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CKAP2 is necessary to ensure the faithful spindle bipolarity in a dividing diploid hepatocyte

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ABSTRACT

Spindle bipolarity is crucial for segregating chromosome during somatic cell division. Previous studies have suggested that cytoskeleton associated protein 2 (CKAP2) is involved in spindle assembly and chromosome segregation. In this study, we show that CKAP2-depleted primary hepatocytes exhibit over-duplicated centrosomes with disjoined chromosomes from metaphase plate. These cells proceed to apoptosis or multipolar cell division and subsequent apoptotic cell death. In addition, a mouse liver regeneration experiment showed a marked decrease in efficiency of hepatic regeneration in CKAP2-depleted liver. These data suggest a physiological role of CKAP2 in the formation of spindle bipolarity, which is necessary for maintaining chromosomal stability.

1. Introduction

Spindle microtubules are highly dynamic structures that continuously push and pull to align the chromosomes on the metaphase plate [1,2]. To ensure proper chromosome segregation, the spindle assembly checkpoint strictly inspects the alignment of chromatid pairs and attachment of the microtubule-kinetochore [3,4]. Previous studies have suggested that disrupting the bipolar spindle structure destabilizes chromosomes and leads to aneuploidy, which has been considered a leading cause of tumorigenesis [5–8]. In this regard, cytoskeleton associated protein 2 (CKAP2) is thought to play an important role for stabilizing chromosome.

CKAP2 is highly expressed in actively dividing cells, such as epithelial and neoplastic cells [9–11]. CKAP2 appears in spindle poles and spindle microtubules from prophase through anaphase and disappears at the end of cytokinesis [12,13]. These spatiotemporal expression patterns suggest a role of CKAP2 in mitotic spindle assembly and function [14,15]. Our previous studies showed that CKAP2 knockdown destabilizes chromosomes, whereas overexpression of CKAP2 causes microtubule bundling and monopolar mitotic spindle [14,16]. Although previous studies have revealed the function of CKAP2, the *in vitro* studies were based on established cell lines, most of which are derived from malignant or transformed cancer cells. Thus, it may be an obstacle to determine the physiological function of CKAP2 during the cell cycle. Here, to rule out the possible genetic aberrations in these established cell lines, we examined the physiological role of CKAP2 by depleting CKAP2 expression in regenerating mouse liver. Our results show that knockdown of CKAP2 caused over-duplicated centrosomes and resulted in impaired liver regeneration, suggesting that CKAP2 is necessary for the fidelity of spindle bipolarity. These data suggest a physiological role of CKAP2 in normal diploid cells and that CKAP2 could be related to centrosome function and the mitotic spindle bipolarity.

2. Materials and methods

2.1. Animals

All animal experiments followed the international instructions for experimental animal use. The experiments were performed on age-matched, male C57/BL6 mice (8 weeks old) with targeted disruption of CKAP2; their wild-type littermates were used as controls. All animals were housed in a temperature and pathogen-free environment under a constant light cycle where they had free access to water and food.

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2.2. Generation of CKAP2-shRNA or miRNA expression vector

The following three target sequences were chosen for silencing of mouse CKAP2 (NM_001004140): shRNA #5, 5′-GATGAGCAGAGATTATTTATTCAAGAGATAAATAATCTCTGCTCATCTTTTTT-3'; shRNA #7, 5′-GAGATGCGTCATATAATCATTCAAGAGATGATTATATGACGCATCTCTTTTTT-3'; and shRNA #9, 5′-GACAAGACTTCACGACTGCTTCAAGAGAGCAGTCGTGAAGTCTTGTCTTTTTT-3'; miRNA, 5′- TATGGTTGTGTTACTCCTGTC-3′. Templates for expressing each shRNA were designed, annealed together, and inserted into the siXpress-mU6 vector (Mirus, Madison, WI, USA) according to the manufacturer's instructions. A negative control Luciferase-shRNA expression vector was provided by the manufacturer. Templates for lentiviral expression of miRNA inserted into Bock-iT™ HiPerform™ Lentiviral Pol II miR RNAi Expression Systems (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

2.3. Hydrodynamic injection

The CKAP2-shRNA expression vector (20 μg) was diluted with about 2.2 ml (injection volume was 10% of mouse body weight) of hydrodynamic EE solution (Mirus) and injected into the tail vein, using a syringe with a 30-gauge needle. The DNA injection was completed within 10 s.

2.4. Partial hepatectomy

Animals were fasted for 12 h before PH. Anesthesia was induced by intraperitoneal administration of 30 mg/kg Zoletile and 10 mg/kg xylazine, and 70% of the total liver mass (left lateral and median lobectomy) was resected or sham surgery was performed as described previously [17].

2.5. Primary hepatocyte culture

Hepatocytes were harvested using two-step collagenase (Sigma) perfusion [18]. Briefly, an animal was anesthetized by Zoletil (30 mg/kg) and Rompun (10 mg/kg). Liver was perfused by sequentially injecting pre-warmed 2.5 mM EGTA pH7.4, and a digestion buffer (0.5 mg/mL collagenase type IV, 66.7 mM NaCl, 6.7 mM KCl, 50 mM HEPES pH7.6 and 4.8 mM $CaCl_2$) into portal vein. The liver was excised in Medium 199/EBSS (Thermo Scientific, Waltham, MA, USA) with 10% fetal bovine serum (Gibco, Waltham, MA, USA), 1 U/mL Penicillin/Streptomycin (Gibco) and 10 nM dexamethasone. The cell suspension was filtered through 70 μm cell strainer (BD, Franklin Lakes, NJ, USA), and the cells were pelleted and resuspended once in 5 ml of fresh medium. The cells were overlaid on 3% of pH 7.4 percoll solution and then centrifuged at 700 rpm by using Allegra 6R centrifuge (Beckman Coulter, Brea, CA, USA). The cells were resuspended in hepatocyte culture medium (Medium 199/EBSS with 10% FBS). Cell viability was assessed by trypan blue exclusion and only preparations of >95% viability was accepted. The cells were seeded at a density of 4×10^5 cells/well in a 6-well plate (Nunc, Rochester, NY, USA). The cells were maintained in a humidified incubator at 37 °C in an atmosphere of 5% CO_2.

2.6. Immunostaining

Liver tissue was fixed with 4% paraformaldehyde in PBS and embedded in paraffin. Antigen was retrieved by boiling for 20 min in 10 mM Sodium citrate, pH 6.0 containing 0.05% Tween 20. The sections were blocked for 10 min in 5% goat serum, 1% BSA in PBS containing 0.25% Tween 20 (PBST) at room temperature. Antibody dilutions were as follows: the following: mouse anti-CKAP2 (1:500), rabbit anti-Ki-67 (1:100), rabbit anti γ-tubulin (1:500), and rabbit anti-cleaved caspase 3 (1:1000), mouse anti α-tubulin (1:5000), Alexa647- conjugaed γ-tubulin (1:100), mouse anti-γ-tubulin (1:500), mouse anti-centrin 2 (1:1000) and rabbit anti-Ninein (1:100). Fluorescence dye-conjugated secondary antibodies were all used at 1:500 dilutions. Fluorescence images were acquired using an Axioplan2 microscope (Carl Zeiss, Oberkochen, Germany) equipped with an AxioCam CCD camera. Confocal images were obtained using the LSM510 META DuoScan confocal microscope (Carl Zeiss).

2.7. Time-lapse microscopy

Live cell images were obtained on a fully motorized Axiovert 200M microscope (Carl Zeiss), equipped with Axiocam HRm. The images were acquired for 24–96 h with 20 min's interval using Axiovision 4.7.2 software (Carl Zeiss). For routine and quantitative analyses, images were acquired using LD plan-Neofluar 20x/0.4 Corr Ph2 (Carl Zeiss).

2.8. TUNEL assay

Apoptotic hepatocytes were identified in mouse liver by the terminal dUTP nick end labeling (TUNEL) assay (Roche Applied Science, Penzberg, Upper Bavaria, Germany). It was performed according to the manufacturer's instructions.

3. Results

3.1. CKAP2 expression in regenerating mouse liver

To examine the temporal expression pattern of CKAP2 in regenerating mouse liver, we performed 70% partial hepatectomy (PHx) and extracted the regenerating tissues at 0, 24, 40, 48, 56, 72 h, and 7 d after surgery. It took about 1 week for the complete liver mass to be restored after 70% PHx. Mitosis occurred between 48 and 72 h after PHx, and the hepatocytes underwent two replication cycles (data not shown). CKAP2 expression level began increasing gradually from 40 to 56 h after PHx and decreased rapidly and disappeared 72 h after PHx in accordance with the Ki67 proliferation marker (Fig. 1A and B). This result indicates that the mitotic figures appeared in the regenerating liver at 40 h - 56 h after PHx.

3.2. Characterization of the shRNA expression vector targeting mouse CKAP2

We subcloned the shRNA targeting the CKAP2 vector (shCKAP2) to knockdown the CKAP2 gene expression. We also used shRNA targeting the luciferase vector (shLuc) as a nonspecific control. The plasmid vectors were delivered to the mouse liver by hydrodynamic injection through the tail vein (See Materials and Methods). The 70% partial PHx was performed at the day after injection, and the residual mouse liver was extracted at 48 h later and analyzed by Western blot. The shCKAP2 effectively reduced mouse CKAP2 protein in the regenerating mouse liver (Fig. 1C).

To understand how depleting CKAP2 affected proliferation of regenerating liver, we induced liver regeneration after injecting the shRNA vector. Liver tissues were extracted at 0, 24, 40, 48, 56, 72 h, and 7 d after PHx. CKAP2-depleted mouse liver exhibited impaired liver regeneration (Fig. 1D). To quantify mass restoration of the liver, we measured residual liver weight at respective time points (Fig. 1E). No weight differences were observed in livers between the CKAP2-depleted and control groups up to 40 h. However, the control group exhibited relatively increased liver mass 56–72 h