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A role for CITED2, a CBP/p300 interacting protein, in colon cancer cell invasion

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Abstract **A thorough understanding of histone acetyltransferase CBP/p300-mediated regulation of gene expression and cell growth is essential to identify mechanisms relevant to the development of histone deacetylase (HDAC) inhibitor-based preventive and therapeutic strategies. We found that knockdown of CBP/p300 interacting coactivator with glutamic acid/aspartic acid-rich tail 2 (CITED2) increased colon cancer cell invasiveness in vitro. Gene expression profiling revealed that CITED2 knockdown induced matrix metalloproteinase-13 (MMP-13) gene expression in colon cancer cells. Butyrate, a naturally occurring HDAC inhibitor, induced CITED2 expression and downregulated MMP-13 expression in RKO cells. Additionally, ectopic expression of CITED2 arrested RKO cell growth. Thus, CITED2 regulates colon cancer invasion and might be a target for HDAC inhibitor-based intervention of colon cancer.**

Keywords: Histone deacetylase inhibitor; Butyrate; Matrix metalloproteinase-13; β-catenin

1. Introduction

Colon cancer is the second leading cause of cancer death in the United States. Colorectal cancer commonly metastasizes to the liver [1], and as with most cancers, it is the metastasis that is mainly responsible for high mortality rates. Thus, elucidation of the mechanisms responsible for initiation, progression and eventually metastasis of colon cancer metastases is required for the ultimate control of this disease. Evolution of the metastatic phenotype requires enhanced cell invasiveness to enable the tumor cell to separate from the primary site and successfully establish a metastatic colony [2]. Unlike the molecular events described for the pathogenesis of primary colon tumors, the genes and pathways responsible for metastasis in these tumors have not been well characterized.

Aberrant gene expression due to epigenetic changes has been postulated to be a driving force underlying tumor progression, and histone deacetylase (HDAC) and DNA methyltransferase inhibitors-based epigenetic therapy has emerged as a reliable approach for the intervention of cancer [3]. Functional CBP/p300 is critical for transcriptional regulation of gene expression and control of cell growth [4,5]. CBP/p300 loss-of-function is associated with a variety of malignant cancers, including colon cancer [6–8]. In the human colon cancer cell line HCT116, p300 deletion leads to aggressive "cancer" phenotypes, including increased migration and invasion in vitro [9].

CBP/p300 interacting coactivator with glutamic acid/aspartic acid-rich tail 2 (CITED2) is a bifunctional protein that belongs to a family of transcriptional cofactors that is characterized by a conserved ED-rich domain at the C-terminus. A functional motif (LPXL) within this domain is necessary and sufficient for binding to the first cysteine–histidine-rich region of CBP/p300 [10]. Initially described as a corepressor of hypoxia-inducing factor 1α (HIF1α) by competing for CBP/p300 binding [11], CITED2 also functions as a coactivator of activator protein 2 (AP-2) [12], PPARα and PPARγ [13], and LIM-homeodomain protein Lhx2 [14] by recruiting CBP/p300. Loss of CITED2 in mice results in embryonic lethality – a consequence of multiple developmental defects [15,16]. Ectopic expression of melanocyte-specific gene related gene (MRG1), an alternatively spliced isoform of CITED2, results in oncogenic transformation in rat fibroblasts [17]. However, it is not clear if CITED2 functions as a tumor-promoter or suppressor. A recent study showed that knockdown of CITED2 in the breast cancer cell line MDA-MB-231 attenuates TGFβ1-mediated upregulation of matrix metalloproteinase-9 (MMP-9) and cell invasiveness in vitro [18]. This study raised the possibility that CITED2 affects tumorigenesis by modulating tumor invasion rather than proliferation. Using CITED2 specific small hairpin (sh) RNA to knockdown CITED2 expression in human colon cancer cells, we observed that CITED2 knockdown induced changes in cell morphology, concomitant with increased cancer cell invasiveness in vitro. Our results suggest a pivotal role for CITED2 in colon cancer cell growth regulation and may have important implications in targeting CITED2 for nutrition-based chemoprevention and chemotherapy for colon cancer.

2. Materials and methods

2.1. Reagents

Monoclonal antibody against $p21^{waf1}$ and rabbit polyclonal antibody against HDAC1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); rabbit polyclonal antibody against acetyl-H2A

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Abbreviations: CITED2, CBP/p300 interacting coactivator with glutamic acid/aspartic acid-rich tail 2; MMP, matrix metalloproteinase; HDAC, histone deacetylase

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was obtained from Cell Signaling (Danvers, MA); mouse monoclonal anti-CITED2 (JA22) was obtained from Novus Biologicals (Littleton, CO); and mouse monoclonal anti-β-catenin was obtained from BD Biosciences Pharmingen. A control scrambled siRNA was obtained from Ambion (Austin, TX).

2.2. Plasmids

The pMK17, which contains 598 bp of human CITED2 proximal promoter, was kindly provided by Dr. Shoumo Bhattacharya (University of Oxford). The plko1 lentiviral vector or plko1-shCITED2 which expresses shRNA targeting the human CITED2 cDNA coding region from +428 to +448 nt (BC004377) were obtained from the Open Biosystems (Huntsville, AL). The lentiviruses were produced at the University of Michigan Vector Core. The pOBT7-CITED2, which contains the full-length human CITED2 cDNA was purchased from the Origene (Rockville, MD). To generate HA-tagged CITED2 expression vector, the following primers were used to amplify full-length CITED2 cDNA using the pOBT7-CITED2 as the template. Forward: 5′-CCGACAAGCTTGCAGACCATATGATGGCAATGAACC-3′; backward: 5′-TTAAGCGTAATCTGGAACATCGTATGGGTAACAGCTCACTCTGCTGGGC-3′. The PCR fragments were digested with Hind III and inserted into the pCMV10 vector and verified by sequencing. The expression of HA-CITED2 was verified by immunoblots.

2.3. Cell culture

The human colon cancer cell line RKO was purchased from the ATCC (Manassas, VA) and cultured in minimum essential medium with 10% fetal bovine serum. To generate CITED2 knockdown stable cell line, RKO cells were transduced with lentiviral empty vector or CITED2 shRNA expressing vector and selected with puromycin at 0.1 μg/ml for 3–4 weeks. The pools of puromycin-resistant cells were used for further analyses. To generate stable cell line that expresses HA-tagged CITED2, cells were transfected with pCMV10 vector or pCMV10-HA-CITED2 vector and selected with G418 at 0.5 mg/ml for 3 weeks. The pools of G418-resistant cells were used for further analyses.

2.4. Reporter assay

Cells cultured in 48-well plates were transfected with human CITED2 reporter pMK17 using FUGENE 6 (Roche). The cells were treated with 2.5 mM sodium butyrate for 20 h prior to performing luciferase reporter assays that were normalized to protein [19]. Luciferase assay was performed on Perkin–Elmer VICTOR^3 1420 Multilabel Counter using the Luciferase Reporter Assay System (Promega).

2.5. Small interfering RNA

RNA interference experiments with small interfering RNA (siRNA) were carried out as described before [20]. The region of CITED2 cDNA targeted for siRNA was: +519 5′-AAGGTTTAACAACTCCCAGTT-3′. A scrambled siRNA (Ambion) was used as control. siRNAs were transfected into cells with Oligofectamine (Invitrogen).

2.6. RNA isolation and RT-PCR analysis

Total RNA was isolated from cells using RNeasy mini kit (Qiagen) following the manufacturer's protocol. First-strand cDNA synthesis was performed using the SuperScript® III First-strand Synthesis System (Invitrogen). The sequences of primers and amplification conditions are available upon request.

2.7. Matrigel invasion assay

Matrigel invasion assays were performed using BD Matrigel Invasion Chamber (6-well plates, 8 μm pore size, BD Biosciences). Cells were first cultured in serum free medium for 20–24 h, then collected and resuspended in medium with 0.1% BSA at a density of 2.5×10^5 cells/ml. Culture medium with 10% FBS was added to the lower chamber and 500 μl of the resuspended cells were added onto the top of the Matrigel. Forty hours later, the non-invaded cells and Matrigel on the topside of the transwell were scrapped off with cotton swab. Cells on the lower surface of the membrane were fixed with methanol, stained with haematoxylin and eosin (H&E) and viewed with Olympus BX60 microscope using SPOT software.

2.8. MMP-13 activity assay

Cells were seeded in 12-well plates at a density of 2×10^5/well. Twelve hours later, cells were washed with PBS and incubated in 0.5 mL serum-free medium for another 24 h. The conditional medium was collected, centrifuged at 10 000 rpm for 5 min; and 25 μl of the supernatant was used to detect MMP-13 activity using the SensoLyte Plus™ 520 MMP-13 Assay Kit (AnaSpec, San Jose, CA) following the manufacturer's instruction. The fluorescence signal was measured by VICTOR^3 Multilabel Counter (Perkin–Elmer) with a filter set of excitation/emission = 495 nm/535 nm.

2.9. Confocal microscopy

Cells were fixed in 3.7% formaldehyde solution for 10 min, treated with 0.1% Triton X-100 for 5 min, and incubated with Alexa fluor 488 phalloidin (Molecular Probes, Eugene, OR) for 30 min at room temperature. The cells were mounted and examined with a confocal microscope (Olympus FV-500) at the University of Michigan Microscopy & Image Analysis Lab.

3. Results and discussion

3.1. CITED2 knockdown induced morphological changes in colon cancer cells

To explore the functions of CITED2 in colonic cells, we used CITED2-specific shRNA to knockdown its expression in the colon cancer cell line RKO. As shown in Fig. 1A and B, both CITED2 mRNA and protein levels were significantly reduced by CITED2 shRNA. The expression of CITED4 was not affected (Fig. 1A), which validated the specificity of CITED2 shRNA. Cells with reduced CITED2 expression induced a flattened morphology compared to the control cells (Fig. 1C). Notably, a flattened appearance is also a feature of *CITED2* (−/−) mouse fibroblasts [21]. To determine whether the morphological changes were associated with alterations in the actin cytoskeleton, the cells were stained with phalloidin. Phalloidin staining revealed cytoskeleton reorganization in CITED2 knockdown RKO cells (Fig. 1D).

3.2. CITED2 knockdown increased colon cancer cell invasiveness in vitro

Actin reorganization is normally associated with changes in cancer cell migration and invasion [22]. Therefore, we assessed whether the morphological changes in CITED2 knockdown cells were accompanied by changes in cell migration and invasion in vitro. Wound healing assays showed that CITED2 knockdown had no affect on cell migration (data not shown). However, the Boyden chamber invasion assay demonstrated that the CITED2 knockdown significantly increased the invasiveness of RKO cells (Fig. 2). Given the importance of Wnt signaling in colon cancer progression, we examined whether the phenotypic changes in CITED2 knockdown cells were accompanied by alterations in the Wnt/β-catenin pathway. As shown in Fig. 3A, reduced levels of CITED2 had no significant affect on the subcellular localization of β-catenin in RKO cells, nor the expression of β-catenin and TCF (Fig. 3B). The lack of induction of the β-catenin pathway correlates with the lack of induction of downstream targets, e.g., cyclin D1 and c-myc (Fig. 3B). Therefore, reduced levels of CITED2 induced phenotypic changes that were independent of the β-catenin/TCF pathway.

3.3. CITED2 knockdown upregulated MMP-13 expression

To identify key genes involved in the phenotypic changes in CITED2 knockdown of the RKO cells, we performed DNA