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ORIGINAL ARTICLE

Inhibition of Nucleostemin Upregulates CDX2 Expression in HT29 Cells in Response to Bile Acid Exposure: Implications in the Pathogenesis of Barrett's Esophagus

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Abstract
Background Barrett's esophagus (BE), a squamous-to-columnar metaplasia, may originate from growth-promoting mutations in metaplastic stem cells. Nucleostemin is a protein highly expressed in undifferentiated embryonic stem cells. The objectives of this study were to explore the potential role of nucleostemin in the pathogenesis of BE
Methods The expression profiles of 30,968 genes were compared between BE and normal esophageal tissues ($n=6$ in each group) by using oligo microarray. Three siRNA plasmid expression vectors against nucleostemin, *pRNAi-1*, *pRNAi-2*, and *pRNAi-3*, were constructed and transfected into HT29 cells. In addition, HT29 cells were exposed to 100–1,000 μM chenodeoxycholic acid (CDC), a bile acid, for 2, 12, and 24 h, and then messenger RNA and protein expressions of nucleostemin and CDX2 were determined by reverse-transcriptase polymerase chain reaction and Western blotting.
Results Four hundred and twenty-six differentially expressed genes were detected in BE; 142 were upregulated and 284 downregulated. Nucleostemin was downregulated while CDX2 was upregulated. *In vitro*, all the recombinant plasmids inhibited the nucleostemin expression in transfected HT29 cells, with *pRNAi-1* being the most effective. CDX2 expression was significantly increased in *pRNAi-1*-transfected HT29 cells, compared with that in the empty plasmid (*pRNAT-U6.1*/*Neo*) transfected or untransfected HT29 cells. In addition, CDX2 expression was increased whereas nucleostemin expression was decreased in a dose- and time-dependent manner in HT29 cells treated with CDC.
Conclusion These findings suggest that the inhibition of nucleostemin expression in "esophageal stem cells" in response to bile acid exposure may be involved in the pathogenesis of BE through upregulating CDX2 expression.

Keywords Barrett's esophagus · Nucleostemin · CDX2 · HT29 cell · Oligomicroarray

Introduction

The incidence of esophageal adenocarcinoma has increased at a rate that is among the highest of all cancers.[1,2] The major risk factor for esophageal adenocarcinoma is the presence of Barrett's esophagus (BE), a premalignant neoplastic lesion that is characterized by intestinal metaplasia replacing the normal squamous esophageal epithelia.[3] The presence of BE increases the overall risk of adenocarcinoma by 40-fold.[4]

Nucleostemin, a newly found p53-binding protein, exists mainly in the nucleoli of stem cells and some various cancer cells but is not expressed in committed and terminally differentiated cells.[5] Nucleostemin helps regulate proliferation of both cancer cells and stem cells and is considered as a useful marker of undifferentiated human adult bone marrow stem cells.[6] It has been demonstrated that nucleostemin is expressed, to a certain extent, in normal esophageal squamous mucosa and increasingly expressed in esophageal squamous carcinoma.[7] However, its role in the pathogenesis of BE and subsequently esophageal adenocarcinoma is yet to be elucidated.

In the present study, we first performed a genome-wide assessment of gene in endoscopic biopsy specimens taken

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from BE patients and those with normal esophageal mucosa, using an oligo microarray method. We observed that CDX2 was expressed, but nucleostemin was not detected in BE tissues. We hypothesized that nucleostemin downregulates CDX2 expression, and the loss of nucleostemin expression in the esophageal "stem cells" may result in activation of CDX2 expression, leading to the intestinal differentiation and subsequent formation of intestinal metaplasia. It has been demonstrated that HT29 cells can be used to serve as an *in vitro* model for the study of the effects of different components of gastroduodenal refluxate on cellular and molecular events in the development of Barrett's esophagus.[8] To test our hypothesis, we further determined the effects of silencing nucleostemin expression on the expression of CDX2 in HT29 cells with the RNAi technique to see whether siRNAs that target nucleostemin transduction would enhance CDX2 expression in vitro. In addition, we observed the expression of nucleostemin and CDX2 in HT29 cells after chenodeoxycholic acid (CDC) exposure.

Materials and Methods

Tissue Specimens

Endoscopic tissue biopsies taken from the BE areas of six patients (n=6) and from six subjects with normal esophageal mucosa (n=6) were provided by the Gastroenterology Research Institute, Southwest Hospital, Third Military Medical University, Chongqing, China. Routine histopathologic examinations were performed to confirm the diagnosis by experienced gastrointestinal pathologists. BE was defined as any columnar-lined mucosa above the gastroesophageal junction, which was further confirmed by Alcian blue staining. Intestinal metaplasia was defined by the presence of barrel-shaped goblet cells in normal gastroesophageal junction.[9]

The study protocol was approved by the Ethic Committee of the Third Military Medical University, and written informed consent was provided by all study subjects.

RNA Preparation

Total sample RNA was extracted by a single-step method. Briefly, the tissues were ground and homogenized using the Trizol reagent (Invitrogen Life Technologies, CA, USA) for extraction of total RNA, according to the instructions of the manufacturer. The integrity of total RNA was checked by 1.2% formaldehyde agarose gel electrophoresis showing the 28S and 18S bands. Total RNA with OD260/OD280>1.8 was used for microarray experiments

Detection of Gene Expression Profiles in Tissue Specimens by Oligomicroarray

Total RNA from BE and matched normal tissue were labeled with cyanine 3-dUTP and cyanine 5-dUTP by direct labeling method (Perkin Elmer Life Sciences, USA: Micromax Direct labeling kit). Labeled probes were denatured at 95°C for 5 min and hybridized with a human oligo microarray (University Health Network, Microarray Center, Toronto, Canada) in a hybridization chamber (Corning Life Sciences, USA) at 65°C water bath for 18 h. Before hybridization, slides were prehybridized in 5× saline–sodium citrate buffer (SSC), 0.1% sodium dodecyl sulfate (SDS), and 1% bovine serum albumin solution at 65°C for 45 min to prevent nonspecific hybridization. After hybridization, the slides were washed in 2× SSC with 0.1% SDS, 0.1× SSC with 0.05% SDS, and 0.1× SSC sequentially for 20 min each and then spin-dried. The microarray image was scanned by Gene Pix 4200A scanner (Axon Instruments Inc., Foster City, CA, USA) and analyzed by Gene Pix Pro 6.0.1.27 software (Axon Instrument). Differentially expressed genes, which were defined as genes with twofold or greater difference in the expression between BE and normal esophageal tissues in four out of the six chips, were further analyzed for functional gene clusters using GeneSpring software GXV. The normalized ratio of Cy5 intensity to Cy3 intensity greater than 2.0 or less than 0.5 was considered as upregulated or downregulated gene expression, respectively.

Cell Line and Culture

Human colon adenocarcinoma cell line, HT29, was obtained from the American Type Culture Collection (Manassas, VA USA). HT29 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (Gibco-BRL, Grand Island, NY, USA), 50 U/mL of penicillin, and 50 μg/mL of streptomycin. The cells were detached from the flasks before subculturing by the removal of the medium and the addition of 1 mL of 0.25% trypsin and incubation at room temperature for 3 to 5 min.

Construction of the siRNA Plasmid Expression Vectors and Transfection of Plasmids

Three siRNAs targeted against nucleostemin were designed by a program available online (www.genscript.com), namely, nucleostemin I (GTGGACAGGTGCCTCATTA), nucleostemin II (ACAGAGGCTTGAAGAACTA), and nucleostemin III (GAAGCTGTACTGCCAAGAA). siRNA-expressing plasmids were constructed by cloning the siRNA sequences into *pRNAT-U6.1*/*Neo* via *Bam*HI and *Hind*III digestion. The plasmids were extracted following the manufacturer's instruction and then sequenced to confirm the correct insertion. The new plasmids were

named *pRNAi-1*, *pRNAi-2*, and *pRNAi-3*, respectively, and the concentration and purity of the plasmids were detected by ultraviolet spectrophotometry. The plasmids were stored at −20°C for subsequent experiments.

HT29 cells were seeded on six-well culture plates and grown to 80~90% confluence before the transfection. The recombinant *pRNAi-1*, *pRNAi-2*, and *pRNAi-3* were used for the transfection in the corresponding experimental groups. Lipofectamine™ 2000 alone was used for the transfection in the blank control group whereas the empty plasmid *pRNAT-U6.1/Neo* was used in the negative control group. The culture medium was replaced with the fresh medium containing calf serum (150 mL/L) at 6 h posttransfection. Forty-eight hours later, the transfected cells were selected by G418 (600 μg/mL; Huamei Biotechnology Company, Beijing, China) until positive clones were discovered after 14 days. The cells were cultured and finally selected by G418 (300 μg/mL) for a further 10 days. Single clones were selected to build a stable transfected cell line.

Treatment of HT29 Cells with CDC

After 70% confluence, HT29 cells were placed in serum-free Roswell Park Memorial Institute 1640 for 24 h and then exposed to 100, 500, and 1,000 μM CDC (Sigma Chemical Co., St. Louis, MO, USA) in serum-free medium for 2, 12, and 24 h, respectively. Cells were harvested at the end of each time point with 0.25% trypsin solution.

Detection of Protein Expression of Nucleostemin and CDX2 in HT29 Cells by Western Blot Analysis

Cells were washed three times with ice-cold sterile phosphate buffer solution (PBS), then lysed in radio-immunoprecipitation assay (Beyotime Co., China) with 10 mM phenylmethylsulfonyl fluoride (Beyotime Co.) for 30 min on ice. The lysate was centrifuged at 16,000×*g* for 15 min at 4°C. Then, the supernatant was transferred to clean microfuge tubes. Protein concentration was measured by the bicinchoninic acid protein assay (Pierce, Rockford, IL, USA), as recommended by the manufacturer.

Proteins (25 μg) were separated by 12% SDS polyacrylamide gel electrophoresis and then transferred to nitrocellulose membrane (0.45 mm). Each membrane was then blocked for 1 h at room temperature with 5% dehydrated skim milk; the membranes were incubated overnight at 4°C with a goat polyclonal antinucleostemin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, 1:200) and a mouse monoclonal anti-CDX2 antibody (Santa Cruz, 1:100), and for the detection of nucleostemin (62 kDa) and CDX2 (33 kDa). β-actin (42 kDa) was also detected with a mouse monoclonal antibody (Sigma, St. Louis, MO, USA) as a loading control. Membranes were washed in 3% dry nonfat milk in PBS containing 0.05% Triton X-100 and incubated with antigoat or antimouse peroxidase-conjugated secondary antibody (Amersham Pharmacia Biotech, Berkshire, UK, 1:10,000) for 30 min. Immunoblots were revealed by using an enhanced chemiluminescence system (Amersham Pharmacia Biotech). Densitometric analyses were performed using Quantity one software (version 4.2.2, Bio-Rad USA).

Detection of mRNA Expression of Nucleostemin and CDX2 in HT29 Cells by Reverse Transcription Polymerase Chain Reaction

Total RNA was extracted from each sample using the Total RNA Extract Kit (Omega) following the manufacturer's instructions. The concentration of RNA was measured by spectrophotometry. Total RNA was reverse-transcribed to complementary CDNA (cDNA) with reverse-transcriptase reagents (Toyobo Co., Japan) according to the manufacturer's protocol. Two-microgram cDNA was amplified in a total volume of 25 μL under the conditions recommended by the manufacturer. The cycling conditions were 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 64°C (for primers of nucleostemin) for 30 s or 60°C (for primers of CDX2) or 58°C (for primers of β-actin), and 72°C for 60 s, and a final extension of 72°C for 10 min. Polymerase chain reaction (PCR) products were separated on a 1.5% agarose gel and viewed by ethidium bromide staining. Amplification of human β-actin served as an internal standard. The gene primers are shown in Table 1.

Statistical Analysis

All data were expressed as mean±standard deviation (SD) and analyzed by analysis of variance. All data were analyzed with SPSS 10.0 software. A P value of <0.05 was considered as statistical significant.

Table 1 Sequence and Size of Primers Used for RT-PCR Amplification of *Nucleostemin*, *CDX2* Gene, and β-*actin* Genes

Gene	Primer	Sequence	Product size (bp)
β-actin	Sense	GTTGCGTTACACCCTTTCTTGACA	446
	Antisense	GCACGAAGGCTCATCATTCAAAA	
Nucleostemin	Sense	GAAACAGAGGCTTGAAGAACTAA	223
	Antisense	GGAGGCTTCGATCACCTTTTTA	
CDX2	Sense	ACCAGGACGAAAGACAAATATCGA	85
	Antisense	TGTAGCGACTGTAGTGAAACTCCTTCT	

Results

Gene Expression Pattern in BE and Normal Tissues

From the original number of 30,968 gene probes, a total of 426 genes were identified to be differentially expressed genes in all six chips; 142 were upregulated and 284 were downregulated in BE compared with the normal esophageal mucosa (Fig. 1). Among these differentially expressed genes, nucleostemin downregulation was 0.34±0.09-fold, while CDX2 upregulation was 3.58±0.97-fold in BE, compared with the normal esophageal mucosa.

Identification of Constructed Recombinant Plasmids and Confirmation of Transfection of the Vectors

The recombinant plasmids were identified to have correct sequences by DNA sequencing analysis, and the resulting sequencing confirmed that the DNA chains had been ligated to the vectors. Efficiency of transfection was evaluated by fluorescence microscopy after transfection of a vector containing the gene encoding a green fluorescent protein at 2 weeks, and nearly 100% of cultured HT29 cells transfected with *pRNAi-1* were positive for the green fluorescent protein (Fig. 2).

Protein and mRNA Expression of Nucleostemin and CDX2 in Transfected HT29 Cells

Nucleostemin protein expression were downregulated significantly in HT29 cells after transfection with *pRNAi-1*, *pRNAi-2*, and *pRNAi-3* (all $P<0.05$; Fig. 3a, b). Since the *pRNAi-1* was the most effective vector, it was selected for further experiment on the effect of RNAi on the expression of CDX2. It was shown that CDX2 protein expression in HT29 cells transfected with *pRNAi-1* was significantly increased, compared with that in HT29 cells transfected with *pRNAT* or untransfected HT29 cells (Fig. 3c, d).

Figure 1 Image of gene expression profiles in Barrett's esophagus tissue.

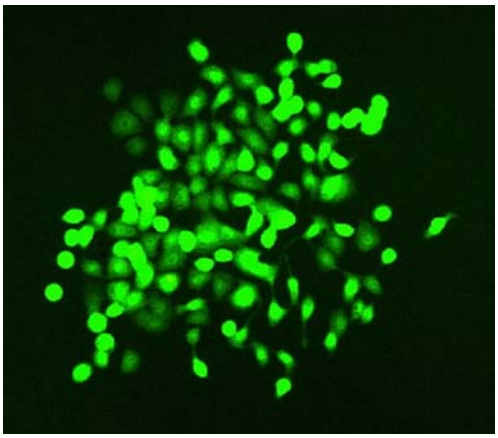


Figure 2 Expression of green fluorescent protein in HT29 cells after transfection with *pRNAi-1* (×200, under a fluorescence microscope).

The expression of *nucleostemin* mRNA was significantly inhibited in HT29 cells transfected with siRNA-expressing vectors, *pRNAi-1*, *pRNAi-2*, and *pRNAi-3*, compared with that in HT29 cells transfected with *pRNAT* or untransfected HT29 cells (all $P<0.05$). It was noticed that *pRNAi-1* was the most effective (Fig. 4a, b). In addition, CDX2 expression in HT29 cells transfected with *pRNAi-1* was noticeably stronger than that in HT29 cells transfected with *pRNAT* or untransfected HT29 cells (Fig. 4c, d).

Protein and mRNA Expression of Nucleostemin and CDX2 in HT29 Cells Exposed to CDC

A low level of CDX2 protein expression was detected in HT29 cells without CDC exposure. CDX2 protein expression was highly upregulated by CDC treatment in a dose- and time-dependent fashion. Although CDC exerted no significant effect of on CDX2 protein expression in HT29 cells at 100 μM for up to 24 h and at 500 μM for up to 12 h, CDX2 protein expression was significantly increased after treatment with 500 μM CDC at 24 h or 1,000 μM CDC at 2 h, with the maximal effect being achieved with 1,000 μM CDC at 24 h. Furthermore, nucleostemin protein expression was decreased in a dose- and time-dependent fashion in HT29 cells treated with CDC (Fig. 5, A1, B1, and C1).

After exposure to CDC, nucleostemin mRNA expression was significantly downregulated but CDX2 mRNA expression was significantly upregulated at all time points (i.e., 2, 12, and 24 h) in a dose- and time-dependent fashion, especially at the concentration of 1,000 μM CDC (Fig. 5, A2, B2, and C2).

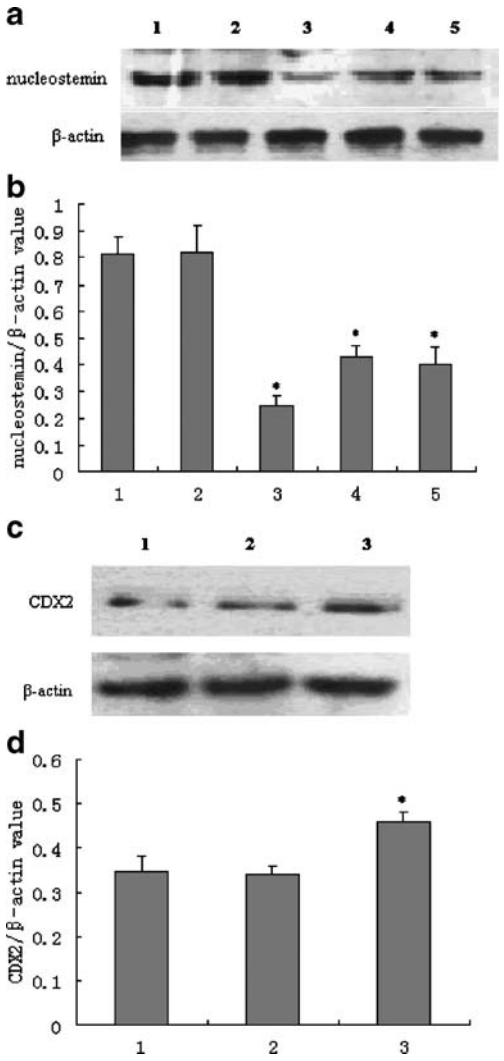


Figure 3 Western blot assay of nucleostemin protein and CDX2 protein expression in HT29 cells. **a** Nucleostemin protein expression shown in the Western blot assay. **b** Nucleostemin protein expression shown in a densitometric analysis. *1*, untransfected HT29 cells; *2*, HT29 cells transfected with *pRNAT*; *3*, HT29 cells transfected with *pRNAi-1*; *4*, HT29 cells transfected with *pRNAi-2*, and *5*, HT29 cells transfected with *pRNAi-3*. **c** CDX2 protein expression in HT29 cells, as shown in Western blot assay. **d** CDX2 protein expression shown in a densitometric analysis. *1*, untransfected HT29 cells; *2*, HT29 cells transfected with *pRNAT*; *3*, HT29 cells transfected with *pRNAi-1*. The densitometric analysis of nucleostemin protein and CDX2 protein over β-actin protein data are expressed as mean±SD of three experiments. *, $P<0.05$, compared with untransfected HT29 cells and HT29 cells transfected with *pRNAT*.

Discussion

Although there is great interest in the pathogenesis of BE, little is known regarding the mechanism of cellular metaplasia or precise cell origin of this lesion. In the present study, we found, for the first time, that nucleostemin is persistently expressed in HT29 cells but is not in biopsy specimen of human BE, and inhibition of nucleostemin expression results in the upregulation of expression of CDX2, a caudal-related homeobox gene and intestinal transcription factor essential for intestinal development or intestinal metaplasia of the esophagus.[10,11] Our results suggest that there is an association between nucleostemin and CDX2 in the development of BE. While the exact mechanisms of the interaction in esophageal cells remain to be elucidated. It is conceivable that inhibition

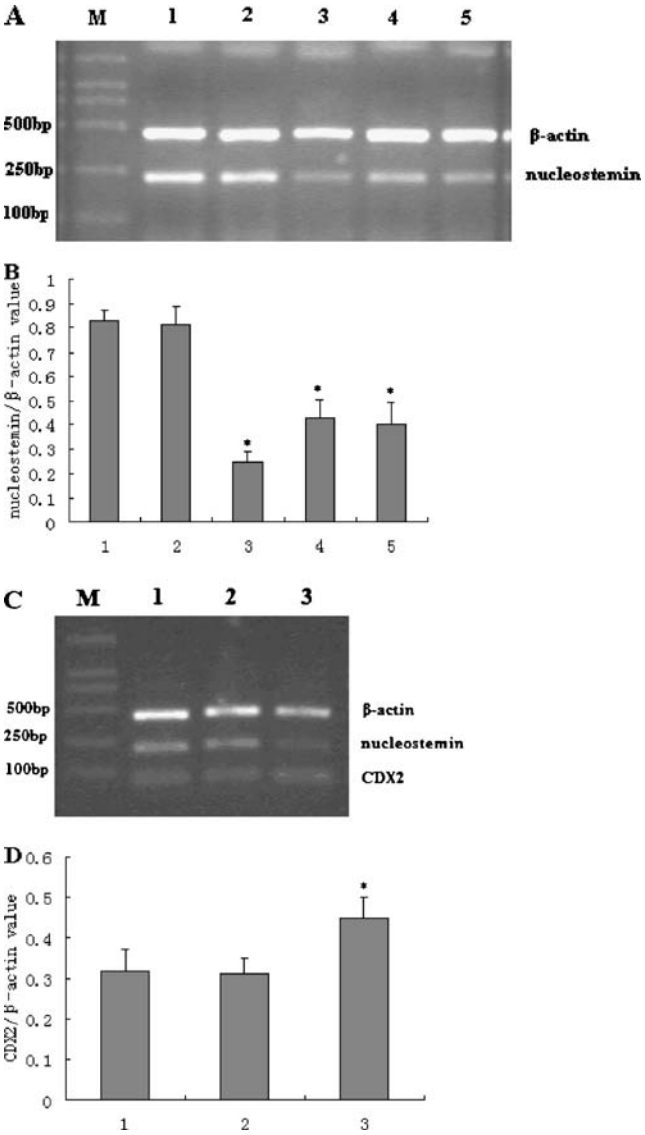


Figure 4 Expression of *nucleostemin* mRNA and *CDX2 mRNA* expression in HT29 cells as detected by RT-PCR (β-actin was used as a control). **a** *M*, DL2000 marker; *1*, untransfected HT29 cells; *2*, HT29 cells transfected with *pRNAT*; *3*, HT29 cells transfected with *pRNAi-1*; *4*, HT29 cells transfected with *pRNAi-2* (for *nucleostemin* mRNA only); and *5*, HT29 cells transfected with *pRNAi-3* (for *nucleostemin* mRNA only). **b** The densitometric analysis of *nucleostemin* mRNA over β-actin mRNA data is expressed as mean±SD of three experiments. **c** *1*, untransfected HT29 cells; *2*, HT29 cells transfected with *pRNAT*; *3*, HT29 cells transfected with *pRNAi-1*. **d** The densitometric analysis of *CDX2* mRNA over β-actin mRNA data is expressed as mean±SD of three experiments. *, $P<0.05$, compared with untransfected HT29 cells and HT29 cells transfected with *pRNAT*.

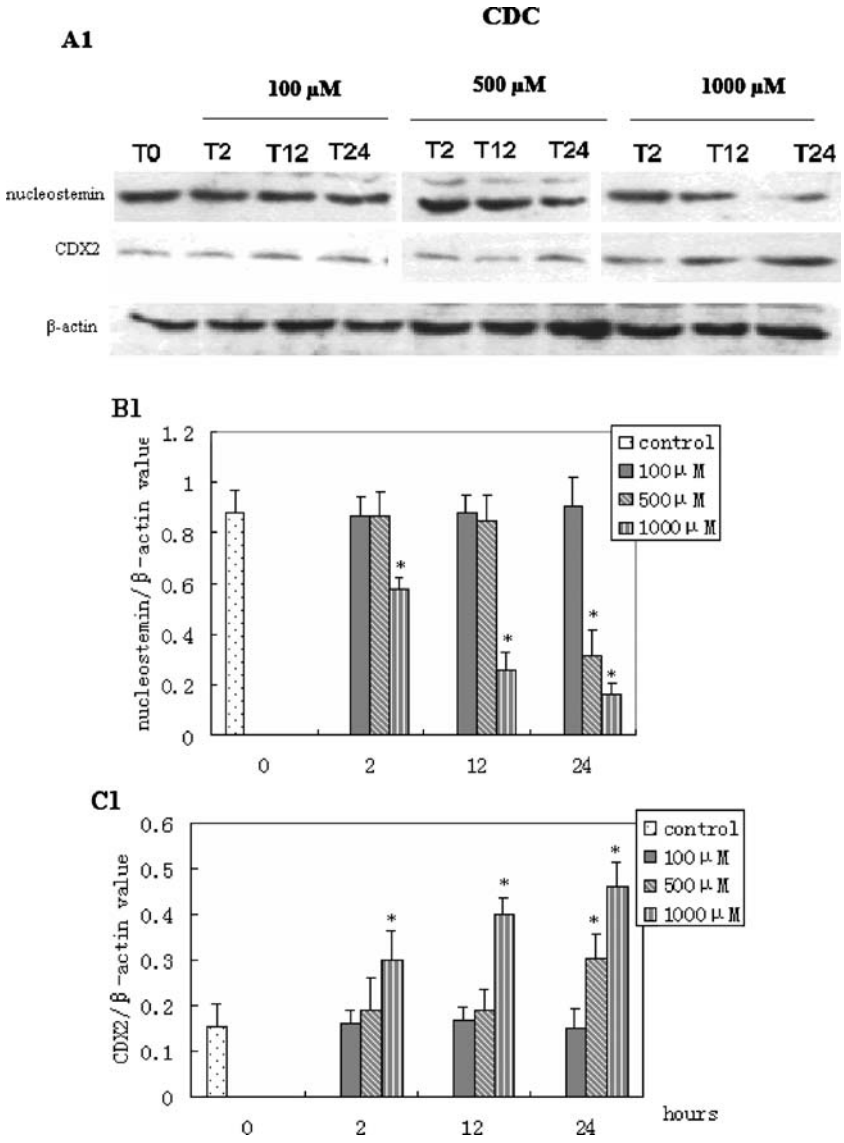


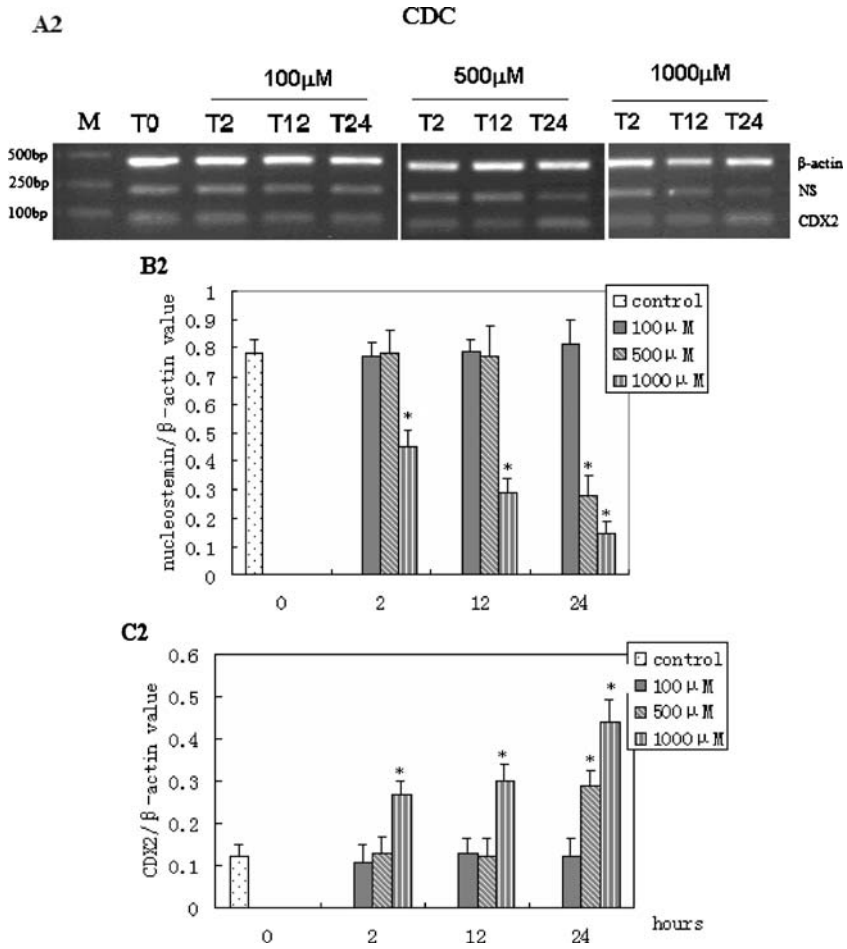
Figure 5 Effects of chenodeoxycholic acid (*CDC*) on the production of nucleostemin and CDX2 in HT29 cells as shown in Western blot assay and RT-PCR. (*A1*) After incubation with various concentrations of CDC (100, 500, or 1,000 mM) for 2, 12, and 24 h, protein (25 μg) was extracted and subjected to Western blot analysis as described in Fig. 3. (*B1*) and (*C1*) Results are expressed as the mean (SD) of three experiments. (*A2*) Effect of chenodeoxycholic acid (CDC) on the mRNA expression of *nucleostemin* and *CDX2* in HT29 cells, as detected by RT-PCR (β-actin was used as a control). *M*, DL2000; *T* indicates time points (i.e., 2, 12, and 24 h). After incubation with various concentrations of CDC (100, 500, or 1,000 μM) for 2, 12, and 24 h. (B2) and (C2) Results are expressed as the mean (SD) of three experiments. *, $P<0.05$, compared with untreated HT29 cells.

of nucleostemin expression in esophageal stem cells promotes the cells to differentiate toward an intestinal epithelial lineage by upregulating CDX2.

It has been well established that chronic gastroesophageal reflux disease (GERD) is the most important etiological factor for BE and adenocarcinoma.[12] It is widely accepted that chronic GERD leads to inflammation and ulceration of the esophageal squamous mucosa, which if persistent and recurrent, leads to columnar metaplasia and eventually to "intestinal" metaplasia. Growing evidence suggests that bile reflux is important in the etiology of BE. In animal studies, bile acids, especially in acid environments, accumulate in esophageal mucosal cells and cause cell membrane and tight junction dissolution.[13] This process allows acid and activated pepsins access to the submucosal region, precipitating more severe injury. Bile acids also increase the gastric fluid pH to 3–5, a range which promotes phenotypic differentiation of cardiac-type mucosa toward specialized intestinal-type glandular epithelium.[14] Several human studies have identified esophageal bile reflux as a risk factor for BE.[15] In addition, studies specifically investigating BE risk and duodenogastric reflux have reported a correlation between bile acid levels in refluxate and the presence of BE.[16]

The molecular and genetic events underlying the pathogenesis of BE, particularly the cell of origin, are poorly understood.[17] Stem cells are present throughout embryonic development as well as in several organs of the adult. They constitute a pool of undifferentiated cells with the remarkable ability to perpetuate through self-renewal while also retaining the potential to terminally differentiate into various mature cell types.[18] Recently, there is *in vitro* and experimental evidence to support the possibility that pluripotent stem cells may be derived from either undifferentiated mesenchymal cells in the lamina propria or the

Fig. 5 continued.



bone marrow.[19,20] Accumulating clinical and experimental studies suggest that the esophageal mucosal gland ducts harbor stem cells capable of differentiating into the columnar epithelium.[21–24] Detailed analysis of mitotic figures in the esophageal epithelium combined with immunohistochemical staining for proliferating cells has also demonstrated that cells in the flat interpapillary basal layer are candidates for esophageal epithelial stem cells.[25] There is also accumulating evidence that the squamous-to-columnar metaplastic sequence occurs through an intermediate, or transitional, phase characterized by the presence of an epithelium that shows combined squamous and columnar features, termed "multilayered epithelium.[21–26]

CDX2 is a nuclear transcription factor that has an important role in the early differentiation and maintenance of the intestinal epithelial phenotype.[27] CDX2 is specifically expressed in the small and large intestines and has been shown to activate other intestinal differentiation genes.[28,29] In normal intestinal epithelium, CDX2 is expressed in most cell lineages.[10] Squamous epithelial cells of normal human esophagus do not express CDX2, while submucosal glands weakly express CDX2 protein in the cytoplasm. In human BE, CDX2 is expressed in both goblet and nongoblet cells.[30–32] In esophageal adenocarcinoma, a high level of CDX2 expression was usually associated with well or moderate differentiation.[33,34] CDX2-mediated expression of cell adhesion proteins such as e-cadherin, LI-cadherin, and claudin-2 appears to play a role both in maintaining intestinal cell morphology and polarity.[35] Recently, CDX2 has been shown to be a useful marker of intestinal metaplasia in the diagnosis of Barrett esophagus.[36]

Because of the difficulty in establishing an appropriate culture model of esophageal stem cells, the effects of bile acids on esophageal stem cells have not been fully tested. We postulate that the nucleostemin may actually arise from stem cells and that these cells are the ones responding to bile acid exposure. To approve this hypothesis, we used the HT29 human colon adenocarcinoma cell line as an *in vitro* model for esophageal stem cells because they have the capacity to differentiate in vitro in response to changes in their extracellular environment and because in their differentiated state the polarized HT29 cells with an apical microvillus border show ultrastructural resemblance to the differentiated cell phenotype of BE.[37,38] Using these cells, we investigated the effect of CDC on the expression of nucleostemin and CDX2 in HT29 cells in vitro and found that exposure to bile

acids inhibits nucleostemin but activates CDX2 expression. In addition, our results also support that HT29 cells may serve as an in vitro model for studying the mechanism underlying the effect of bile acids or other gastroduodenal refluxate components on cellular and molecular biology of BE.

Identification of stem-cell-specific proteins and elucidation of their novel regulatory pathways may help in the development of protocols for the control of the self-renewal and differentiation of the stem cells.[39] Nucleostemin is a newly discovered nucleolar protein present in both embryonic and adult stem cells and also in several human cancer cell lines.[5] This protein is abundantly expressed while the cells are proliferating in an early multipotential state, but it almost disappears at the start of differentiation. Thus, it has been considered that it may be involved in the regulation of proliferation of these cells and can be used as a marker of undifferentiated human adult bone marrow stem cells.[6] Nucleostemin may play an essential role in the specification and/or maintenance of intestinal progenitor cells. Characterization of the zebra fish phenotype will likely provide additional insight into the functional role of nucleostemin in the intestine.[40] The fact that nucleostemin expressed in HT29 cells, but not in the differentiated cells of adult BE, suggests that HT29 cells share a common characteristics with esophageal stem cells and any factors that results in the loss of nucleostemin expression would lead to the intestinal differentiation and the subsequent development of BE. This is in agreement with observation in a study of rodent stem cells that nucleostemin expression was downregulated in mature and terminally differentiated cells, compared with their precursor neural stem cells.[5]

The key steps in the molecular pathogenesis of BE are still largely unknown. It has been shown that the intestinal transcription factor, CDX2, may play a key role in the early columnar differentiation of what are presumably the esophageal stem cells known to be present in the basal layer of esophageal epithelium.[41] In the present study, we observed that CDX2 was overexpressed in the BE biopsy tissue but weakly expressed in HT29 cells, which is consistent with previous observations.[42] Moreover, exposure to a bile acid, CDC, induced CDX2 expression in HT29 cells. These findings suggest that the activation of CDX2 in response to bile acids is associated with the pathogenesis of BE.

CDX2 expression has been reported to be regulated by phosphatase and tensin homolog deleted from chromosome 10, tumor necrosis factor α, and butyrate in colon cancer cells, such as Caco-2 and HT-29.[43,44] It has been reported that chronic acid exposure upregulated the expression of CDX2 in primary squamous epithelial cells of mouse esophagus and in cultured rat esophageal keratinocytes and human esophageal epithelial cells, and the nuclear factor kappa B (NF-κB) pathway plays a critical role in this process.[41,45,46] It has been known that bile acids upregulate both CDX2 and MUC2, a goblet cell-specific factor, in normal esophageal and cancer cell lines and activate the NF-κB and p38 MAPK pathways, which further activate CDX2 expression to regulate downstream genes.[44,47–49] In the present study, the inhibition of nucleostemin activated the expression of CDX2 in HT29 cells. All these data demonstrate that multiple regulatory factors including nucleostemin may have contributed to CDX2 activation in human esophageal epithelial cells in response to gastroesophageal reflux. The potential mechanisms of interaction would be that nucleostemin activates CDX2 promoter via NF-κB and stimulates production of CDX2 in HT29 cells, and thus we could use the mutation analysis of CDX2 promoter to identify the NF-κB binding sites that are responsible for the nucleostemin-induced activation of CDX2 in future studies.

The extracellular environment is known to play an important role in cell proliferation and differentiation. Bile acids upregulate both intestinal differentiation factor CDX2 and goblet cell-specific gene MUC2 in normal esophageal and cancer cell lines.[49] Bile-acid-stimulated expression of the farnesoid X receptor enhances the immune response in BE.[50] Results from mutation analysis of CDX2 promoter suggested that two NF-κB binding sites were responsible for the bile-acid-induced activation of the CDX2 promoter.[41] In the present study, we found that CDC exposure upregulated CDX2 gene expression and downregulated nucleostemin gene expression in a dose- and time-dependent manner in HT29 cells. These findings support the role of bile acids in the pathogenesis of BE.

Conclusion

There is an increased nucleostemin expression but decreased CDX2 expression in BE tissues. *In vitro*, inhibition of nucleostemin results in an increased expression of CDX2. In addition, CDC dose-dependently increases CDX2 production and decreases nucleostemin production in HT-29 cells. These findings suggest that the inhibition of nucleostemin expression in "esophageal stem cells" may be involved in the pathogenesis of BE through upregulating CDX2 expression. Further studies are needed to investigate whether the inhibition of nucleostemin results in the activation of the CDX2 promoter via a transcription factor binding site (e.g., NF-κB).

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