# miR-26a regulates mouse hepatocyte proliferation via directly targeting the 3' untranslated region of CCND2 and CCNE2

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**BACKGROUND: The deficiency of liver regeneration needs to be addressed in the fields of liver surgery, split liver transplantation and living donor liver transplantation. Researches of microRNAs would broaden our understandings on the mechanisms of various diseases. Our previous research confirmed that miR-26a regulated liver regeneration in mice; however, the relationship between miR-26a and its target, directly or indirectly, remains unclear. Therefore, the present study further investigated the mechanism of miR-26a in regulating mouse hepatocyte proliferation.**

**METHODS: An established mouse liver cell line, Nctc-1469, was transfected with Ad5-miR-26a-EGFP, Ad5-anti-miR-26a-EGFP or Ad5-EGFP vector. Cell proliferation was assessed by MTS, cell apoptosis and cell cycle by flow cytometry, and gene expression by Western blotting and quantitative real-time PCR. Dual-luciferase reporter assays were used to test targets of miR-26a.**

**RESULTS: Compared with the Ad5-EGFP group, Ad5-anti-miR-26a-EGFP down-regulated miR-26a and increased proliferation of hepatocytes, with more cells entering the G1 phase of cell cycle (82.70%±1.45% vs 75.80%±3.92%), and decreased apoptosis (5.50%±0.35% vs 6.73%±0.42%). CCND2 and CCNE2 were the direct targeted genes of miR-26a. miR-26a down-regulation up-regulated CCND2 and CCNE2 expressions and down-regulated p53 expression in Nctc-1469 cells. On the contrary, miR-26a over-expression showed the opposite results.**

**CONCLUSIONS: miR-26a regulated mouse hepatocyte proliferation by directly targeting the 3' untranslated regions of cyclin D2/cyclin E2; miR-26a also regulated p53-mediated apoptosis. Our data suggested that miR-26a may be a promising regulator in liver regeneration.**

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**KEY WORDS:** microRNA; miR-26a; gene expression; hepatocyte; proliferation; regulation

## Introduction

The healthy adult liver has enormous regenerative capacity. Under normal circumstances, adult liver cells are quiescent, and divide only one to two times a year in mice. Nevertheless, adult hepatocytes have the capacity to divide many times in response to partial hepatectomy. After 70% partial hepatectomy, hepatocytes immediately enter and progress the cell cycle by a highly synchronized method, so liver regeneration complete liver reconstruction in 7 to 10 days after 70% partial hepatectomy in rodents.[1-6] Therefore, the mice are often used as experimental models to study mechanisms of liver regeneration. Although miRNAs have been displayed to post-transcriptionally regulate gene expression that orchestrate cell proliferation in various biological events, such as cancer, their roles in liver regeneration are still unclear.

miRNAs regulate a plenty of biological events, including cell differentiation, proliferation, apoptosis, metabolism and even carcinogenesis.[7-11] It was reported that miR-26a was involved in numerous cell activities,[12-14] and especially presented an anti-proliferative property in human hepatocellular carcinoma.[13] Another study suggested that members of the miR-26a family inhibited tumorigenesis in B lymphoma cells.[15] Our previous study showed that miR-26a regulates liver regeneration after 70% partial hepatectomy in mice during liver

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regeneration,[16] but the mechanism is not completely clear. Hence it is important to study the role of miR-26a and its direct or indirect target genes in liver disease. The present study aimed to further elucidate the mechanism of miR-26a in the proliferation of mouse hepatocyte.

## Methods

### Vector construction

At first, anti- or pri-miR-26a sequences were individually introduced into a pShuttle-IRES-hrGFP-1 vector (Agilent Technologies, USA). After linearization with PmeI and pAdWasy-1 (Agilent Technologies, USA), the pShuttle-IRES-hrGFP-1 vector was recombined into a pAdEasy-IRES-hrGFP-1 vector. Then, a 293AD cell line (Cell Biolabs, San Diego, CA, USA)[17] was transfected with the pAdEasy-IRES-hrGFP-1 vector, and liquid supernatant including viral particles was isolated and collected. The viral particles including Ad5-anti-miR-26a-EGFP or Ad5-miR-26a-EGFP vector were established.

### Cell culture and transient transfection

An established mouse liver cell line, Nctc-1469 (ATCC, Virginia, USA),[18] was obtained from the SuJi biotech company (Guangzhou, China). The Nctc-1469 cells were cultured in DMEM (Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco) under a humidified atmosphere containing 5% $CO_2$ at 37 ℃. Transfections with Ad5-anti-miR-26a-EGFP ($2.5\times10^{10}$ IU/mL), Ad5-miR-26a-EGFP ($2.12\times10^{10}$ IU/mL) or Ad5-EGFP ($4.5\times10^{10}$ IU/mL) were conducted using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. All experiments repeated three times independently.

### Transfection efficiency assessment

The Ad5-miR-26a-EGFP vector was diluted to different concentrations of $2.12\times10^{10}$ IU/mL, $2.12\times10^{8}$ IU/mL, and $2.12\times10^{6}$ IU/mL with PBS, respectively. Similarly, the Ad5-anti-miR-26a-EGFP vector was diluted into $2.5\times10^{10}$ IU/mL, $2.5\times10^{8}$ IU/mL and $2.5\times10^{6}$ IU/mL, respectively. Each vector was transfected to Nctc-1469 cells. Three days later, the cells were collected, and miR-26a expression was tested by qRT-PCR. All experiments repeated three times independently.

### Cell proliferation by MTS assay

The Nctc-1469 cells were transfected with Ad5-miR-26a-EGFP, Ad5-anti-miR-26a-EGFP or Ad5-EGFP in the 24-well plates, and re-seeded in 96-well plates at a density of 1000 cells per well at 48 hours after transfection. At the indicated time points (24, 48, 72, 96, 120 hours) after re-seeding in 96-well plates, 10 μL MTS was added to the culture medium, and incubated for 4 hours. The absorbance at 490 nm of each sample was recorded by microplate reader (Thermo Fisher Scientific, USA). All experiments repeated three times independently.

### Cell cycle analysis by flow cytometry

The Nctc-1469 cells were cultured in 6-well plates, at a density of $2\times10^{6}$ cells per well, were transfected with Ad5-miR-26a-EGFP, Ad5-anti-miR-26a-EGFP or Ad5-EGFP. After 72 hours, the cells were collected and fixed with 70% ethanol for 30 minutes, and then washed with ice-cold PBS twice. The cells were spun down and re-suspended using RNase-containing PBS (1:100 in dilution) on ice before staining with propidium iodide and analyzed using a flow cytometer (FACSCalibur, BD, USA). All experiments repeated three times independently.

### Cell apoptosis analysis by flow cytometry

The Nctc-1469 cells were cultured in 6-well plates, and were transfected with Ad5-miR-26a-EGFP, Ad5-anti-miR-26a-EGFP or Ad5-EGFP. At 48, 72 and 120 hours after transfection, the cells were collected for apoptotic analysis by flow cytometry analysis. The annexin V detection kit was used to detect apoptotic cells. Data acquisition and analysis were performed using a FACSCalibur Cytometer (BD, USA). For each analysis, $1\times10^{5}$ cells were scanned. All experiments repeated three times independently.

### Western blotting analysis

Cell samples were homogenized in lysis buffer (Promega, USA), incubated for 30 minutes on ice, then centrifuged for 15 minutes at 14 000×g. All buffers were treated with a protease inhibitor cocktail (Konchem, China). Equal amounts of protein were separated on 12%-15% SDS-PAGE and transferred to a PVDF membrane (Millipore, USA). The antibodies included anti-p53 (Santa cruz, USA), and anti-GAPDH (Kangcheng, China). Immunoblots were developed using anti-rabbit-HRP secondary antibodies (Dako, CA, USA), followed by detection with immobilon Western chemilimunescent HRP substrate (Millipore, USA). GAPDH was used as a referenced gene. All experiments repeated three times independently.

### Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from prepared liver cells with Trizol (Invitrogen, Carlsbad, CA, USA). Reagent and cDNA were synthesized according to the manufacturer's protocol (MBI Fermentas). qRT-PCR was performed