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Axis of serotonin -pERK-YAP in liver regeneration

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ABSTRACT

Background and aim: Serotonin and YAP exhibit a vital role in regulating cell proliferation and wound-healing response. The aim of the study was to investigate whether 5-HT could promote liver regeneration by activating YAP.

Methods: PH models were established by WT and TPH1−/− mice. ELISA, RT-PCR, western blot, immunohistochemistry, flow cytometry and MTT assay were used to assess the level of 5-HT and YAP and proliferation after PH.

Results: We found that 5-HT level was lower in the serum and liver of TPH1−/− mice. After PH, TPH1−/− mice, lacking in 5-HT, demonstrated worse regenerative ability and suffered more severe liver injury. Additionally, YAP expression was also lower in TPH1−/− mice. Moreover, we found that YAP expression was prominent within the first three days following PH. Similarly, 5-HT could promote cell proliferation by upregulating YAP expression in L-O2 cells. As predicted, using YAP-siRNA sharply reduced the proliferative capacity mediated by 5-HT. Further study also indicated that ERK participated in the regulation of YAP induced by 5-HT. By using an ERK inhibitor, the YAP expression and cell proliferation induced by 5-HT were both suppressed. Although YAP-siRNA was used to block YAP expression, pERK and ERK expression were not affected. Taken together, these data showed that 5-HT contributed to liver regeneration by regulating YAP expression, which at least in part, was by activation of pERK.

Conclusion: A role of the 5-HT-pERK-YAP axis in liver regeneration emerged from our study and might be a potential target to promote regeneration and injury repair.

1. Introduction

Liver is a unique organ that has the potential to fully recover function in response to injury or surgical resection [1]. This strong regenerative capacity of the liver makes it possible to remove up to a maximum of 70% of this organ during hepatectomy [2]. Remarkably, the residual liver can regenerate its volume in a few months. Liver regeneration is a complex process that involves a large number of cytokines, growth factors, and several pathways.

5-HT, which is mainly stored and distributed in platelets outside the central nervous system, acts as a vital mediator in physiological and pathological functions [3]. Landmark studies have confirmed the critical role of platelet-derived 5-HT in liver regeneration after PH in mice [4–6]. A delayed regenerative response was proved to be associated with a deficiency in circulating 5-HT in mice [7]. In addition, a mitogenic effect of 5-HT on hepatocytes in vitro was demonstrated in several studies [8,9].

The Hippo signaling pathway has been implicated in controlling liver size partly through regulating the activity of the transcriptional coactivator—YAP [10]. Suppression of the Hippo pathway induces liver overgrowth and tumorigenesis by increasing YAP expression and location of YAP to the nucleus [11]. On the contrary, YAP can be phosphorylated as p-YAP, an inactivated form of YAP, localized to the cytoplasm with the activation of the Hippo pathway [12]. In addition, our previous findings have revealed that 5-HT can promote hepatoma cell proliferation through activating YAP by upregulating pERK level [13]. Thus, our study gave a new insight into the molecular mechanisms by which 5-HT affected the malignant biological behavior of hepatoma

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cells via the 5-HT-5-HT_{2B}R-pERK-YAP axis. Although liver has the ability to regenerate, only a few studies are available describing the mechanisms of regeneration, especially the effects of serotonin and YAP on the regeneration process.

In this study, we investigated whether 5-HT could promote liver regeneration by activating YAP, and the underlying mechanisms. We used the murine model of PH based on $TPH1^{-/-}$ mice, which lacked of 5-HT, to assess the impact of platelet-derived 5-HT on liver regeneration in vivo. Additionally, the L-O2 cell line was used to explore the mechanisms of liver regeneration. Our findings demonstrate that the 5-HT-pERK-YAP axis promotes liver regeneration in vivo and in vitro.

2. Materials and methods

2.1. Animals and partial hepatectomy model

Male wild-type C57BL/6 mice (4–5 weeks old, 20–25 g, normal in 5-HT) were obtained from the Animal Breeding Center of Central South University. The $TPH1^{-/-}$ mice (C57BL/6 background, 4–5 weeks old, 20–25 g, lack of 5-HT) were kindly provided by Professor Jingyao Zhang of the First Affiliated Hospital of Xi'an Jiaotong University. All mice were housed in a controlled environment in pathogen-free cages with 12 h light-dark cycles and constant temperature. Mice were caged three to a cage as standard practice and were adapted to the environment for 7 days before use. The standard diet and pure water were provided ad libitum for all mice. Animal care was conducted according to the Guide for the Care and Use of Laboratory Animals enacted by the US National Institutes of Health. The study was approved by the Animal Research Committee of Center of Central South University.

The animals were subjected to a 70% PH as previously described [14]. In brief, the median and left lateral hepatic lobes of the mice were resected to build the 70% PH model. The mice were then sacrificed at 1st, 2nd, 3rd and 7th day (5 animals per time point per group). Body weight and remnant liver weight were measured. Synchronously, blood samples and liver specimens were collected for further tests.

2.2. Blood examination

Blood samples were taken from the inferior vena cava at different time points and were centrifuged at room temperature at 1610 ×*g* for 3 min. Then the obtained serum was analyzed via standard laboratory techniques in the clinical laboratory of the second Xiangya Hospital to assess the ALT (alanine aminotransferase) and AST (aspartate transaminase) levels. The level of 5-HT in the serum and liver tissues was measured by a 5-HT ELISA Kit according to the manufacturer's protocol (Abnova, Taiwan, China).

2.3. Assessment of BrdU (5-bromo-2′-deoxyuridine)

Mice were injected intraperitoneally with 100 mg/kg BrdU (Sigma-Aldrich, St. Louis, USA) dissolved in PBS (phosphate buffer saline), 2 h before being humanely killed. After euthanasia, the liver samples were fixed in formalin and embedded in paraffin, sliced into serial of 4 μm thicknesses, and then, samples were incubated with the mouse anti-BrdU antibody (1:200, Abcam). BrdU-positive cells were counted across five random fields at 200× magnification.

2.4. Immunohistochemistry

Liver tissue sections were incubated with Ki-67 (ab15580, 1:500, Abcam), BrdU (ab6326, 1:200, Abcam) or YAP (ab205270, 1:500, Abcam) antibodies using a streptavidin peroxidase-conjugated method. Evaluation of the immunohistochemical staining was assessed by two independent pathologists who unware of the treatment groups. The assessment criteria were based on the percentage of positive cells and staining intensity as well as the final score for each microscopic field [15]. The slides were photographed in five random fields under an inverted microscope.

2.5. Western blot assay

Protein was extracted from L-O2 cell lines and liver tissue lysates with proteinase inhibitors and phosphatase inhibitors according to the manufacturer's instructions. The procedure of the western blot assay was similar to that previously reported [16]. β-Actin was used as an internal reference. The following antibodies were used for western blot assay: anti-YAP (ab205270, 1:1000, Abcam), anti-p-YAP(#4911, 1:1000, Cell Signaling Technology); anti-CTGF (ab6992, 1:1000, Abcam), anti-β-Actin (60008-1-Ig, 1:5000, Proteintech), anti-cyclin E (ab71535, 1:2000, Abcam), anti-Cdk2 (ab32147, 1:3000, Abcam), anti-p27 (ab62364, 1:1000, Abcam), anti-p21 (ab109199, 1:1000, Abcam), anti-p44/42 MAPK (ERK) (16443-1-AP, 1:2000, Proteintech), anti-phospho-p44/42 MAPK(ERK1/2) (#4370, 1:2000, Cell Signaling).

2.6. Cell culture

The human immortalized normal hepatocyte cell line (L-O2) was purchased from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Hyclone, South Logan, UT, USA) containing 10% fetal bovine serum (Gibco, Grand Island, NY, USA) at 37 °C in 95% air and 5% CO_2 as described previous [17]. Based on our different set of experiments, L-O2 cells were exposed to the following culture conditions. The cells in control group were culture in serum-free medium. The cells in 5-HT group were cultured in serum-free medium containing 100 μM 5-HT. The cell in 5-HT + YAP-siRNA group were transfected with YAP-siRNA and then cultured in serum-free medium containing 100 μM 5-HT. The cells in 5-HT + PD032 group were pre-treated in the medium containing 1uM PD0325901 for 30 min before addition of 100 μM 5-HT. All the group were cultured for 12 h after administration to assess the indicators of liver regeneration. Before administration, the cells were incubated with DMEM without FBS for synchronization.

2.7. siRNA transfection

Small interfering RNA (siRNA) oligonucleotides against YAP and control siRNA were designed and synthesized by the GenePharma Corporation (Shanghai, China). L-O2 cells were cultured in serum free DMEM for 24 h and transfected with a mixture of siRNA using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA). 6ul Lipofectamine 2000 dissolved in 300ul DMEM and 24ul YAP-siRNA dissolved in 300ul DMEM were mixed. And the mixture was added into a 6 well culture plate which already had 2.4 ml DMEM in each well. The culture medium was changed after 8 h of transfection. And after treatment for another 12 h, the knockdown efficiency was determined by RT -PCR and western blot analysis. Three independent transfection experiments were performed. The siRNA sequences used were as follows:

YAP-siRNA #1 5′-CCACCAAGCUAGAUAAAGA-3′;
YAP-siRNA #2 5′-GAACAUAGAAGGAGAGGAG-3′;
Control siRNA 5′-GCAAGCUGACCCUGAAGUUCAU-3′.

2.8. Quantitative real-time PCR

Total RNA from L-O2 cells was extracted by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The procedure was performed as described previously [18]. Fold changes in mRNA expression were calculated by the relative quantification method ($2^{-\Delta\Delta Ct}$). The following primer sequences were used:

YAP, (forward) 5′-GCTACAGTGTCCCTCGAACC-3′ and (reverse)