RESEARCH ARTICLE

Hath1 Inhibits Proliferation of Colon Cancer Cells Probably Through Up-regulating Expression of Muc2 and p27 and Down-regulating Expression of Cyclin D1

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Abstract

Previous studies showed that *Math1* homologous to human *Hath1* can cause mouse goblet cells to differentiate. In this context it is important that the majority of colon cancers have few goblet cells. In the present study, the potential role of *Hath1* in colon carcinogenesis was investigated. Sections of paraffin-embedded tissues were used to investigate the goblet cell population of normal colon mucosa, mucosa adjacent colon cancer and colon cancer samples from 48 patients. *Hath1* and Muc2 expression in these samples were tested by immunohistochemistry, quantitative real-time reverse transcription -PCR and Western blotting. After the recombinant plasmid, pcDNA3.1(+)-*Hath1* had been transfected into HT29 colon cancer cells, three clones were selected randomly to test the levels of *Hath1* mRNA, Muc2 mRNA, *Hath1*, Muc2, cyclin D1 and p27 by quantitative real-time reverse transcription-PCR and Western blotting. Moreover, the proliferative ability of HT29 cells introduced with *Hath1* was assessed by means of colony formation assay and xenografting. Expression of *Hath1*, Muc2, cyclin D1 and p27 in the xenograft tumors was also detected by Western blotting. No goblet cells were to be found in colon cancer and levels of *Hath1* mRNA and *Hath1*, Muc2 mRNA and Muc2 were significantly down-regulated. *Hath1* could decrease cyclin D1, increase p27 and Muc2 in HT29 cells and inhibit their proliferation. *Hath1* may be an anti-oncogene in colon carcinogenesis.

Keywords: *Hath1* - colon carcinoma - etiology - colon carcinoma cell line - cell cycle regulation

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Introduction

Accompanying changes of foods, the morbidity of colon cancer becomes higher and higher in China (Shin et al., 2006). It is to be regretted that the etiology of colon adenocarcinoma is yet unclear and remains to be determined. Polygenic causes such as oncogene *K-ras*, *c-myc*, EGFR and anti-oncogene APC, DDC, p53 have shown effect on colon tumorigenesis (Markowitz et al., 2002).

Hath1 is homologous to the Drosopila atonal and mouse *Math1*, its location is at 4q22 and its CDS has 1065bp (Akazawa et al., 1995). *Hath1* encodes *Hath1* which is a basic helix-loop- helix (bHLH) transcription factor. Previous studies in the developing brain and inner ear of *Math1* knockout mice have shown that cerebellar granule neurons and inner ear hair cells of mice fail to differentiate, respectively (Bermingham et al., 1999; Gazit et al., 2004; Fritzsch et al., 2005; Jones et al., 2006). Misexpression of *Math1* in postnatal and adult mice inner ears is capable of inducing terminal differentiation of inner ear hair cells (Zheng et al., 2000; Shou et al., 2003; Kawamoto et al., 2003). More importantly, targeted deletion of *Math1* also resulted mice intestinal secretory cells, including goblet cells, in failure of differentiate (Yang et al., 2001) and *Hath1* can induce Muc2 expression (van de Wetering et al., 2002). However, a potential role for *Hath1* in colon cancer is not yet clear.

In the course of human colon carcinogenesis, goblet cells have gradually decreased. At the beginning of colon tumorigenesis, the first pathological change is the formation of aberrant crypt focus (ACF) and the numbers of goblet cells begin to decrease (Pinto et al., 2003; Grady, 2004). In the stage of adenoma, colon adenomas have all the fewer goblet cells, even goblet cell has disappeared (Hamilton, 1992). In colon cancer, goblet cells are few or disappearance (Hanski et al., 1997; Ponz de Leon et al., 2001).

Main component of mucinous secreted by colon goblet cells is mucin 2 (Muc2) (Pinto et al., 2003; Grady, 2004). In the course of human colon carcinogenesis, Muc2 expression has decreased while the numbers of

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goblet cells reduced (Hamilton, 1992; Hanski et al., 1997). The majority of colon carcinomas produce a little mucin except for mucinous adenocarcinoma and signet-ring cell carcinoma (Hanski et al., 1997; Wong et al., 2002). Some studies have shown that Muc2 was concerned with colon tumors. Mice lacking the Muc2 reportedly developed intestinal adenomas (Velcich et al. 2002; Yang et al., 2005). Otherwise, Muc2 expression reportedly was regulated by Wnt/β-catenin signaling pathway, the latter has been proved to play an important role in colon carcinogenesis (Yang et al., 2001).

In a word, *Hath1*, Muc2 and goblet cell constitutes a chain which maybe have some effect on colon carcinogenesis.

It is known to all that fundamental characteristic of tumor cell is proliferation lost to control which is caused by disorder of regulation of cell cycle. Cyclin D1 is a positive regulation factor of G1/S check point and can induce cell from G1 stage into S stage (Kang et al., 2002). Studies have shown that cyclin D1 is concerned with tumorigenesis and development in some tumor, so cyclin D1 is considered as a proto-oncogene (Blok et a1., 2000). Another cell cycle regulation factor, p27 can inhibit various kinds of CDK-cyclin complexes so prevents cell from G1 stage into S stage (Bryja et al., 2004). Previous Studies indicated that p27 also involved in tumorigenesis and development in some tumor (Slingerland et al., 2000). To research the potential role of *Hath1* in colon carcinogenesis, in the present study, we investigated its expression and Muc2 level in colon cancer. Then, we transfected *Hath1* into colon cancer cells line and tested *Hath1* mRNA, Muc2 mRNA, *Hath1*, Muc2, cyclin D1 and p27 expressions. Finally, we observed whether introduction of *Hath1* would alter the proliferative ability of colon cancer cells by in vitro and in vivo studies.

Materials and Methods

Collection of clinic samples

Colon samples were from 48 patients in our hospital with colon carcinoma not including cases with mucinous adenocarcinoma and signet-ring cell carcinoma. We collected samples of normal mucosa (>10cm from the margin of the cancer), mucosa adjacent to cancer (<1cm from the margin of the cancer) and cancer tissue from the same patient. One part of each sample was used to manufacture paraffin tissue sections and another was conserved in liquid nitrogen.

Histochemical and Immunohistochemical Analysis

To confirm whether there is a reduction in goblet cell population in colon cancer, Sections of paraffin-embedded tissues from normal colon mucosa, mucosa adjacent colon cancer and colon cancer were stained with H&E. Each slide contains three types of sections from one of 48 individual colon adenocarcinoma cases. Goblet cells were counted in five fields (magnification ×100), the result was expressed as mean ± SD. We used S-P immunohistochemistry to detect *Hath1* and Muc2 expression in colon samples. After deparaffinage and rinsing sections in PBS, sections then were left in citrate (0.01mol/L) buffer for 20 minutes at 95°C for antigen retrieval. Peroxidase block was applied and sections were rinsed in 1×PBS and blocked in 10% normal donkey serum for 10 minutes at room temperature. The primary antibodies used were rabbit anti-*Hath1* (1:200; Chemicon, USA) and rabbit anti-Muc2 (1:100; Maixin, China). The primary antibody was added on sections and incubation was performed for 1 hour at room temperature. Then, we added ready-to-use biotinylated anti-rabbit secondary antibody (Maixin, China) on sections and incubated sections for 10 minutes at room temperature to detect anti-*Hath1* and anti-Muc2. Then the sections were counterstained with H.E. We used image analysis software to analyze the value of positive area density of ten campus visualis each section. Results were expressed as mean±SD and q test was used for statistical analysis.

Construction of pcDNA3.1(+)-Hath1

It is known that *Hath1* expression is high in normal colon mucosa, so we used Total RNA of normal colon mucosa as templates to synthesize cDNA of *Hath1* (1065bp) by reverse transcription (primer, 5'AAGCTTA TGTCCCGCCTGCTGCATGCAGAAGAGT 3'). Then *Hath1* genes were amplified by PCR method (forward primer, 5'AAGCTTATGTCCCGCCTGCT GCATGCAG AAGAGT 3'; reverse primer, 5'GA ATTCCTAACTTGC CTCATCCGA 3') and PCR amplifications also were examined by agarose gel electrophoresis to ensure that bands were only visible at the expected molecular weights. We used Agarose gel DNA purification kit (Takara, Japan) to purify *Hath1* and DNA ligation kit (Takara) to construct pM19-T-*Hath1*, then used BcaBEST primer M13-47 and BcaBEST primer RV-M to perform sequencing for pM19-T-*Hath1*. When the purified DNA was confirmed that its sequence was identical with *Hath1*, we connected it with pcDNA3.1(+) after treating pM19-T-*Hath1* and pcDNA3.1(+) with EcoRIand Hind III.

Cell Culture and Cell Transfection

Colorectal cancer cell line HT29 came from Shanghai institute of biochemistry and cell biology, China. The medium was RPMI1640 containing 10% fetal bovine serum (Sijiqing, China), 100U/ml penicillin and 100U/ml streptomycin. Conditions of cell culture were 37°C, 5%CO2 and saturated humidity. Cell transfection was performed with Infectamine 2000(Invitrogen, USA) according to manufacturer's instructions using 0.8 μg DNA of pcDNA3.1(+) or pcDNA3.1(+)-*Hath1* per well in 24-well plates. After 24 hours, G418 was added to the media (400 μg/ml), and drug selection was continued for 2 weeks. Three clones were randomly selected from each transfection for additional characterization. When a clone transfected pcDNA3.1(+)-*Hath1* was confirmed expressing higher *Hath1* mRNA, Muc2 mRNA (quantitative real-time reverse transcription-PCR) and higher *Hath1* (Western blot), we assured this clone was the cell strain expressing *Hath1* stably.

Quantitative Real-time Reverse Transcription -PCR

Quantitative real-time reverse transcription-PCR was used to analyse *Hath1* mRNA and Muc2 mRNA.

Total RNA was prepared according to manufacturer's instructions of Total tissue/cell RNA extraction kit (Omega, USA) from samples of normal colon mucosa, colon mucosa adjacent to cancer, colon adenocarcinoma, HT29 cells untransfected, HT29 cells transfected pcDNA3.1(+) and HT29 cells transfected pcDNA3.1(+)-*Hath1*. Quantitative real-time reverse transcription-PCR was performed through fluorescence engomphosis and according to instructions of SYBR ExScript RT-PCR kit (Takara, Japan). Beta actin was used as control gene. Random 6mers primer applied to reverse transcription and the following specific primers were used in PCR for *Hath1*: forward primer, 5'TCCCACTTTGCAGGGCATC 3'; reverse primer, 5'CAGCTGTTCCCGCACTTTCA 3'; Muc2: forward primer, 5'TGTGTTCACGGGAATGCT GAG 3', reverse primer, 5'TGCAGGCGATGACGTTGAG 3'; Beta actin: forward primer, 5'TGGCACCCAGCACA ATGAA 3', reverse primer, 5'CTAAGTCATAGTCCGCC AGAAGCA 3'. Expression levels of genes of interest were normalized to Beta actin. Initial real-time PCR amplifications also were examined by agarose gel electrophoresis to ensure that bands were only visible at the expected molecular weights. Data were collected from three cultures from each of the experimental groups and were expressed as mean ± SD and q test was used for statistical analysis.

Western Blot

We advantaged of Western blot to detect *Hath1*, Muc2, cyclin D1 and p27. The levels of *Hath1*, cyclin D1 and p27 in HT29 cells untransfected, HT29 cells transfected pcDNA3.1(+), HT29 cells transfected pcDNA3.1(+)-*Hath1* and xenograft tumors were detected by Western blotting analysis. Protein extracts were obtained by homogenizing samples in a cell lysis buffer containing 20 mmol HEPES, 25% glycerol, 0.42 mmol NaCl, 15 mmol MgCl2, 0.2 mmol EDTA, 0.5 mmol phenylmethylsulfonyl fluoride and 0.5 mmol dithiothreitol, then by two cycles of centrifugation at 12 000×g for 15 minutes. Protein concentration was determined by Bradford Assay Kit (Bio-Rad, USA). Extracted protein was separated by 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis, and transferred to polyvinylidene fluoride membranes (Dupont, USA). These membranes were washed with 0.1% Tween20-PBS and incubated with 5% dry non-fat skimmed milk powder in 0.1% Tween20-PBS for 1 hour, then with rabbit antihuman *Hath1* polyclonal antibody (diluted 1:500 ; Chemicon, USA) or rabbit antihuman cyclin D1 monoclone antibody (diluted 1:200; Maixin, China) or rabbit antihuman p27 monoclone antibody (diluted 1:200; Maixin, China), and horseradish peroxidase–conjugated goat anti-rabbit IgG (diluted 1:5000; Santa Cruz, USA) for 1 hour. Finally, the immune complexes were developed with an Enhanced Chemiluminescence Detection Kit (Pierce, USA) and the membranes were then immediately exposed to autoradiographic film (Kodak, USA). The relative amount of *Hath1*, cyclin D1 and p27 was quantified from relative optical density of the band by image analysis system (Bio-Rad Gel Doc 2000, USA). A rabbit antihuman GAPDH antibody (diluted 1:1000; Abcam, USA) was used to assess the levels of protein loaded per lane. Data were expressed as mean ± SD and q test was used for statistical analysis.

Colony Formation Assay and Xenograft Experiment

Assays to determine colony formation in soft agar by tumor cell lines were performed using 24-well plates. The bottom layer was consisted of 0.8ml culture medium containing 0.5% (w/v) agar (Takara, Japan) and 20% fetal bovine serum. Similarly, the top layer also had 0.8ml culture medium containing 20% fetal bovine serum, but the concentration of agar was 0.25% (w/v). HT29 cells untransfected, HT29 cells transfected pcDNA3.1(+) and HT29 cells transfected pcDNA3.1(+)-*Hath1* were diluted with culture medium so as to the cell density was 103 cells/ml. Before the top layer was added into the well, we put in 100 cells to top layer and mixed them thoroughly. Experiments were performed in triplicate. After 2 weeks of incubation, all of colonies containing above 50 cells were counted. Data collected from each experimental group were expressed as mean ± SD and q test was used for statistical analysis. For HT29 colon cancer cell xenograft experiments, five female Balb/C nude mice (ages 4 weeks; Center of experimental animals of Chongqing Medical Sciences University, Chongqing, China) were inoculated s.c. with 1.5×10^6 HT29 cells per group. The xenograft experiment groups included group of HT29 cells untransfected, group of clone transfected pcDNA3.1(+) and group of clone transfected pcDNA3.1(+)-*Hath1*. Mice were monitored for 30 days after inoculation, and tumor measurements were taken on day 5, 10, 15, 20, 25 and 30. Tumor volume was calculated based on two dimensions, measured using calipers, and was expressed in cubic millimeters according to the formula: $V = 0.5a \times b^2$, where 'a' and 'b' are the long and the short diameters of the tumor, respectively. Data collected from each experimental group were expressed as mean ± SD, and q test statistical analysis was used. This study was conducted in accordance with administration rules of experimental animals instituted by Chinese experimental Animal Committee.

Statistical analysis

Data were presented as mean±standard deviation and analyzed by q test using SPSS11.0. A $P<0.05$ was considered statistically significant.

Results

Goblet Cell Population Is Dramatically Reduced in Colon Adenocarcinomas

We observed sections of paraffin-embedded tissues stained with H&E to recognize and count goblet cells. In normal colon, goblet cells have a goblet-like morphology readily identifiable by H&E staining (black arrowhead, Figure 1A). Sections of mucosa adjacent colon cancer samples showed that there were hyperplastic change and reduction of goblet cells (Figure 1B). Sections obtained from colon adenocarcinoma display cells that appeared crowded without goblet-like features (Figure 1C). Goblet cells were counted in five fields (magnification ×400). We could not find any goblet cell

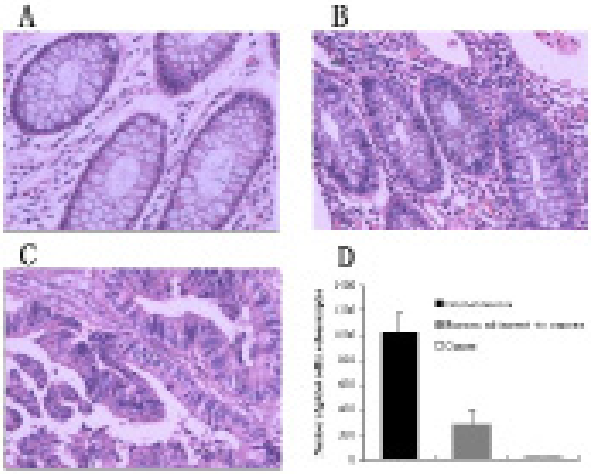

Figure 1. Noticeable Reduction of Goblet Cell Populations in Colon Carcinomas. Sections of paraffin-embedded tissues from normal mucosa (A), mucosa adjacent to cancer (B) and colon adenocarcinomas (C) are stained with H&E. D, the number of goblet cell in the three kinds of colon samples

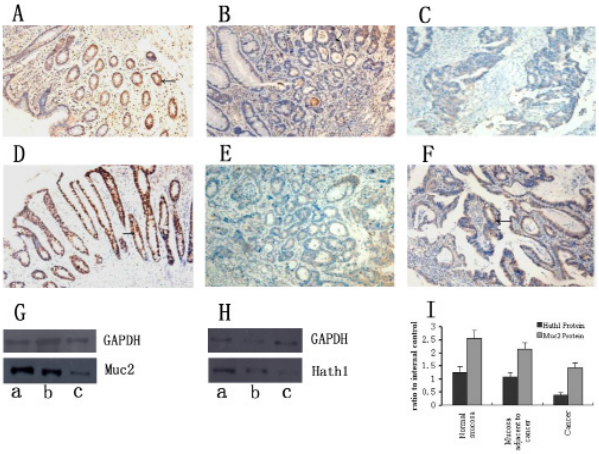

Figure 2. Down-regulation of *Hath1* and Muc2 in Human Colon Carcinomas. A, B, C, representative images of immunohistochemical detection of *Hath1* in normal mucosa, mucosa adjacent to cancer and colon adenocarcinoma respectively (magnification ×100). D, E, F, representative images of immunohistochemical detection of Muc2 in normal mucosa, mucosa adjacent to cancer and colon adenocarcinoma respectively. (magnification×100). G, Western blot detection of Muc2 in normal mucosa (a), mucosa adjacent to cancer (b) and colon adenocarcinoma (c). H, Western blot detection of *Hath1* in normal mucosa (a), mucosa adjacent to cancer (b) and colon adenocarcinoma (c). I, results of Western blot indicated that Muc2 and *Hath1* level in colon cancer was obviously reduced (q test; * to compare with group of human normal colon mucosa or group of mucosa adjacent to coloncancer, $P<0.05$)

Table 1. *Hath1* mRNA and Muc2 mRNA Expression in Colon Samples (n=48)

Group	Relative expression *Hath1* mRNA (χ±s)	Relative expression of Muc2 mRNA (χ±s)
Normal mucosa	6.34±1.87	$2.37\times10^{5}\pm7.84\times10^{4}$
Mucosa adjacent to cancer	3.32±1.25	$5.22\times10^{4}\pm6.13\times10^{3}$
Cancer	0.36±0.13	8.31±0.85

Table 2. Relative Expression of *Hath1* mRNA and Muc2 mRNA in Different HT29 Cells (n=6)

Group	Relative expression of *Hath1* mRNA(χ±s)	Relative expression of Muc2 mRNA(χ±s)
Untransfected HT29	$2.0\times10^{-2}\pm7.63\times10^{-3}$	$2.46\times10^{-4}\pm1.0\times10^{-4}$
Transfected pcDNA3.1(+) clone1	$1.96\times10^{-2}\pm6.62\times10^{-3}$	$2.43\times10^{-4}\pm9.57\times10^{-5}$
Transfected pcDNA3.1(+)-*Hath1* clone1	507.83±115.93*	$3.41\times10^{-2}\pm9.93\times10^{-3}$**
Transfected pcDNA3.1(+) clone2	$1.58\times10^{-2}\pm5.47\times10^{-3}$	$2.75\times10^{-4}\pm8.44\times10^{-5}$
Transfected pcDNA3.1(+)-*Hath1* clone2	523.56±109.74*	$3.12\times10^{-2}\pm9.53\times10^{-3}$**
Transfected pcDNA3.1(+) clone3	$1.81\times10^{-2}\pm6.42\times10^{-3}$	$2.56\times10^{-4}\pm8.79\times10^{-5}$
Transfected pcDNA3.1(+)-*Hath1* clone3	497.52±104.34*	$3.68\times10^{-2}\pm8.81\times10^{-3}$**

**Hath1* mRNA expression in HT29 cells stably expressing pcDNA3.1(+)*Hath1*, HT29 cells untransfected and HT29 cells stably expressing pcDNA3.1(+). Quantitative real-time reverse transcription-PCR analysis of *Hath1* mRNA expression was performed. Results shown the *Hath1* mRNA expression was significant up-regulated compared with HT29 cells untransfected and HT29 cells stably expressing pcDNA3.1(+)(q test, P<0.01); **Muc2 mRNA expression in HT29 cells stably expressing pcDNA3.1(+) *Hath1*, HT29 cells untransfected and HT29 cells stably expressing pcDNA3.1(+). Quantitative real-time reverse transcription-PCR analysis of *Hath1* mRNA expression was performed. Results shown the Muc2 mRNA expression was significant up-regulated compared with HT29 cells untransfected and HT29 cells stably expressing pcDNA3.1(+) (q test, P<0.01)

in sections of colon adenocarcinoma. Examination of 48 samples by histochemical methods revealed that there is a significant reduction of the number of goblet cells in colon adenocarcinoma sections (0) compared with normal colon mucosa (1019.80±124.9) and mucosa adjacent colon cancer sections (275.60±64.65) (P<0.01, Figure 1D).

Hath1 and Muc2 Expression is Decreased in Colon Adenocarcinoma

We detected *Hath1* mRNA and Muc2 mRNA expression in colon samples by quantitative real-time reverse transcription-PCR (Table 1). Data shown that *Hath1* mRNA expression in colon cancers (0.36±0.13) was significantly down-regulated compared with normal colon mucosa (6.34±1.87, $P<0.01$) and mucosa adjacent to cancer (3.32±1.25, $P<0.01$). Muc2 mRNA expression in colon cancers (8.31±0.85) also was significantly down-regulated compared with normal colon mucosa ($2.37\times10^{5}\pm7.84\times10^{4}$, $P<0.01$) and mucosa adjacent to cancer ($5.22\times10^{4}\pm6.13\times10^{3}$, $P<0.01$). We also investigated *Hath1* and Muc2 expression in colon samples by immunohistochemistry. *Hath1* positive expression displayed buffy grains in cytoplasm and/or nucleus (Figure 2A, 2B, 2C), and Muc2 positive expression manifested buffy grains in cytoplasm (Figure 2D, 2E, 2F). The results of immunohistochemistry indicated that the expression of *Hath1* and Muc2 in colon cancer was dramatically reduced compared with their matched samples. Importantly, Western blot detections also shown that the expression of *Hath1* and Muc2 in colon cancer (0.317±0.075) was obviously decreased compared with normal colon mucosa (1.205±0.096, $P<0.01$) and mucosa

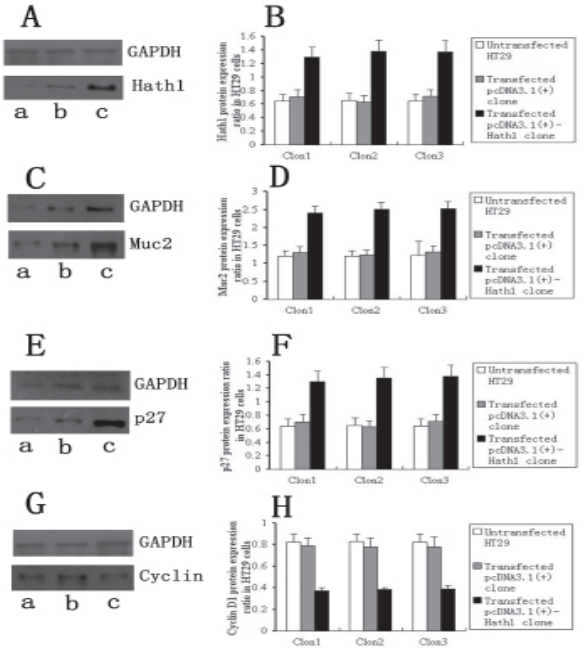

Figure 3. *Hath1* Down-regulates Expression of Cyclin D1, but up-regulates Expression of p27 and Muc2. With all the picture, lane (a) stand for untransfected HT29, lane (b) stand for transfected pcDNA3.1(+) clone, lane (c) stand for transfected pcDNA3.1(+)- *Hath1* clone. A, C, E and G are the Western blot detection of *Hath1*, Muc2, p27 and cyclin D1 expressing, respectively, in HT29 cells untransfected, HT29 cells transfected pcDNA3.1(+). B, D, F and H are the Western blot data of A, C, E and G respectively, showed with histograms

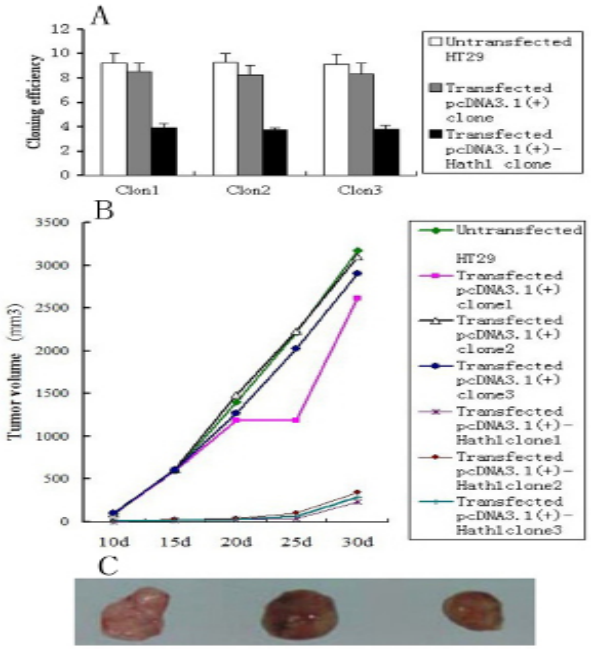

Figure 4. *Hath1* Inhibit Anchorage-independent Growth and Xenograft of HT29 Colon Cancer Cells. A, colony formation assay on HT29 cells transfected pcDNA3.1(+), pcDNA3.1(+)-*Hath1* and HT29 cells untransfected. B, Xenograft of HT29 cells transfected pcDNA3.1(+), pcDNA3.1(+)-*Hath1* and HT29 cells untransfected measured during a 30-day period. C, representative pictures of xenograft tumour

adjacent to cancer (1.017±0.094, P<0.01). the Muc2 expression was singnificantly reduced in colon cancer (1.152±0.144) compared with normal colon mucosa (1.543±0.157, P<0.01) and mucosa adjacent to cancer (2.035±0.113, P<0.01) (Figure 2G, 2H, and 2I).

Hath1 Up-regulates p27 Expression and Down-regulates cyclin D1 Expression in HT29 Colon Cancer Cells

To determine whether *Hath1* was successful to be transfected into HT29 cells, we detected Muc2 Mrna, *Hath1* mRNA, Muc2 and *Hath1* expression in HT29 cells. Data shown that these indexes were significantly higher in HT29 cells transfected pcDNA3.1(+)-*Hath1* compared with HT29 cells untransfectd and HT29 cells transfected pcDNA3.1(+) (Table 2; Figure 3A, 3B, 3C, 3D) (P<0.05). It meant that *Hath1* had been successful to be transfectd into HT29 cells and shown its physiological functions. Western blot experiments indicated p27 expression in HT29 cells transfected pcDNA3.1(+)-*Hath1*(clone1 1.352±0.110, clone2 1.388±0.075, clone3 1.363±0.073) was up-regulation compared with HT29 cells untransfected (0.578±0.086; P<0.05) and HT29 cells transfected pcDNA3.1(+)(clone1 0.545±0.066, clone2 0.590±0.077, clone3 0.520±0.074; P<0.05) (Figure 3E, 3F).

However, cyclin D1 expression(clone1 0.352±0.056, clone2 0.365±0.029, clone3 0.372±0.062) was simultaneously down-regulation compared with HT29 cells untransfected (0.830±0.062; P<0.05) and HT29 cells transfected pcDNA3.1(+)(clone1 0.790±0.045, clone2 0.775±0.059, clone3 0.802±0.067; P<0.05) (Figure 3G, 3H).

Table 3. Cloning Efficiency of Different HT29 Cells in Soft Agar (n=6)

Group	cloning efficiency(%)	*P* Value
Untransfected HT29	9.33±1.21	
Transfected pcDNA3.1(+) clone1	8.50±1.05	
Transfected pcDNA3.1(+)-*Hath1* clone1	3.83±0.75	P<0.01*
Transfected pcDNA3.1(+) clone2	8.17±1.47	
Transfected pcDNA3.1(+)-*Hath1* clone2	3.5±1.05	P<0.01*
Transfected pcDNA3.1(+) clone3	8.33±1.47	
Transfected pcDNA3.1(+)-*Hath1* clone3	3.67±1.49	P<0.01*

*To compare with group of untransfected HT29 or group transfected pcDNA3.1(+) clone

Hath1 Can Suppress Anchorage-Independent Growth of HT29 Colon Cancer Cells in Soft Agar and Xenografts in Athymic Nude Mice

In our efforts to determine the effect of *Hath1* expression on anchorage-independent growth of HT29 colon cancer cells, we performed colony formation assay in soft agar. The assay was performed in triplicate, and colonies whose cell number was more than 50 were counted from whole fields. After 14 days in culture, we found that there was an approximately 3-fold reduction in the number of colonies formed from HT29 cells stably expressing pcDNA3.1(+)- *Hath1*(clone1 3.83±0.75, clone2 3.50±01.05, clone3 3.67 ±1.49) compared with HT29 cells untransfected (9.33±1.21; P<0.01) and HT29 cells transfected pcDNA3.1(+)(clone1 8.50±1.05, clone2 8.17±1.47, clone3 8.33±1.49; P<0.01) (Table 3; Figure 4A).

We extended our study by assessing the growth of these HT29 stable clones in a xenograft experiment. HT29 stably transfected cells were injected s.c. into athymic nude mice and we monitored for tumor growth. Data

showed the tumor formation of HT29 cells expressing *Hath1* had been significantly inhibited (Figure 4B, 4C).

Discussion

Previous studies have shown that *Hath1* rodent homologue *Math1* is required for development of secretory cells, including goblet cells, in the embryonic mouse intestine (Yang et al., 2001).Moreover, in the course of human colon carcinogenesis, goblet cells have gradually decreased and the Muc2 expression has reduced simultaneously (Hamilton, 1992; Hanski et al., 1997; Ponz de Leon et al., 2001; Pinto et al., 2003; Grady, 2004). These led us to hypothesize that *Hath1* is involved in human colon carcinogenesis. One study published by Leow et al indicated that the expression of *Hath1* mRNA and Muc2 mRNA are down-regulated in human colon cancer (not including mucinous cancer and signet ring carcinoma) and forced expression *Hath1* in colon cancer cells results in inhibiting the proliferation of colon cancer cells (Leow et al., 2004). But the samples were small (12 colorectal cancer samples and two clones of colon cancer cells with high level of *Hath1* expression) and there were not the quantitative analysis of the expresion of *Hath1* and Muc2. In our study, we took advantage of 48 colon cancer samples and three clones of colon cancer cells overexpressing *Hath1* to reveal the expresion changes of *Hath1* and Muc2 in stages of transcription and translation. Additionally, in xenograft experiment, we not only assessed the growth of xenograft tumors, but also analyzed quantitatively the level in the xenografts of *Hath1*, Muc2, cyclin D1 and p27 by Western blot to better understand the role that *Hath1* plays in colon cancer.

In the present study, we observed that the expression of Muc2 mRNA and *Hath1* mRNA in the human colon cancer and mucosa adjacent to cancer was dramatically lower than those in normal colon mucosa ($P<0.01$, Table 1). The results are consistent with Leow's study. We also observed that the expression of Muc2 and *Hath1* in the human colon cancer and mucosa adjacent to cancer was also significantly lower than those in normal colon mucosa ($P<0.01$, Figure 2). Moreover, the expression of *Hath1* and Muc2 correlated positively with the population of goblet cells (Figure 1). Our data shows, accompanying with the decrease of goblet cell population, at the levels of transcription and translation, the expression of *Hath1* and Muc2 is down-regulated in the human colon cancer and mucosa adjacent to cancer. The results indicate that *Hath1* maybe concern with goblet cell differentiation and colon carcinogenesis.

To better understand the relation between *Hath1* and Muc2, we transfected *Hath1* into HT29 cell, a colon cancer cell line with low level *Hath1* expression, then detected the level of Muc2 expression. The results showed that the levels of Muc2 mRNA and Muc2 correlate positively with the levels of *Hath1* mRNA and *Hath1* (Table 2, Figure 3 A, B, C, D). These observations hint at *Hath1* is the up-stream gene of Muc2 and maybe involve in goblet cell differentiation. A previous study shows that the Muc2 promoter contains three proximal E-box motifs that have been identified as binding sites for *Hath1*. Mutation of these sites diminished the stimulatory effect of *Hath1*, suggesting that *Hath1* may activate MUC2 transcription via direct interaction with the MUC2 promoter (Park et al., 2006).

Previous studies had shown a role for *Hath1* as a terminal differentiation factor involved in a series of cells including intestinal secretory cells [8-11]. So we reason that *Hath1* maybe have a suppression function for colon cancer cell proliferation. To test this possibility, we measured the growth rate of HT29 cell forced expression *Hath1* in vitro and in vivo. The colony formation assay in soft agar showed an approximately 3-fold reduction in the number of colonies formed from HT29 cells overexpressing *Hath1* compared with the controls (Figure 4A). We also observed that the tumor volume in nude mice transplanted HT29 cells overexpressing *Hath1* was smaller than control groups (Figure 4 B, C).

The majority of oncogenes and anti-oncogenes what belong to transcription factors can facilitate or inhibit canceration by controling the cell cycle. Accordingly, we suppose that *Hath1* maybe also inhibit the cell cycle and result in the differentiation and repress the canceration.

As a cell cycle regulator, cyclin D1 can promote cell from G1 stage into S stage. The amplification and/or overexpression of cyclin D1 can be found in a series of tumous. Toncheva et al reported that the expression of cyclin D1 was obviously higher in colorectum cancer than that in normal colorectum tissue (Toncheva et al., 2004).

As a cyclin dependent kinase inhibitor, p27 can arrest the cells from G1stage into S stage, so inhibit the cells proliferation (Newland et al., 1999). The levels of p27 were significantly lower than those in normal colon tissue (Loda et al., 1997). Combining with the compounds of cyclin D1-CDK4 and cyclin D1-CDK2, p27 inhibit the activity of CDK and arrest the cells from G1 stage into S stage (Sherr, 1996; Park et al., 1999). Therefore, we chose p27 and cyclinD1, which they are interactive and express abnormally in colon cancers, to investigate whither their expression is regulated by *Hath1*.

With Western blot analyses, we observed that the expression of cyclin D1 is down-regulated and the expression of p27 is up-regulated in HT29 cells overexpressing *Hath1* and in xenograft tumors with high level of *Hath1* expression (Figure 3 E, F, G, H). These results indicated that the suppression of *Hath1* for proliferation of HT29 cells maybe come true through the regulation of cyclin D1 and p27, probably other cell cycle regulators. But the mechanisms are unclear.

Park ET reported recently that the expression of *Hath1* and Muc2 was up-regulated in mucinous adenocarcinomas and signet-ring cell carcinomas of colon [29]. The results also support the view that *Hath1* can up-regulate the expression of Muc2. However, it is opposite to our findings in general adenocarcinomas of colon. We presume the cause of the difference due to differences of etiopathogenesis between general adenocarcinomas and adenocarcinomas containing abundant mucus. Although, at present, we cannot determine the status of *Hath1* in carcinogenesis of colon, but there are evidences that the down-regulation of the expression of Hath concerned with carcinogenesis of general colon adenocarcinomas. But, in

adenocarcinomas containing abundant mucus, changes of some gene promote the carcinogenesis and up-regulate simultaneously the expression of *Hath1* and Muc2, thus make the cancers have the pathologic characteristic containing abundant mucus.

Accordingly, we can conclude: (1) Expression of *Hath1* and Muc2 is down-regulated in human colon cancer in levels of transcription and translation. (2) *Hath1* can inhibit the proliferation of HT29 cells. (3) Up-regulating expression of *Hath1* can result in down-regulation of cyclin D1 and up-regulation of p27. (4) Muc2 is a downstream gene of *Hath1*. In a word, down-regulation of *Hath1* may concern with colon cancer. *Hath1* may be an anti-oncogene in colon carcinogenesis.

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