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Knockout of NCOA5 impairs proliferation and migration of hepatocellular carcinoma cells by suppressing epithelial-to-mesenchymal transition

Jingcai He [a, b], Wan Zhang [a, b], Aimin Li [a, b], Fengsheng Chen [a, b, **], Rongcheng Luo [a, b, *]

[a] Cancer Center, Southern Medical University, Guangzhou, Guangdong, 510315, China
[b] Integrated Hospital of Traditional Chinese Medicine, Southern Medical University, Guangzhou, Guangdong, 510315, China

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ABSTRACT

Nuclear receptor coactivator 5 (NCOA5) plays important roles in the development of a variety of malignancies. However, the underlying mechanisms remain obscure. In this study, we successfully generated the NCOA5 knockout hepatocellular carcinoma (HCC) cells by CRISPR/Cas9 - mediated genome editing and found that knockout of NCOA5 inhibited the proliferation and tumor microsphere formation of HCC cells significantly. Moreover, the migration ability of NCOA5 knockout HCC cells declined. Mechanistic analyses indicated that knockout of NCOA5 can suppress the epithelial - mesenchymal transition (EMT) in HCC cells. In conclusion, our findings provide a mechanistic insight into the role of NCOA5 in HCC progression.

1. Introduction

Hepatocellular carcinoma (HCC), the dominant form of primary liver cancer [1], is the sixth most common malignancy in the world and ranks third in cancer-related causes of death [2]. Although great advancements have been made in the treatment of HCC, the outcomes of HCC remain unfavorable. The poor prognosis is primarily due to the distant metastases and recurrences [3]. Therefore, further elucidation of the molecular mechanisms involved in HCC progression is urgently required.

The nuclear receptor coactivator 5 (NCOA5), also called coactivator independent of AF2 (CIA), is known to modulate ERα-mediated transcription [4,5]. Recent studies have shown that NCOA5 is involved in tumorigenesis and prognosis of several cancers. In esophageal squamous cell carcinoma (ESCC) and papillary thyroid carcinoma (PTC) [6,7], NCOA5 low expression is associated with tumor progression, whereas NCOA5 high expression indicates unfavorable survival in luminal breast cancer, colorectal cancer (CRC) and osteosarcoma [8–10]. These results revealed that the roles of NCOA5 in cancers are context dependent. In previous study, we found that NCOA5 deficiency is a common risk factor in glucose intolerance in HCC [11]. However, the molecular mechanisms underlying the roles of NCOA5 in HCC development remain unclear.

In this study, we employed CRISPR/cas9 and generated two NCOA5-knockout hepatocellular carcinoma (HCC) cells lines. We found that knockout of NCOA5 inhibited the cell proliferation, the tumor microspheres formation and migration in HCC cells. Mechanistically, knockout of NCOA5 suppressed epithelial - mesenchymal transition (EMT) in HCC cells. In conclusion, our results demonstrate that knockout of NCOA5 suppressed EMT in HCC cells, leading to impaired cell proliferation and migration.

2. Materials and methods

2.1. Cell culture

Human HCC cell lines (Huh-7, HepG2, Bel-7402, Bel-7404, LM3, SK-Hep-1) and human immortalized liver epithelial cell (LO2) were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco, Waltham, MA, USA) supplemented with 10% Fetal Bovine Serum (Gibco, Waltham, MA, USA). All cells were maintained at 37 °C with 5% CO_2.

* Corresponding author. Cancer Center, Integrated Hospital of Traditional Chinese Medicine, Southern Medical University, Guangzhou, Guangdong, 510315, China.
** Corresponding author. Cancer Center, Integrated Hospital of Traditional Chinese Medicine, Southern Medical University, Guangzhou, Guangdong, 510315, China.
E-mail addresses: fsc0126@163.com (F. Chen), luorc02@vip.163.com (R. Luo).

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2.2. Targeting strategy with Cas9 and off target analysis

The CRISPR/Cas9 virus vector was constructed by Shanghai Genechem Co., Ltd (Shanghai, China). For targeting, 5×10^4 LM3 cells were transfected with 2 μL Cas9 - puro lentiviruses (titer: 5E+8 TU/mL) in 24-well plate, then puromycin (0.1%) was added to select positive cells after transfection for 7 days. Next, the positive cells were transfected with single guide RNAs (sgRNAs) targeting NCOA5. Sequencing was used for mutation identification. The primers used for PCR amplification and sequencing were listed in the Supplementary Table 1.

Seven potential off-target sites (POTs) for sgRNAs were predicted to analyze site-specific cleavage by the CRISPR/Cas9 system according to an online design tool (http://crispr.cos.uni-heidelberg.de). PCR products of the POTs were confirmed by sequencing. Primers for POTs determination were listed in Supplementary Table 2.

2.3. CCK8 assay and EDU

Cell Counting Kit-8(CCK8) assay was used to evaluate the rate of cell proliferation in vitro. Cells were seeded in 96-well plates at a density of 5000 cells/well, and incubated for 7 days. 10 μL of CCK8 (Dojindo, Kumamoto, Japan) was added to each well and incubated for 1 h. The absorbance value (OD) of each well was measured at 450 nm.

Proliferating cells were examined using the Cell-Light EdU Apollo 567 In Vitro Imaging Kit (RiboBio, Guangzhou, China) according to the manufacturer's protocol. In brief, cells were incubated with 10 μM EdU for 2 h before fixation with 4% paraformaldehyde, and then permeabilized by 0.3% Triton X-100 and stained by EdU. Cell nuclei were stained with 5 μg/mL DAPI (4′, 6-diamidino-2-phenylindole, Sigma, St. Louis, MO, USA) for 5 min. The number of EdU-positive cells was counted under a microscope in five random fields (× 200) to calculate the proportion of cells that incorporated EdU.

2.4. Tumor microspheres formation assay

Single cells were cultured in suspension in ultra-low adhesion 6-well plate (5000 cells/well) with serum free DMEM/F12 medium (Gibco, Waltham, MA, USA) supplemented with 20 ng/mL human recombinant EGF(Peprotech, Rocky Hill, NJ, USA), 20 ng/mL human recombinant bFGF(Peprotech, Rocky Hill, NJ, USA), 10 ng/mL recombinant Human LIF(Peprotech, Rocky Hill, NJ, USA), B27 (Gibco, Waltham, MA, USA), 500 U/mL penicillin and 500 μg/mL streptomycin(Invitrogen, Carlsbad, CA, USA). Cells were replenished with supplemented medium every second day.

2.5. Transwell and wound healing assay

For the in vitro cell migration assay, 5×10^4 cells in 100 μL DMEM medium without FBS were seeded on a fibronectin coated polycarbonate membrane and then inserted in a transwell apparatus (Costar, Corning, NY, USA). In the lower chamber, 500 μL DMEM with 10% FBS was added as chemoattractant. After incubated for 12 h at 37 °C with 5% CO_2, the upper chamber was washed with PBS, and cells on the top surface of the insert were removed with cotton swabs. Cells adhering to the lower surface were fixed with methanol, stained with Giemsa solution (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the instruction and counted under a microscope in five randomly selected fields (× 200).

4×10^5 cells were seeded in 6-well dishes and incubated overnight yielding confluent monolayers for wounding. Wounds were made using a pipette tip and rinsed softly with PBS for three times to remove the floating cells. Photographs were taken immediately (time zero) and 24 h, 48 h, 72 h and 96 h after wounding for the cells, respectively. The area of injury line was measured through Image J (National Institutes of Health) and the results were expressed as area of injury line related to 0 h.

2.6. RNA-sequencing

Total RNA was extracted with a TRIzol-based protocol. Libraries were prepared according to the instructions for the Illumina TruSeq RNA Sample Prep kit. Sequencing was performed on a MiSeq instrument. The experiments were performed at the Annoroad Gene Technology Co., Ltd (Beijing, China). Data were analyzed with RSEM software.

2.7. Data availability

RNA-Seq data has been deposited to the Gene Expression Omnibus (GEO) repository under the accession number GSE111829.

2.8. Western blots

Proteins were analyzed with SDS-PAGE and then transferred to PVDF membrane (Millipore, Billerica, MA, USA). The blots were probed with the primary antibodies against NCOA5 (Abcam, Cambridge, MA, USA), E-cad (Cell Signaling Technology, Danvers, MA, USA), N-cad (Proteintech Group, Rosemont, PA, USA) and β-Actin (CWBIO, Beijing, China), followed by HRP (horseradish peroxidase) labeled secondary antibodies (CWBIO, Beijing, China). The hybridization signal was detected using enhanced chemiluminescence (ECL) (Millipore, Billerica, MA, USA).

2.9. Statistical analysis

All the quantified data represented an average of three times. Data are represented as mean ± SD. One-way analysis of variance or two-tailed Student's t-test was used for comparisons between groups. Differences were considered statistically significant when $P < 0.05$.

3. Results

3.1. Generation of NCOA5-knockout HCC cell lines using CRISPR/Cas9

Previously, we reported that NCOA5 is a haploinsufficient tumor suppressor. Here, we examined NCOA5 expression in several HCC cell lines by western blot and found NCOA5 was highly expressed in LM3 compared to other HCC cell lines (Huh-7, HepG2, Bel-7402, Bel-7404, SK-Hep-1) and the human immortalized liver epithelial cells LO2 (Fig. 1 A). To better understand the role of NCOA5 in LM3, we inactivated NCOA5 by CRISPR/Cas9 mediated gene editing. Two sgRNAs were designed to target on exon 3 (target 1) and exon 5 (target 2) of *NCOA5* respectively (Fig. 1 B). NCOA5 knockout LM3 cells referred to as KO-1 and KO-2 cells were verified by western blot, and we confirmed that they carried a 12 bp deletion and a 1 bp deletion by genome sequencing respectively (Fig. 1C, D). To test whether off-target effects occurred in KO-1 and KO-2 cells, a total of 7 potential off-target sites (POTs) were predicted by the CRISPR design online tool (http://crispr.cos.uni-heidelberg.de). All POTs were amplified by PCR and sequenced. Sequencing results showed that no mutation was detected in these POTs (Fig. S1 A, B), indicating that the Cas9/sgRNA system did not induce undesirable off-target effects in NCOA5 knockout LM3 cell lines.

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