Experimental Cell Research 354 (2017) 78–84

Contents lists available at ScienceDirect

Experimental Cell Research

journal homepage: www.elsevier.com/locate/yexcr

APC^{Cdh1} controls cell cycle entry during liver regeneration

Ranran Cheng[a,b,1], Xin Liang[a,b,1], Quancheng Zhao[a,b,1], Zhusheng Lian[b,c], Lichun Tang[b,c], Chen Qiu[b,c], Huan Chen[b,c,*], Pumin Zhang[a,d,**]

[a] Anhui Medical University, Hefei 230032, China
[b] State Key Laboratory of Proteomics, Beijing Proteome Research Center, Beijing Institute of Radiation Medicine, Beijing 102206, China
[c] National Center for Protein Sciences (Beijing), Beijing 102206, China
[d] Department of Molecular Physiology and Biophysics, Baylor College of Medicine, Houston, TX 77030, USA

ARTICLE INFO

Keywords:
Cdh1
Replication
Liver regeneration

ABSTRACT

Cdh1 is one of the two adaptor proteins of anaphase-promoting complex/cyclosome (APC/C), an E3 ubiquitin ligase controlling mitosis and DNA replication. To date, the *in vivo* functions of Cdh1 have not been fully explored. In order to characterize Cdh1 in liver regeneration, we generated a conditional knock-out mouse model. Our data showed that loss of Cdh1 leads to increased and extended S phase progression possibly due to the upregulation of cyclin D1. Moreover, the increased DNA replication resulted in activated DNA damage response. Interestingly, the final liver weight after partial hepatectomy in the Cdh1 depleted mice did not differ from that of the controls, implying that Cdh1 is not required for the competence of hepatocytes to regenerate itself.

1. Introduction

Ubiquitin-mediated proteolysis of cell cycle regulators is essential for cell cycle progression. SCF (Skp1/CUL1/F-box protein) and APC/C (anaphase promoting complex/cyclosome) are two main E3-ubiquitin ligase complexes that have been well studied for their role in cell cycle regulation [1–3]. The biological function and activity of APC/C depends on two related adaptor proteins, Cdc20 and Cdh1, which bind and activate the APC/C at distinct cell cycle stages [4–6]. The APC/C^{Cdc20} complex initiates the transition from metaphase to anaphase, whereas in late mitosis and the subsequent G1 phase, Cdc20 is replaced by dephosphorylated Cdh1 to form the APC/C^{Cdh1}complex triggering the ubiquitination of several cell cycle regulators (including cyclin A, cyclin B and aurora kinases) [1,2,5]. As a conserved player in G0/G1 regulation from yeast to human, the biological relevance of Cdh1 in cell cycle control has been extensively studied. In mammals, genetic ablation of Cdh1 leads to embryonic lethality [7]. In addition to its role in cell cycle control, Cdh1 is also involved in axon growth, the morphology and plasticity of synapses, and learning and memory [8–10]. A recent study have established Cdh1 as a haploinsufficient tumor suppressor [11]. Substrates of APC/C^{Cdh1} are often deregulated in tumor cells, including Skp2, Ets2 and Polo-like-kinases [11,12].

In adult animals, liver is one of the organs that constitutively express Cdh1. However, the biological role of Cdh1 in the adult liver and the involvement of Cdh1 in liver regeneration have not been determined. Liver regeneration is an orchestrated process involving cell-cycle-dependent oscillations of cyclin protein levels and multiple signal transduction pathways. After partial hepatectomy, for instance, hepatocytes undergo the transition from G0 into G1 phase almost immediately, and then enter the cell cycle within the next 24 h. The regeneration process stops when the liver regains its original size [13]. In the present study, we used conditional Cdh1 knockout mice to investigate the function of Cdh1 in liver regeneration.

2. Material and methods

2.1. Generation of Cdh1 conditional knockout mice

Mice containing the *Cdh1* sequence between two lox P sites (*Cdh1*floxed mice) were generated and genotyped as described previously [11]. Transgenic mice that carry the Cre coding sequence plus the Rosa 26 promoter were purchased from The Jackson Laboratory.

Abbreviations: APC/C, anaphase-promoting complex/cyclosome; SCF, Skp1/CUL1/F-box protein; PHx, partial hepatectomy; QRT-PCR, quantitative reverse transcription-PCR; Chk1, checkpoint kinase 1

* Corresponding author at: State Key Laboratory of Proteomics, Beijing Proteome Research Center, Beijing Institute of Radiation Medicine, Beijing 102206, China.
** Corresponding author at: Department of Molecular Physiology and Biophysics, Baylor College of Medicine, Houston, TX 77030, USA.
E-mail addresses: chenhuan@smmu.edu.cn (H. Chen), pzhang@bcm.edu (P. Zhang).
[1] These authors contributed equally to this article.

http://dx.doi.org/10.1016/j.yexcr.2017.03.038
Received 21 December 2016; Received in revised form 15 March 2017; Accepted 17 March 2017
Available online 18 March 2017
0014-4827/

To generate *Cdh1* knock-out mice, the *Cdh1* floxed mice were first crossed with the Rosa 26-Cre mice, then the resultant *Cdh1* and *Cre* heterozygotes were crossed with *Cdh1* floxed mice to produce mice that were *Cdh1* double floxed and Cre heterozygotes. All animal studies were approved by the Institutional Animal Care and Use Committee of the Beijing Proteome Research Center.

2.2. Animal model

Tamoxifen injections were performed as described previously, to induce the expression of Cre recombinase; the Cre-recombinase then catalyzed the recombination between LoxP sites to generate the *Cdh1* knockout (KO) [14]. An aliquot of 600 mg tamoxifen-free base (Sigma, Cat. No. T5648) was dissolved in 900 µl ethanol and suspended in 5.1 ml sunflower oil. For intraperitoneal injection of mice (50 mg/kg), the 100 mg/ml tamoxifen solution was diluted 2.5 times in oil. For 2/3 hepatectomy (PHx), the large left and the two median lobes were ligated and removed. In sham-operated animals, the large left and the two median lobes were exposed and replaced without any removal of tissue. For each experiment, data were obtained from 3 to 6 animals.

2.3. Western blot analysis

Homogenization of tissues was performed in RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China), supplemented with phosphatase and protease inhibitors. Then 20 µg protein per lane was loaded on a 8% and 12% gel and transferred to a PVDF membrane (Millipore Corp., Bedford, MA, USA). For detection of proteins antibodies against Cdh1 (Proteintech Group, Chicago, IL), Ets2 (CST, Cell Signaling Technology, Danvers, MA, USA), Aurora A (CST), skp 2 (CST), p27 (CST), cyclin D1 (CST) and actin (Sigma, Sigma Chemical Co., St. Louis, MO) were used.

2.4. BrdU and Edu staining

For BrdU staining, 100 mg/kg BrdU was injected intra-peritoneally 1 h before the animal was sacrificed. Tissues were fixed in 4% paraformaldehyde overnight, dehydrated, and embedded in paraffin. Then 5-µm paraffin sections were cut, rehydrated and incubated in 4N HCl for 20 min, followed by 1× Trypsin for 30 min. Unspecific binding was blocked by incubation in 5% BSA in PBST, and sections were incubated with anti-BrdU-antibody (Sigma) overnight, then incubated with secondary antibody (Jackson ImmunoResearch Lab., West Grove, PA, USA). After three washes in PBST, slides were stained with 1 µg/ml DAPI and mounted. For dual pulse labeling using EdU and BrdU (Invitrogen, Carlsbad, CA, USA), EdU was used for the first pulse and BrdU for the second pulse. EdU (50 mg/kg) was injected intraperitoneally 2 h before and BrdU (100 mg/kg) was injected 1 h before the animal was sacrificed. After the DNA denaturation step in the BrdU protocol, the cells were first labeled by click chemistry for the detection of EdU, and then subjected to an antibody labeling protocol for the detection of BrdU.

2.5. TUNEL assay

For the detection of apoptotic cells, the In Situ Cell Death Detection Kit (Roche, Indianapolis, IN, USA) was used according to the manufacturer's instructions. The apoptotic cells were detected by FITC end-labeling of fragmented DNA and were counterstained with DAPI for 2 min. Apoptotic cells were then imaged with a fluorescent microscope and counted.

2.6. Immunohistochemistry and immunofluorescence

Tissues for immunohistochemical (IHC) analysis were frozen in liquid nitrogen and embedded in OCT (Tissue-tek, Sakura Fine Tek, Torrance, CA, USA). Then 10-µm cryosections were cut and fixed in 4% paraformaldehyde for 10 min. Unspecific binding was blocked in 5% BSA in PBST for 1 h. After blocking, the primary antibodies were used: anti-pH3 antibody (CST) and γH2AX (Millipore). Secondary antibody (Jackson ImmunoResearch) was then applied after 3 washes with PBS buffer. The stained cells were observed and counted with a fluorescent microscope. For each slide, at least six areas were examined and counted.

2.7. RNA isolation, reverse transcription and real-time PCR

Total RNA was isolated using the Trizol reagent (Invitrogen) according to the manufacturer's instructions. For reverse transcription 2 µg RNA extract was reverse transcribed using the M-MLV Reverse Transcriptase Kit (Promega Corp., Madison, Wis). The SYBR Green Realtime PCR Master Mix (Toyobo Co., Ltd., Tokyo, Japan) was used for quantitative reverse transcription-PCR (QRT-PCR). Each sample was assayed in triplicate for each gene. The relative expression levels of target gene mRNA expression levels were compared to the levels of GAPDH by using the comparative cycle threshold (Ct) method. PCR primers are listed below:

GAPDH-F, 5′-AGGTCGGTGTGAACGGATTTG- 3′,
GAPDH-R, 5′-TGTAGACCATGTAGTTGAGGTCA- 3′;
Cyclin D1-F, 5′-GGAGCTGCTGCAAATGGAAC –3′,
Cyclin D1-R, 5′-GCTTGTTCTCATCCGCCTCT –3′;
Cyclin D2-F, 5′-GACAACTCTGTGAAGCCCCA –3′,
Cyclin D2-R, 5′-AACTTGAAGTCGGTAGCGCA –3′;

2.8. Statistical analysis

For statistical analysis, the Student's *t*-test for parametric variables was used (two-tailed). All tests were performed at least three times, and a P value of less than 0.05 was considered statistically significant.

3. Results

3.1. Generation of Cdh1 conditional knockout mice

It was reported that constitutive Cdh1 homozygous knockout mice results in embryonic lethality. Therefore, in order to assess the biological role of Cdh1 in the liver of adult mice, a conditional knockout model was used. Here we utilized the cre-lox system [15] to ablate the expression of Cdh1. The Cdh1 protein is encoded by the *Fzr1* gene. Mice expressing a loxP tag flanking exons 2 and 3 in the *Fzr1* allele [11] were crossed with mice expressing the Cre recombinase under the control of a Rosa 26 promoter (Fig. S1A). The Rosa 26-Cre transgenic line contains the Rosa 26 promoter- Crerecombinase flanked by two hormone-binding domains of the murine estrogen receptor. Thus, the Cre is kept inactive until induced by the injection of tamoxifen, allowing Cre-mediated recombination. Expression of the cre allele and the floxed *Fzr1* gene was confirmed using specific PCR primers (Fig. S1B). Six-week-old mice heterozygous for the Tg-Cre transgene and carrying one floxed allele and one Δ allele of Cdh1 were injected six times with tamoxifen. *Fzr1*$^{+/+}$ *Tg-Cre* mice were used as controls. First, we detected the Cre-mediated knockout of Cdh1 after tamoxifen treatment in genome level by analysis of the DNA sequence (Fig. S1C); and then confirmed the deficiency of Cdh1 by westernblot analysis. As revealed in Fig. S1D, robust reduction of Cdh1 expression was detected in the liver and kidney tissues of the Cdh1 KO mice when compared with their control littermates after tamoxifen treatment for 6 days.