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Regulation of mice liver regeneration by early growth response-1 through the GGPPS/RAS/MAPK pathway

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ABSTRACT

Background & Aims: Liver regeneration (LR) consists of a series of complicated processes in which several transcription factors play important roles. Among them, the early growth response 1 gene (EGR-1) is rapidly induced in response to liver resection. Previous studies have shown that EGR-1−/− mice exhibit delayed hepatocellular mitotic progression after partial hepatectomy (PH). The mechanism underlying the EGR-1 regulated LR is still unknown. Our aim is to elucidate the underlying mechanism.
Methods: Mice infected with adenoviral vectors expressing GFP, EGR-1 or dominant negative EGR-1 (dnEGR-1) were subjected to 2/3 PH. The serum starvation recovery cell model was chosen to mimic the regeneration process for the in vitro studies. Cell proliferation and signaling pathways downstream of geranylgeranyl diphosphate synthase (GGPPS) were examined in the regenerating liver and serum starvation recovery cell model.
Results: Loss of function of EGR-1 significantly inhibited liver recovery and the expression of cyclin D1, cyclin E, and proliferating cell nuclear antigen (PCNA). The expression of GGPPS and the activity of the downstream RAS/MAPK pathway were inhibited in dnEGR-1-infected liver, which was consistent with the serum-induced cell model. In addition, loss of function of EGR-1 aggravated liver damage with increased serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels.
Conclusions: EGR-1-induced GGPPS plays a vital role in the LR after PH through the RAS/MAPK signaling.
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1. Introduction

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The liver has the unique ability to regenerate in response to liver injury with reentry of highly differentiated hepatocytes into the cell cycle stimulated by the release of growth factors and cytokines. 70% PH is the best animal model used to study regulatory mechanisms of cell growth and proliferation (Higgins and Anderson, 1931). In mice, the complete regeneration process takes approximately 7 days, during which the hepatocytes first divide within 36 to 48 h post-PH, followed by proliferation of non-parenchymal cells (NPC) (Michalopoulos, 2007). PH lead to a rapid change in gene expression and DNA-binding activity of transcription factors, such as EGR-1, CEBP/β, c-Fos, c-Myc and CREM. They increase significantly and subsequently trigger the transcription of downstream genes to regulate the regenerative process (Greenbaum et al., 1998; Haber et al., 1993; Servillo et al., 1997).

Abbreviations: LR, liver regeneration; EGR-1, early growth response 1; PH, partial hepatectomy; dnEGR-1, dominant negative EGR-1; GGPPS, geranylgeranyl diphosphate synthase; PCNA, proliferating cell nuclear antigen; AST, aspartate aminotransferase; ALT, alanine aminotransferase; NPC, non-parenchymal cells; GGPP, geranylgeranyl pyrophosphate; GFP, green fluorescent protein; siRNA, small-interfering RNA; FBS, fetal bovine serum; FCM, flow cytometer; GGTase, geranylgeranyltransferase.

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EGR-1 is an immediate-early transcription factor that can be rapidly induced by many extracellular signaling molecules. At least 80 genes are induced or repressed by the binding of EGR-1 to its target DNA sequences (Adamson and Mercola, 2002). TNF-α, Cdc20, PRL-1 and TGF-β, which are regulated by EGR-1 have been implicated in early event of hepatic regeneration (Liao et al., 2004; Pritchard and Nagy, 2005; Taub et al., 1999; Xiao et al., 2005). It has been reported that EGR-1 promoter activity and its mRNA levels increase at 48 and 72 h after 1/3 PH (Dussmann et al., 2011). Further research showed that mice lacking EGR-1 display a phenotype of delayed hepatocellular mitotic progression after 2/3 PH, characterized by an increased activation of the p38 MAPK and inhibition of hepatocellular metaphase-to-anaphase mitotic progression (Liao et al., 2004). Microarray-based gene expression analysis has shown that the expression of Cdc20 gene, a key regulator of the mitotic anaphase-promoting complex, is significantly reduced in EGR-1 null mice (Liao et al., 2004). However, the specific molecular mechanism underlying this observation is poorly understood.

GGPPS is a member of the trans-prenyltransferases family that catalyzes the formation of geranylgeranyl pyrophosphate (GGPP). Our previous work has shown that GGPPS is a target of EGR-1 and participates in the regulation of chronic insulin resistance and cigarette smoke-induced pulmonary inflammatory response (Shen et al., 2011a,b). GGPPS is used for the geranylgeranylation of RAS, Rho, RAC, and the subunit of several heterotrimeric G proteins (Matsumura et al., 2004; McTaggart, 2006). Particularly in regenerating liver, the RAS-dependent MAPK signaling cascade is important for the regulation of cell cycle and plays a key role in G1 phase progression (Kunimoto et al., 2009; Ng et al., 1992). Therefore, we hypothesized that EGR-1 may directly induce GGPPS/RAS/MAPK signaling cascade to regulate the timing of LR.

2. Materials and methods

2.1. Animals

Male C57BL/6 mice were kept under standard conditions with a 12 h light–dark cycle and were fed regular laboratory chow with free access to standard food and water. All animal protocols used were reviewed and approved by the Animal Care Committee of Nanjing University in accordance with Institutional Animal Care and Use Committee guidelines.

2.2. Replication-defective recombinant adenovirus

Human EGR-1 and dnEGR-1 cDNA were obtained as a gift from Professor J.M. Baraban. Adenovirus vectors containing a green fluorescent protein (GFP) cassette as an infection marker were constructed using the AdEasy adenoviral vector system. Recombinant adenoviruses were amplified in 293A cells and purified using CsCl gradient centrifugation. Adenovirus expressing human GGPPS small-interfering RNA (siRNA) and the scrambled sequence was constructed by Shanghai Sunbio Biomedical Technology CO., LTD, China.

2.3. Administration of adenovirus

Since the introduced gene and GFP was maximal at 3 days after administration and lasted for 1 week (Supplementary Fig. 1). Mice were subjected to tail vein injection with PBS containing 1.6×10^9 purified adenovirus particles three days before PH.

2.4. PH

8–12-week-old mice were subjected to 70% PH according to the method of Higgins and Anderson (Higgins and Anderson, 1931). After laparotomy, the left and median liver lobes were surgically removed. Mice were killed at defined times points after PH. For RNA and protein analysis, liver samples were snap-frozen in liquid nitrogen. For histological analysis, livers were fixed in 4% paraformaldehyde overnight at 4 °C and stored in 70% ethanol at 4 °C until further processed.

2.5. Histology and immunofluorescence

Fixed liver samples were embedded in paraffin and 5 μm sections were used for Hematoxylin & Eosin (H&E) staining. Hepatocyte DNA replication was assessed by immunostaining with Ki67 (Abcam). To visualize nuclei, slides were counterstained with DAPI (0.2 μg/ml). Ki67-positive cells were visualized by fluorescence microscopy. The Ki67-positive hepatocytes were counted at a minimum of 2,000 hepatocytes per sample.

2.6. BrdU incorporation assay

Mice were given intraperitoneal injection of BrdU (50 mg/g body wt, diluted in saline) (Sigma) 2 h before killed. The remnant liver was fixed, sectioned, and stained with an antibody against BrdU. The BrdU-positive hepatocytes were counted in a minimum of 1,000 to 1,500 hepatocytes per sample.

2.7. Measurement of serum AST and ALT

Serum samples were separated and stored at −80 °C until further use. The levels of serum AST and ALT were measured by Nanjing Jiancheng Biological Technology, Inc., China. Enzyme activities were expressed as unit per liter (U/L).

2.8. Cell culture

WRL-68 (Human embryonic liver cell line) was obtained from the ATCC. Primary cultured hepatocytes were isolated from male C57BL/6 mice by perfusion with collagenase as described previously (Benveniste et al., 1988). Cells were cultured with 10% heat-inactivated fetal bovine serum (FBS).

2.9. Western blotting

Protein isolated from cells or mouse liver was separated by 10–15% SDS-PAGE, transferred onto Immobilon-P membranes. Membranes were probed with primary and secondary antibodies, processed with ECL and exposed to an X-ray film.

2.10. RAS geranylgeranylation measurements

WRL-68 cells were seeded into 100 mm-dishes, infected with the indicated adenovirus, and treated with the indicated inhibitor. After lysing cells with 500 μl RIPA buffer, the lysates were centrifuged for 15 min at $12{,}000 \times g$ at 4 °C. RAS geranylgeranylation was measured as our previous work (Shen et al., 2011b). Briefly, protein was diluted to 1 mg/ml and mixed with an equal volume of 4% Triton X-114 for 5–10 min at 37 °C. The resulting aqueous upper phase contained the enriched intracellular RAS, while the organic lower phase contained the highly enriched membrane-associated RAS. Following the standard immunoprecipitation protocol, samples were subjected to western blotting analysis.

2.11. Real-time PCR

Total RNA was isolated from liver tissues using TRIzol reagent according to the manufacturer's protocol (Invitrogen) and reverse

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