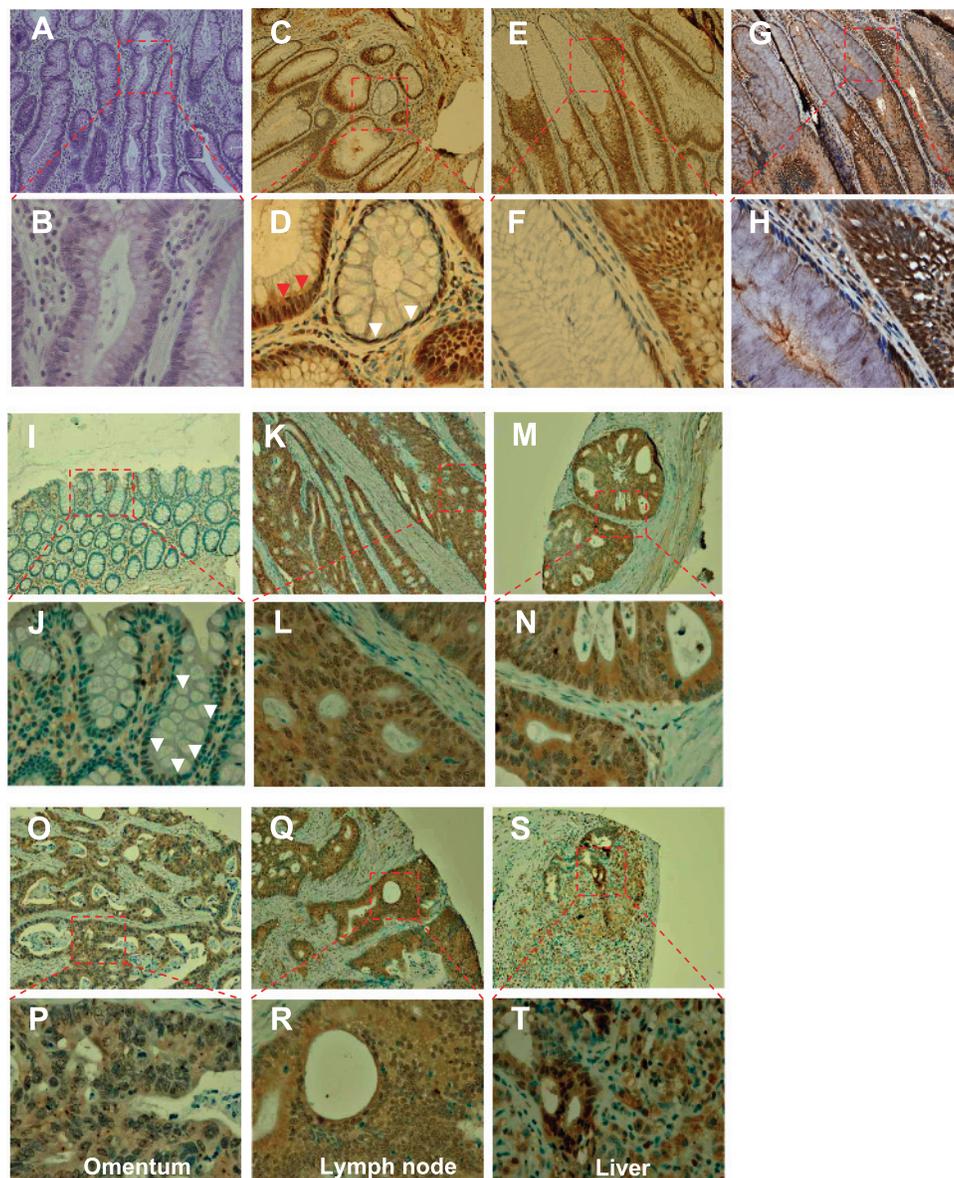


Figure 1. Immunohistochemical analysis of GATA6 expression in normal colon, polyps, primary adenocarcinomas, and metastatic tumors. Sections of normal colon were stained with nonimmune goat antibody (A, B) or goat GATA6 antibody (I, J). In normal colon, GATA6 was expressed within the nuclei of crypt epithelial cells and, to a lesser extent, within the differentiated epithelial cells at the top of the crypt (deep brown to black nuclear stain indicated by white arrowheads in panel J). (C, D, E, and F) GATA6 staining in polyps. White and red arrowheads in panel D indicate normal and dysplastic regions, respectively. uPA staining in a section adjacent to panels E and F is shown in panels G and H. GATA6 staining in primary adenocarcinomas is shown in panels K, L, M, and N. Staining in metastatic tumors from omentum (O, P), lymph node (Q, R), and liver (S, T). After immunostaining, sections were counterstained with hematoxylin. Original magnification: (A, C, E, G, I, K, M, O, Q, S) $\times 100$. Boxed areas from these images are shown at a magnification of $\times 400$ in panels B, D, F, H, J, L, N, P, R, and T, respectively.

mucosa in all three pairs of specimens analyzed (Figure 2). An additional fast-migrating isoform of GATA6 of approximately 45 kDa was enriched in colon cancer specimens.

GATA6 Expression Is Related to uPA Expression and Colorectal Cancer Cell Invasion

To correlate GATA6 expression with colon cancer invasiveness, we analyzed GATA6 protein levels in more invasive HT29 subclones. HT29 colon cancer cell line is nonclonal and consists of a mixture of cells, some of which are more invasive [39,40]. The more invasive subclones were isolated based on their ability to rapidly invade and

cross the Matrigel-coated invasion chambers (unpublished observations). GATA6 protein was strongly upregulated in more invasive subclones compared with the parental HT29 cells (Figure 3A). In addition to cells selected for invasiveness *in vitro*, we examined whether GATA6 was upregulated in colon cancer cells selected for invasiveness *in vivo*. For this purpose, we chose KM12 and KM12L4 cells. Compared with KM12 cells, KM12L4 cells are highly invasive and metastatic. KM12 cells were established from a primary human colon cancer. KM12L4 cells were obtained by four repeated passages of KM12 cells through the spleen of nude mice and selected for increased metastasizing ability to liver [41]. GATA6 and uPA were

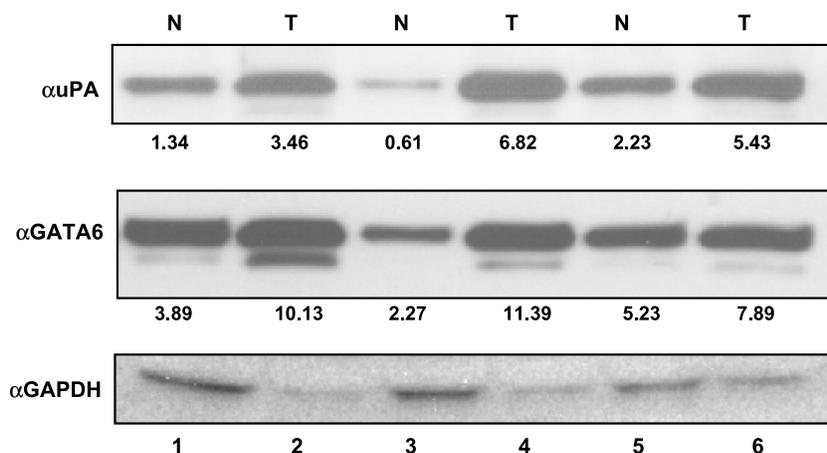


Figure 2. uPA is upregulated in GATA6 overexpressing colorectal tumors. Protein extracts (25 μg) from paired cancerous (T) and adjacent mucosa (N) were analyzed by Western blot analysis with uPA antibody (top panel), GATA6 antibody (middle panel), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (bottom panel). Signals were quantified by densitometry. uPA and GATA6 signals normalized to the respective GAPDH loading control signals are shown below the top and the middle panels.

upregulated in KM12L4 cells compared with parental KM12 colon carcinoma cells (Figure 3B).

GATA6 Is Required for uPA Gene Expression and Cell Invasion

On the basis of the correlation between uPA production and GATA6 overexpression in resected human colorectal tumors and more invasive colon cancer cell lines, we examined whether GATA6 regulates uPA gene expression by knocking down GATA6 expression in HCT116 colon cancer cells. HCT116 cells were selected for these experiments because these cells are invasive and express high levels of uPA. Compared with the control siRNA-transfected cells, GATA6 siRNA-transfected cells showed a dose-dependent down-regulation of uPA protein expression, suggesting that GATA6 is a potential regulator of uPA gene expression (Figure 4A).

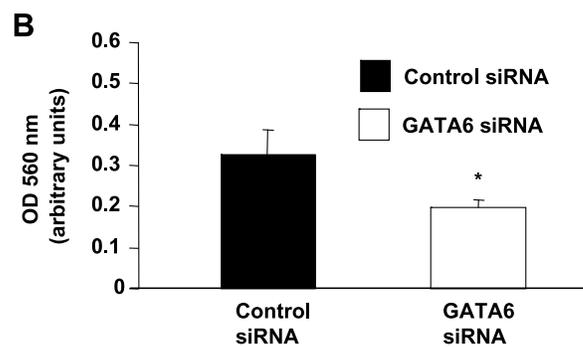
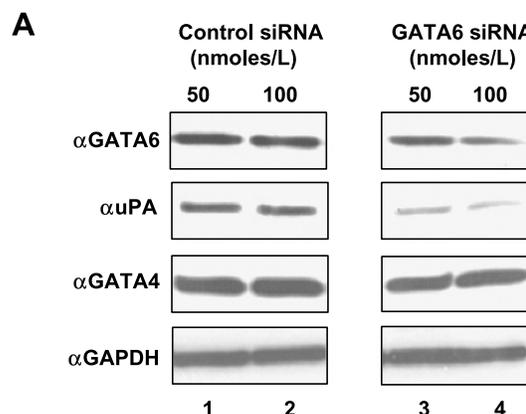


Figure 4. GATA6 is required for uPA expression and colon cancer cell invasion. (A) Protein extracts prepared from HCT116 colon cancer cells transfected with increasing concentrations of control siRNA (left panels) or GATA6 siRNA (right panels) were analyzed by Western blot analysis with GATA6 antibody (first panel), uPA antibody (second panel), GATA4 antibody (third panel), and GAPDH antibody (fourth panel). (B) HCT116 cells seeded in matrix-coated invasion chambers were transfected with 100 nM control siRNA (column 1) or GATA6 siRNA (column 2). At 48 hours later, cells that invaded the matrix and crossed the membrane were quantified colorimetrically. *n* = 3, **P* < .01 compared with control siRNA transfection.

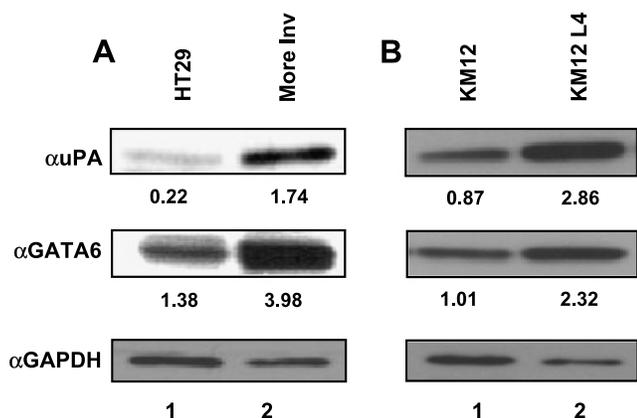


Figure 3. GATA6 expression is related to uPA expression and colorectal cancer cell invasion. Protein extracts from parental HT29 and more invasive HT29 clones (A) and less metastatic KM12 cells and highly metastatic KM12L4 cells (B) were probed with uPA (top panels) and GATA6 (middle panels) antibody. Stripped blots reprobed with GAPDH internal control antibody are shown in the bottom panels. Signals were quantified by densitometry. uPA and GATA6 signals normalized to the respective GAPDH loading control signals are shown below the top and the middle panels.

Because the expression of uPA, an important protease involved in cancer cell invasion, depended on GATA6, we examined whether the invasive phenotype of colon cancer cells is affected when GATA6 is downregulated. HCT116 cells plated in matrix-coated invasion chambers were transfected with GATA6 siRNA or control siRNA, and the ability of transfected cells to invade the matrix and cross the chamber was determined colorimetrically. Invasion by GATA6 siRNA-transfected cells was reduced by 50% compared with the control siRNA-transfected cells, suggesting that GATA6 levels regulate the invasive phenotype of colon cancer cells (Figure 4B).

GATA6 Activates uPA Promoter Activity

uPA gene expression is known to be regulated by transcriptional, posttranscriptional, and epigenetic mechanisms [22,23,42]. To determine whether GATA6 regulates uPA gene expression at the transcriptional level, we cotransfected GATA6 siRNA or the control siRNA along with a 2.5-kb uPA promoter-luciferase reporter into HCT116 cells. Compared with the control siRNA, GATA6 siRNA strongly downregulated the uPA promoter activity, suggesting that GATA6 may regulate uPA gene expression at the transcriptional level (Figure 5A). A relatively lower decrease in luciferase activity with increasing doses of control siRNA could be related to the often reported nonspecific targeting or the toxicity associated with control siRNA.

To examine whether GATA6 activates the uPA promoter, we cotransfected a 2.5-kb uPA-luciferase reporter along with the GATA6

expression vector or empty control vector into HCT116 cells. Expression of GATA6 but not the empty vector activated the uPA promoter by five-fold (Figure 5B). To delineate the region(s) of the uPA promoter that mediate(s) activation by GATA6, we used a series of uPA promoter deletion mutants. Deletion of the promoter to -2020 slightly enhanced GATA6 mediated activation. A further deletion to -520, which deletes the uPA enhancer, reduced the basal as well as GATA6-induced activity by approximately 50%. These results suggested that, although the uPA promoter sequences to -520 is sufficient for GATA6 induced activation, sequences located between -2020 and -520, which contains several consensus and nonconsensus GATA-binding sites, are important for the maximal basal and GATA6-induced activity of the uPA promoter.

GATA6 Activation of uPA Promoter Requires Sp1

A further deletion from -520 to -86 resulted in a slight increase in GATA6 activation of the uPA promoter (Figure 6A). Although the -520 promoter has potential nonconsensus GATA binding sites located at -430 and -336, the -86 uPA promoter does not contain discernible GATA binding sites. This construct contains five Sp1 binding sites spanning approximately 50 nucleotides, previously shown to be essential for the activity of the uPA promoter [27]. Deletion of all the Sp1 binding sites in the construct -30 completely abolished GATA6-induced activation of the uPA promoter, suggesting that GATA6 may target Sp1 family members to regulate uPA

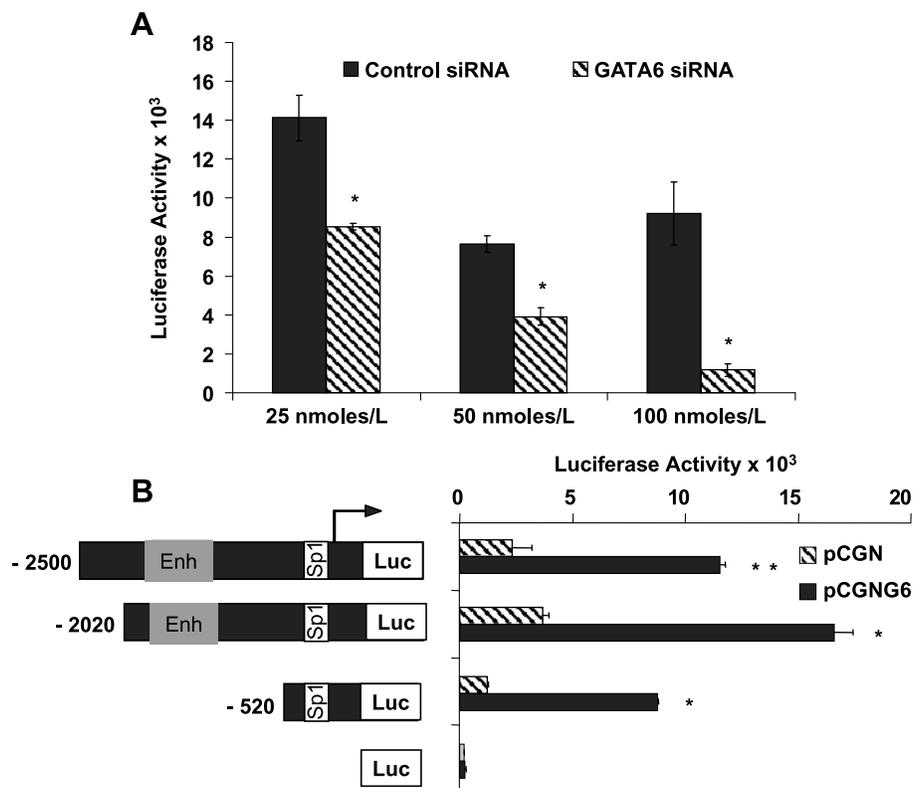


Figure 5. GATA6 activates uPA gene expression. (A) HCT116 cells were transfected with the 2.5-kb uPA promoter-luciferase reporter along with increasing concentrations of control siRNA or GATA6 siRNA. Luciferase values were read 36 hours after transfection. $n = 3$, $*P < .01$ compared with control siRNA transfections. (B) HCT116 cells were transfected with the 2.5-kb uPA promoter-luciferase and deletion mutants of uPA promoter-luciferase reporter constructs along with the GATA6 expression vector or pCGN empty vector. Deletion constructs are diagrammatically shown on the left with numbers corresponding to nucleotide coordinates. Enh indicates enhancer; Luc, luciferase; Sp1, Sp1 binding sites. $n = 3$, $*P < .01$, $**P < .05$.

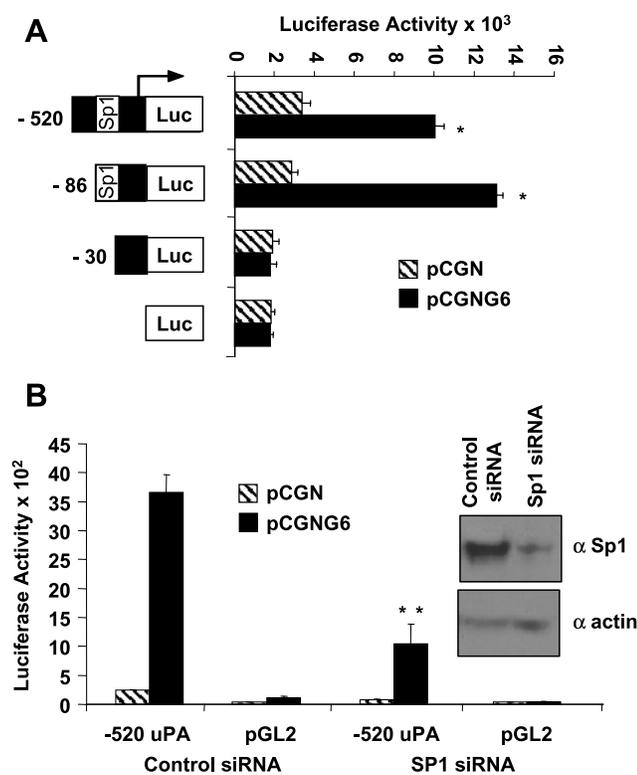


Figure 6. Sp1 and Sp1 binding sites in the uPA promoter are required for GATA6 activation of the uPA promoter. (A) HCT116 cells were transfected with the indicated uPA promoter deletion mutants driving luciferase reporter along with the GATA6 expression vector or pCGN empty vector. Deletion constructs are diagrammatically shown on the left with numbers corresponding to nucleotide coordinates. Luc indicates luciferase; Sp1, Sp1 binding sites. $n = 3$, $*P < .01$. (B) HCT116 cells were transfected with 100 nM control siRNA or Sp1 siRNA. At 36 hours later, siRNA-transfected cells were split into 24-well plates and transfected with -520 uPA promoter luciferase or pGL2 basic promoter–enhancer less reporter with GATA6 expression vector or pCGN empty vector. $n = 3$, $**P < .05$. Protein lysates from control or Sp1 siRNA-transfected cells were analyzed by Western blot analysis with Sp1 or internal standard actin antibodies, and a representative gel is shown in the inset.

promoter activity (Figure 6A). Although both Sp1 and the related family member, Sp3, can bind to these Sp1 binding sites *in vitro*, only Sp1 but not Sp3 binds to these sites *in vivo* in uPA-producing cells [27]. To investigate whether Sp1 is required for the GATA6-activated uPA promoter activity, we used RNAi to knockdown Sp1 expression in HCT116 cells. GATA6 activation of the -520 uPA-luciferase construct was significantly reduced in Sp1 siRNA-transfected cells compared with the control siRNA-transfected cells (Figure 6B). Together, these results suggest that GATA6 activation of the uPA promoter is mediated by Sp1 and the Sp1 binding sites in the uPA promoter proximal region.

GATA6 Binds to uPA Regulatory Regions In Vivo

Because endogenous uPA expression requires GATA6 and the uPA promoter is activated by GATA6, we examined whether GATA6 binds to the uPA promoter *in vivo* by ChIP analysis. Chromatin from uPA-producing HCT116 cells was isolated and immunoprecipitated with goat GATA6 antibody. A nonimmune goat antibody and acetylated histone H3 antibodies were used as negative and positive controls, re-

spectively. The chromatin immunoprecipitates were analyzed by semi-quantitative PCR with primers spanning -290 to -650 and -910 to -1214 regions of uPA 5' flanking region. These regions contain several consensus and nonconsensus potential GATA6 binding sites. Results of ChIP analysis indicated that GATA6 binds over the promoter proximal -650 to -290 and promoter distal -1214 to -910 regions of the uPA promoter (Figure 7, A and B). Because 86 bp of the promoter

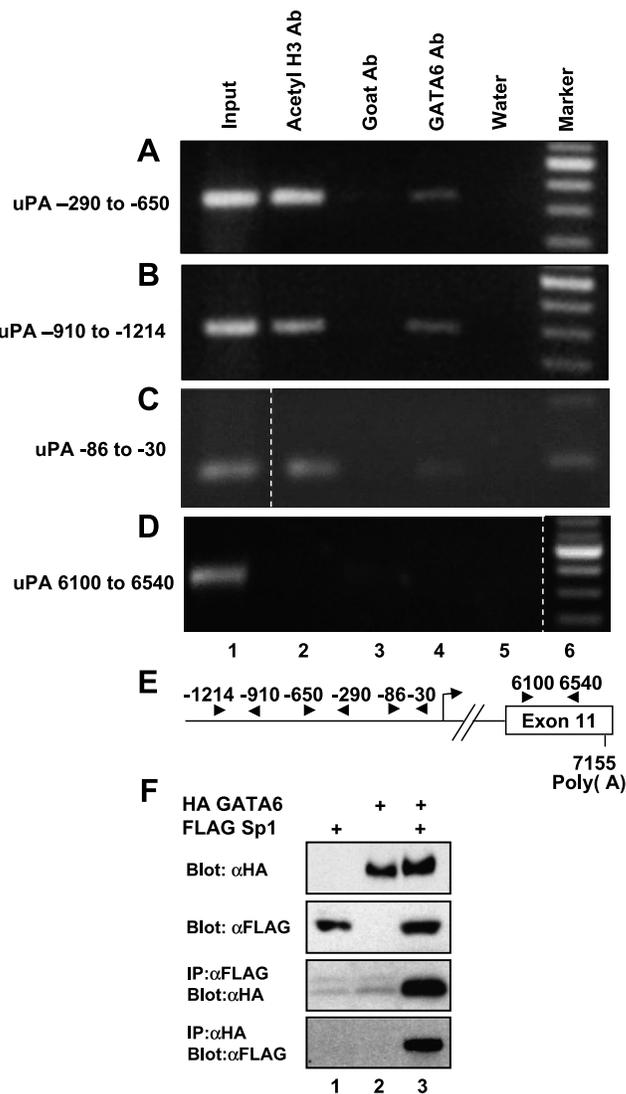


Figure 7. (A-E) GATA6 binds to uPA regulatory regions *in vivo*. Cross-linked chromatin from HCT116 cells was immunoprecipitated with acetylated histone 3 antibody (lane 2), control nonimmune goat antibody (lane 3), and GATA6 antibody (lane 4) and analyzed by PCR using the indicated uPA primers. The input control (5%) is in lane 1, and water control is in lane 5. Position of PCR primers relative to the uPA cap site (arrow) and the polyadenylation signal (poly[A]) within the terminal exon 11 of the uPA gene is shown diagrammatically in panel E. (F) GATA6 and Sp1 physically interact. Five hundred micrograms of lysates from HCT116 cells transfected with FLAG epitope-tagged Sp1 (lane 1), HA epitope-tagged GATA6 (lane 2), or both (lane 3) was immunoprecipitated with FLAG antibody and probed with HA antibody (third panel) or immunoprecipitated with HA antibody and probed with FLAG antibody (bottom panel). Five percent of inputs were probed with HA antibody (top panel) or FLAG antibody (second panel).

region contained five Sp1 binding sites and no discernible GATA binding sites was sufficient for GATA6 activation of the uPA promoter, we examined whether GATA6 can bind to this region of the uPA promoter by analyzing the GATA6 ChIP using primers spanning -86 to -30 regions of the uPA promoter. As shown in Figure 7C, this region containing Sp1 binding sites and no discernible GATA sites was bound by GATA6, albeit weakly, suggesting that GATA6 may bind to the uPA promoter independent of GATA binding sites and by recruitment through Sp1 binding sites. GATA6 recruitment was specific to the regulatory promoter region of uPA because a primer pair spanning the nonregulatory exon 11 region of uPA (+6100 and +6540) was not amplified in GATA6 ChIP (Figure 7D).

GATA6 Physically Interacts with Sp1

The requirement for Sp1 protein and Sp1 binding sites in the proximal promoter for GATA6 activation of uPA promoter suggested that GATA6 may be recruited to the uPA promoter through physical interaction between these two proteins. To examine this possibility, we performed coimmunoprecipitation experiments in HCT116 cells by transfecting FLAG epitope-tagged Sp1 or HA epitope-tagged GATA6 either alone or in combination. Sp1 protein was present in the immunoprecipitate of GATA6 only when coexpressed with GATA6 (Figure 7F, bottom panel). Reciprocally, GATA6 was detected in the immunoprecipitate of Sp1 on coexpression with Sp1, confirming that Sp1 and GATA6 proteins physically interact with each other (Figure 7F, third panel).

Discussion

Our results show that GATA6 is overexpressed in preneoplastic lesions and primary and metastatic colorectal tumors. Whereas dysregulated GATA6 expression in preneoplastic lesions suggests a role for GATA6 in the earliest events that lead to colonic neoplasia, continued expression of GATA6 in primary and metastatic tumors suggests a role for GATA6 in the progression of colon cancer. However, the mechanisms by which overexpressed GATA6 participates in the initiation and/or progression of colorectal cancers is not known. GATA6 binding sites are present in the regulatory regions of members of the wnt family of secreted glycoproteins such as wnt2, 4, 6, 7b, and 8b, and GATA6 regulates the expression of wnt2, 7b, and 8 and the wnt receptor Fzd2 [43–46]. Dysregulated expression of GATA6 in preneoplastic colorectal lesions may lead to overexpression of target wnts, which may trigger the wnt signaling pathway implicated in the pathogenesis of colorectal cancers [47]. Continued overexpression of GATA6 in benign and malignant lesions may help in maintaining the activated wnt- β -catenin signaling during progression of colorectal cancers. In addition to wnts, GATA6 also regulates the expression of the members of the transforming growth factor β family of proteins such as BMP4. The BMP4 promoter contains consensus GATA binding sites, and these sites are essential for GATA6-induced activation of the BMP4 promoter [48]. BMP4 is overexpressed in malignant and metastatic colorectal cancers compared with benign lesions and normal mucosa [49]. A recent genome-wide study has shown the association between BMP4 and colorectal cancer [50]. BMP4 activates the Smad signaling pathway and induces epithelial-mesenchymal transition and uPA production in colorectal cancer cells and promotes cell migration and invasion. In support of this, a recent study has shown that forced expression of BMP4 in HCT116 colon cancer cells induces uPA gene expression and enhanced cell migration and invasion [49]. GATA6 and

the related protein, GATA4, physically and functionally interact with Smads and play an essential role in transforming growth factor β signal transduction [4]. Thus, it is conceivable that the overexpression of GATA6 will serve dual functions: leading to excessive production of BMP4 and participating in the activation of BMP4-induced Smad pathway signaling, leading to cell migration and invasion.

Although our studies have established a positive correlation between GATA6 overexpression and uPA gene expression using human tumors and gain or loss of GATA6 function in colorectal cancer cell lines, GATA6 may affect colon cancer cell migratory and invasive properties independent of uPA. Other extracellular matrix degrading proteases such as matrix metalloproteinases 2 and 9 are regulated by GATA2 in endothelial cells [51]. Because all six members of the GATA family bind to the same core GATA sequence, and GATA6 is the only member of this family expressed in colorectal cancers, the expression of these matrix metalloproteinase in colorectal tumors may be regulated by GATA6. GATA6 may also use pathways independent of proteases to influence the cell migratory properties. GATA6 and the related GATA4 proteins are targeted by small GTPase RhoA pathway, an evolutionarily conserved pathway that regulates the migratory behavior of normal and cancerous cells [36].

GATA6 is potentially both a tumor suppressor and a tumor promoter. GATA6 activates the expression of tumor suppressor, Dab2, and regulates the activity of LKB tumor suppressor protein [52,53]. Loss of GATA6 protein function either because of epigenetic silencing of GATA6 gene or GATA6 protein exclusion from the nuclei has been reported in ovarian cancers [9,54]. Whereas GATA6 was expressed in normal adrenal cortex, GATA6 expression was downregulated in adrenocortical tumors [15]. Further, GATA6 was downregulated in hyperplastic neointimal smooth muscle cells and forced expression of GATA6 restored normoplasia [55]. A recent study has revealed tumor suppressor activity of GATA6 in astrocytomas [10]. In contrast to the tumor-suppressing activity of GATA6 in these tissues, GATA6 promotes tumorigenesis in the esophagus, pancreas, and intestines [12,17,18,34,35]. Unlike ovarian cancers in which GATA6 is inactivated through nuclear exclusion, in aggressive colon adenocarcinomas, GATA6 staining is strongly cytoplasmic, suggesting a potential role for cytoplasmic GATA6 in promoting colon carcinogenesis. Promoting uncontrolled cell proliferation and/or conferring resistance to apoptosis are commonly used mechanisms by which oncoproteins cause transformation. GATA6 does not seem to promote either. A recent study by Shureiqi et al. [34] demonstrated that knockdown of GATA6 in HCT116 colon cancer cells did not affect cell proliferation and apoptosis. Thus, it is likely that GATA6 induces migratory and invasive behavior by upregulating extracellular matrix-degrading proteases, promoting invasion and metastasis, and confers survival advantages in foreign niches to establish secondary tumor foci.

GATA6 acts as both a transcriptional activator and a repressor depending on the context of the regulatory regions of the target genes and the cell type. GATA6 activates enteric epithelial-expressed genes, such as *lactase*, and represses the expression of 15-lipoxygenase [56,57]. In addition, combinatorial interaction of GATA6 with various ubiquitous and cell-type-enriched factors may determine whether GATA6 acts as an activator or a repressor. Although GATA6 is an activator of uPA gene expression in colon cancer cells, GATA6 negatively regulates uPA gene expression in endothelial cells by binding to the -692 to -687 GATA binding site [31]. In HCT116 colon cancer cells, GATA6 binds to regions of uPA that contain multiple consensus and nonconsensus potential GATA sites located between -290 to -650 and -910

to -1214. In addition, GATA6 activated the uPA promoter containing Sp1 binding sites and bound to this region *in vivo*, although no discernible GATA binding sites were present within this region, suggesting that GATA6 may be recruited to the uPA promoter by tethering to DNA-bound Sp1. In support of this possibility, coimmunoprecipitation experiments demonstrated a physical interaction between GATA6 and Sp1. Sp1 has been shown to recruit other transcription factors, such as retinoic acid receptor α and retinoid X receptor α , to the uPA promoter [29]. GATA6 physically and functionally interacts with Sp1 to activate expression of various genes such as *cytochrome p450c17* and *aquaporin-5* [6,7]. Although *cytochrome p450c17* promoter contains consensus GATA binding sites that bind GATA6, GATA6 seems to regulate this promoter primarily through the Sp1 binding sites, suggesting that GATA6 can regulate gene expression both dependent and independent of the GATA binding sites [6]. The promoter proximal Sp1 sites are indispensable for uPA gene expression in uPA-producing cancer cells, and binding of Sp1 to these Sp1 sites is highly dependent on Sp1 phosphorylation [27,58]. It would be interesting to examine whether GATA6 can both positively and negatively regulate uPA gene expression in colorectal cancer cells by using distinct uPA promoter elements and interacting with a distinct set of accessory factors and if the transcriptional activating and repressing activities of GATA6 are impaired during colorectal carcinogenesis.

In conclusion, our results show that the dysregulated expression and subcellular distribution of GATA6 occurs at the earliest stages of colorectal tumorigenesis and continues through the later metastatic stages and contributes to cell invasiveness by regulating the expression of the uPA protease.

Acknowledgments

The authors thank Michael Ittmann for reagents and suggestions.

References

- Molkentin JD (2000). The zinc finger-containing transcription factors GATA-4, -5, and -6. Ubiquitously expressed regulators of tissue-specific gene expression. *J Biol Chem* **275**, 38949–38952.
- Divine JK, Staloch LJ, Haveri H, Jacobsen CM, Wilson DB, Heikinheimo M, and Simon TC (2004). GATA-4, GATA-5, and GATA-6 activate the rat liver fatty acid binding protein gene in concert with HNF-1 α . *Am J Physiol Gastrointest Liver Physiol* **287**, G1086–G1099.
- Fang R, Olds LC, and Sibley E (2006). Spatio-temporal patterns of intestine-specific transcription factor expression during postnatal mouse gut development. *Gene Expr Patterns* **6**, 426–432.
- Belaguli NS, Zhang M, Rigi M, Aftab M, and Berger DH (2007). Cooperation between GATA4 and TGF- β signaling regulates intestinal epithelial gene expression. *Am J Physiol Gastrointest Liver Physiol* **292**, G1520–G1533.
- Boudreau F, Rings EH, van Wering HM, Kim RK, Swain GP, Krasinski SD, Moffett J, Grand RJ, Suh ER, and Traber PG (2002). Hepatocyte nuclear factor-1 α , GATA-4, and caudal related homeodomain protein Cdx2 interact functionally to modulate intestinal gene transcription. Implication for the developmental regulation of the sucrase-isomaltase gene. *J Biol Chem* **277**, 31909–31917.
- Fluck CE and Miller WL (2004). GATA-4 and GATA-6 modulate tissue-specific transcription of the human gene for P450c17 by direct interaction with Sp1. *Mol Endocrinol* **18**, 1144–1157.
- Zhou B, Francis TA, Yang H, Tseng W, Zhong Q, Frenkel B, Morrissey EE, Ann DK, Minoo P, Crandall ED, et al. (2008). GATA-6 mediates transcriptional activation of aquaporin-5 through interactions with Sp1. *Am J Physiol Cell Physiol* **295**, C1141–C1150.
- Agnihotri S, Wolf A, Picard D, Hawkins C, and Guha A (2009). GATA4 is a regulator of astrocyte cell proliferation and apoptosis in the human and murine central nervous system. *Oncogene* **28**, 3033–3046.
- Capo-Chichi CD, Roland IH, Vanderveer L, Bao R, Yamagata T, Hirai H, Cohen C, Hamilton TC, Godwin AK, and Xu XX (2003). Anomalous expression of epithelial differentiation-determining GATA factors in ovarian tumorigenesis. *Cancer Res* **63**, 4967–4977.
- Kamnasaran D, Qian B, Hawkins C, Stanford WL, and Guha A (2007). GATA6 is an astrocytoma tumor suppressor gene identified by gene trapping of mouse glioma model. *Proc Natl Acad Sci USA* **104**, 8053–8058.
- Kobayashi S, Lackey T, Huang Y, Bisping E, Pu WT, Boxer LM, and Liang Q (2006). Transcription factor gata4 regulates cardiac *BCL2* gene expression *in vitro* and *in vivo*. *FASEB J* **20**, 800–802.
- Kwei KA, Bashyam MD, Kao J, Ratheesh R, Reddy EC, Kim YH, Montgomery K, Giacomini CP, Choi YL, Chatterjee S, et al. (2008). Genomic profiling identifies *GATA6* as a candidate oncogene amplified in pancreaticobiliary cancer. *PLoS Genet* **4**, e1000081.
- Perlman H, Suzuki E, Simonson M, Smith RC, and Walsh K (1998). GATA-6 induces p21(Cip1) expression and G₁ cell cycle arrest. *J Biol Chem* **273**, 13713–13718.
- Rojas A, Kong SW, Agarwal P, Gilliss B, Pu WT, and Black BL (2008). GATA4 is a direct transcriptional activator of Cyclin D2 and Cdk4 and is required for cardiomyocyte proliferation in anterior heart field-derived myocardium. *Mol Cell Biol* **28**, 5420–5431.
- Vuorenoja S, Rivero-Muller A, Kiiveri S, Bielinska M, Heikinheimo M, Wilson DB, Huhtaniemi IT, and Rahman NA (2007). Adrenocortical tumorigenesis, luteinizing hormone receptor and transcription factors GATA-4 and GATA-6. *Mol Cell Endocrinol* **269**, 38–45.
- Miller CT, Moy JR, Lin L, Schipper M, Normolle D, Brenner DE, Iannettoni MD, Orringer MB, and Beer DG (2003). Gene amplification in esophageal adenocarcinomas and Barrett's with high-grade dysplasia. *Clin Cancer Res* **9**, 4819–4825.
- Kimchi ET, Posner MC, Park JO, Darga TE, Kocherginsky M, Karrison T, Hart J, Smith KD, Mezhir JJ, Weichselbaum RR, et al. (2005). Progression of Barrett's metaplasia to adenocarcinoma is associated with the suppression of the transcriptional programs of epidermal differentiation. *Cancer Res* **65**, 3146–3154.
- Fu B, Luo M, Lakkur S, Lucito R, and Iacobuzio-Donahue CA (2008). Frequent genomic copy number gain and overexpression of GATA-6 in pancreatic carcinoma. *Cancer Biol Ther* **7**, 1593–1601.
- Steeg PS (2006). Tumor metastasis: mechanistic insights and clinical challenges. *Nat Med* **12**, 895–904.
- Choong PF and Nadesapillai AP (2003). Urokinase plasminogen activator system: a multifunctional role in tumor progression and metastasis. *Clin Orthop Relat Res* **415**, S46–S58.
- Iizumi M, Liu W, Pai SK, Furuta E, and Watabe K (2008). Drug development against metastasis-related genes and their pathways: a rationale for cancer therapy. *Biochim Biophys Acta* **1786**, 87–104.
- Crippa MP (2007). Urokinase-type plasminogen activator. *Int J Biochem Cell Biol* **39**, 690–694.
- Shiou SR, Datta PK, Dhawan P, Law BK, Yingling JM, Dixon DA, and Beauchamp RD (2006). Smad4-dependent regulation of urokinase plasminogen activator secretion and RNA stability associated with invasiveness by autocrine and paracrine transforming growth factor- β . *J Biol Chem* **281**, 33971–33981.
- Verde P, Boast S, Franze A, Robbiati F, and Blasi F (1988). An upstream enhancer and a negative element in the 5' flanking region of the human urokinase plasminogen activator gene. *Nucleic Acids Res* **16**, 10699–10716.
- Nerlov C, Rorth P, Blasi F, and Johnsen M (1991). Essential AP-1 and PEA3 binding elements in the human urokinase enhancer display cell type-specific activity. *Oncogene* **6**, 1583–1592.
- Nerlov C, De CD, Pergola F, Caracciolo A, Blasi F, Johnsen M, and Verde P (1992). A regulatory element that mediates co-operation between a PEA3-AP-1 element and an AP-1 site is required for phorbol ester induction of urokinase enhancer activity in HepG2 hepatoma cells. *EMBO J* **11**, 4573–4582.
- Ibanez-Tallon I, Ferrai C, Longobardi E, Facetti I, Blasi F, and Crippa MP (2002). Binding of Sp1 to the proximal promoter links constitutive expression of the human uPA gene and invasive potential of PC3 cells. *Blood* **100**, 3325–3332.
- Kojima S, Hayashi S, Shimokado K, Suzuki Y, Shimada J, Crippa MP, and Friedman SL (2000). Transcriptional activation of urokinase by the Kruppel-like factor Zf9/COPEB activates latent TGF- β 1 in vascular endothelial cells. *Blood* **95**, 1309–1316.
- Suzuki Y, Shimada J, Shudo K, Matsumura M, Crippa MP, and Kojima S (1999). Physical interaction between retinoic acid receptor and Sp1: mechanism for induction of urokinase by retinoic acid. *Blood* **93**, 4264–4276.
- Kopf E and Miskin R (2005). A RUNX/AML-binding motif residing in a novel 13-bp DNA palindrome may determine the expression of the proximal promoter of the human uPA gene. *J Thromb Haemost* **3**, 2057–2064.

- [31] Sokabe T, Yamamoto K, Ohura N, Nakatsuka H, Qin K, Obi S, Kamiya A, and Ando J (2004). Differential regulation of urokinase-type plasminogen activator expression by fluid shear stress in human coronary artery endothelial cells. *Am J Physiol Heart Circ Physiol* **287**, H2027–H2034.
- [32] Chen B, Yates E, Huang Y, Kogut P, Ma L, Turner JR, Tao Y, Camoretti-Mercado B, Lang D, Svensson EC, et al. (2009). Alternative promoter and GATA5 transcripts in mouse. *Am J Physiol Gastrointest Liver Physiol* **297**, G1214–G1222.
- [33] Hellebrekers DM, Lentjes MH, van den Bosch SM, Melotte V, Wouters KA, Daenen KL, Smits KM, Akiyama Y, Yuasa Y, Sanduleanu S, et al. (2009). GATA4 and GATA5 are potential tumor suppressors and biomarkers in colorectal cancer. *Clin Cancer Res* **15**, 3990–3997.
- [34] Shureiqi I, Zuo X, Broaddus R, Wu Y, Guan B, Morris JS, and Lippman SM (2007). The transcription factor GATA-6 is overexpressed *in vivo* and contributes to silencing 15-LOX-1 *in vitro* in human colon cancer. *FASEB J* **21**, 743–753.
- [35] Haveri H, Westerholm-Ormio M, Lindfors K, Maki M, Savilahti E, Andersson LC, and Heikinheimo M (2008). Transcription factors GATA-4 and GATA-6 in normal and neoplastic human gastrointestinal mucosa. *BMC Gastroenterol* **8**, 9.
- [36] Charron F, Tsimiklis G, Arcand M, Robitaille L, Liang Q, Molkentin JD, Meloche S, and Nemer M (2001). Tissue-specific GATA factors are transcriptional effectors of the small GTPase RhoA. *Genes Dev* **15**, 2702–2719.
- [37] Fletcher G, Jones GE, Patient R, and Snape A (2006). A role for GATA factors in *Xenopus* gastrulation movements. *Mech Dev* **123**, 730–745.
- [38] Skelly MM, Troy A, Duffy MJ, Mulcahy HE, Duggan C, Connell TG, O'Donoghue DP, and Sheahan K (1997). Urokinase-type plasminogen activator in colorectal cancer: relationship with clinicopathological features and patient outcome. *Clin Cancer Res* **3**, 1837–1840.
- [39] Hafez MM, Infante D, Winawer S, and Friedman E (1990). Transforming growth factor β 1 acts as an autocrine-negative growth regulator in colon enterocytic differentiation but not in goblet cell maturation. *Cell Growth Differ* **1**, 617–626.
- [40] Hafez MM, Hsu S, Yan Z, Winawer S, and Friedman E (1992). Two roles for transforming growth factor β 1 in colon enterocytic cell differentiation. *Cell Growth Differ* **3**, 753–762.
- [41] Morikawa K, Walker SM, Jessup JM, and Fidler IJ (1988). *In vivo* selection of highly metastatic cells from surgical specimens of different primary human colon carcinomas implanted into nude mice. *Cancer Res* **48**, 1943–1948.
- [42] Pakneshan P, Szyf M, and Rabbani SA (2005). Hypomethylation of urokinase (uPA) promoter in breast and prostate cancer: prognostic and therapeutic implications. *Curr Cancer Drug Targets* **5**, 471–488.
- [43] Alexandrovich A, Arno M, Patient RK, Shah AM, Pizzey JA, and Brewer AC (2006). Wnt2 is a direct downstream target of GATA6 during early cardiogenesis. *Mech Dev* **123**, 297–311.
- [44] Katoh M and Katoh M (2007). Conserved POU/OCT- and GATA-binding sites in 5'-flanking promoter region of mammalian WNT8B orthologs. *Int J Oncol* **30**, 1273–1277.
- [45] Weidenfeld J, Shu W, Zhang L, Millar SE, and Morrisey EE (2002). The WNT7b promoter is regulated by TTF-1, GATA6, and Foxa2 in lung epithelium. *J Biol Chem* **277**, 21061–21070.
- [46] Zhang Y, Goss AM, Cohen ED, Kadzik R, Lepore JJ, Muthukumaraswamy K, Yang J, DeMayo FJ, Whitsett JA, Parmacek MS, et al. (2008). A Gata6-Wnt pathway required for epithelial stem cell development and airway regeneration. *Nat Genet* **40**, 862–870.
- [47] de Lau W, Barker N, and Clevers H (2007). WNT signaling in the normal intestine and colorectal cancer. *Front Biosci* **12**, 471–491.
- [48] Nemer G and Nemer M (2003). Transcriptional activation of BMP-4 and regulation of mammalian organogenesis by GATA-4 and -6. *Dev Biol* **254**, 131–148.
- [49] Deng H, Makizumi R, Ravikumar TS, Dong H, Yang W, and Yang WL (2007). Bone morphogenetic protein-4 is overexpressed in colonic adenocarcinomas and promotes migration and invasion of HCT116 cells. *Exp Cell Res* **313**, 1033–1044.
- [50] Houlston RS, Webb E, Broderick P, Pittman AM, Di Bernardo MC, Lubbe S, Chandler I, Vijayakrishnan J, Sullivan K, Penegar S, et al. (2008). Meta-analysis of genome-wide association data identifies four new susceptibility loci for colorectal cancer. *Nat Genet* **40**, 1426–1435.
- [51] Han X, Boyd PJ, Colgan S, Madri JA, and Haas TL (2003). Transcriptional up-regulation of endothelial cell matrix metalloproteinase-2 in response to extracellular cues involves GATA-2. *J Biol Chem* **278**, 47785–47791.
- [52] Morrisey EE, Musco S, Chen MY, Lu MM, Leiden JM, and Parmacek MS (2000). The gene encoding the mitogen-responsive phosphoprotein Dab2 is differentially regulated by GATA-6 and GATA-4 in the visceral endoderm. *J Biol Chem* **275**, 19949–19954.
- [53] Setogawa T, Shinozaki-Yabana S, Masuda T, Matsuura K, and Akiyama T (2006). The tumor suppressor LKB1 induces p21 expression in collaboration with LMO4, GATA-6, and Ldb1. *Biochem Biophys Res Commun* **343**, 1186–1190.
- [54] Caslini C, Capo-Chichi CD, Roland IH, Nicolas E, Yeung AT, and Xu XX (2006). Histone modifications silence the GATA transcription factor genes in ovarian cancer. *Oncogene* **25**, 5446–5461.
- [55] Mano T, Luo Z, Malendowicz SL, Evans T, and Walsh K (1999). Reversal of GATA-6 downregulation promotes smooth muscle differentiation and inhibits intimal hyperplasia in balloon-injured rat carotid artery. *Circ Res* **84**, 647–654.
- [56] Fitzgerald K, Bazar L, and Avigan MI (1998). GATA-6 stimulates a cell line-specific activation element in the human lactase promoter. *Am J Physiol* **274**, G314–G324.
- [57] Shureiqi I, Jiang W, Fischer SM, Xu X, Chen D, Lee JJ, Lotan R, and Lippman SM (2002). GATA-6 transcriptional regulation of 15-lipoxygenase-1 during NSAID-induced apoptosis in colorectal cancer cells. *Cancer Res* **62**, 1178–1183.
- [58] Benasciutti E, Pages G, Kenzior O, Folk W, Blasi E, and Crippa MP (2004). MAPK and JNK transduction pathways can phosphorylate Sp1 to activate the uPA minimal promoter element and endogenous gene transcription. *Blood* **104**, 256–262.

Supplementary Information

Subcloning of More Invasive HT29 Cells

HT29 cells (1.0×10^5) were plated in matrix-coated 12-well Boyden chamber inserts. After 48 hours of culturing, noninvading cells were removed from the interior of inserts using sterile cotton swabs. Cells that invaded the matrix and crossed over to the lower surface of the membrane were recovered by treatment with trypsin and considered as more invasive cells.

Table W1. List of Primers Used for Generating Deletion Mutants of uPA.

-2020 primer:	5'-TCC <u>CCCCGGG</u> AGGGTGTACGCTTCATAAC-3'
-1500 primer:	5'-TCC <u>CCCCGGG</u> CTTCCTTCTGTCACTCTCAG-3'
-510 primer:	5'-TCC <u>CCCCGGG</u> CTGAACAGACGCACAGAGGG-3'
-86 primer:	5'-TCC <u>CCCCGGG</u> CGCTGCAAGACAGGGGAGGGA-3'
-30 primer:	5'-TCC <u>CCCCGGG</u> TGATATAGAGCAGGCCCGCG-3'
+34 primer:	5'-CCT <u>CCCCGGG</u> CTCCGGGGTCCGGTCTCCG-3'

Top strand sequence is shown for the -2020, -1500, -510, -86, and -30 primers. Bottom strand sequence is shown for the +34 primer. *Sma*I site used for cloning of PCR products into *Sma*I site of pGL2 basic vector is underlined.

Table W2. List of Primers Used for ChIP Analysis of uPA.

uPA ChIP -1214 (T) primer:	5'-GTCTGCTTATCCTCAGGCAG-3'
uPA ChIP -910 (B) primer:	5'-TCTGTTCTCACCTCTGAGTAG-3'
uPA ChIP -650 (T) primer:	5'-CCTTGCCCTTCCCCCTCTGA-3'
uPA ChIP -290 (B) primer:	5'-AGACTCTCTACCTCGCCCAG-3'
uPA ChIP -86 (T) primer:	5'-CGCTGCAAGACAGGGGAGGGA-3'
uPA ChIP -25 (B) primer:	5'-CAGGGCCCCGCCGGCGCCG-3'
uPA ChIP +6100 (T) primer:	5'-CCTCGTCTGTCCCTCCAAGG-3'
uPA ChIP +6540 (B) primer:	5'-ACTCCTGCAGGCTTCACTCCA-3'

Top strand (T) sequence is shown for the -1214, -650, -86, and +6100 forward primers. Bottom strand (B) sequence is shown for the -910, -290, -25, and +6540 reverse primers.