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Upregulation of hepatic melanocortin 4 receptor during rat liver regeneration

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ABSTRACT

Background: Melanocortin 4 receptor (MC4R) is predominantly recognized to mediate energy metabolism and anti-inflammation through the central nervous system. However, the expression of MC4R has recently been identified in rat liver and was shown to be upregulated during acute phase response. This study aims to investigate potential roles of MC4R in liver regeneration.

Materials and methods: Rat partial hepatectomy (PH) was performed, and MC4R expression was analyzed at different time points after resection. Sham-operated animals (SH) served as controls. *In vitro* primary hepatocytes (HCs) were isolated from normal rat liver and stimulated with α-melanocyte–stimulating hormone (MC4R agonist). Real-time polymerase chain reaction, Western blot, and immunofluorescence staining were applied to detect gene expression.

Results: Up to 8 h after PH, hepatic messenger RNA of proinflammatory cytokines interleukin 6 and tumor necrosis factor α reached peak values. Between 8 and 72 h after PH, rat liver regeneration was extremely active as assessed by the regeneration indices labeled by Ki-67. Immunofluorescence staining indicated that MC4R was mostly expressed in hepatocyte nuclear factor 4^+ cells (HCs) and upregulated during rat liver regeneration. Concurrently, the expression of hepatic MC4R protein was significantly higher in PH than in SH animals, and phosphorylated extracellular signal–regulated kinase 1/2 was remarkably increased in PH compared with SH animals ($P < 0.05$, respectively). *In vitro* experiments showed that the expression of proliferating cell nuclear antigen was significantly higher in HCs treated with α-melanocyte–stimulating hormone than in control HCs, which was correlated to the increase of phosphorylated extracellular signal–regulated kinase 1/2 and reduction of phosphorylated signal transducer and activator of transcription 3 ($P < 0.05$, respectively).

Conclusions: MC4R is predominantly expressed in HCs and upregulated during rat liver regeneration. *In vitro* stimulation of HC MC4R is associated with a modulation of extracellular signal–regulated kinase and signal transducer and activator of transcription 3 pathways regulating liver regeneration.

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Introduction

Melanocortin receptor 4 (MC4R), a G-protein–coupled receptor, plays a pivotal role in the regulation of energy homeostasis and anti-inflammation [1]. MC4R is mechanistically regulated by α-melanocyte-stimulating hormone (α-MSH, MC4R agonist) and agouti-related peptide (AgRP, MC4R inverse agonist), both of which are derived from proopiomelanocortin [1]. MC4R is conventionally considered to be expressed in the central nervous system and exerts its biological effects to peripheral organs [2]. However, recent studies identified the expression of MC4R in liver and adipose tissues as well [3,4].

Genes involved in direct energy generating processes, such as oxidative phosphorylation, electron transport, and adenosine triphosphate synthesis, were found upregulated in both liver and adipose tissue of α-MSH-treated pigs homozygously expressing missense mutations in MC4R [4]. Interestingly, in mice, central administration of a MC4R antagonist (SHU9119) or AgRP robustly increased feeding behavior, indicating that antagonism of MC4R is an important orexigenic signal [5]. Knockout of MC4R and proopiomelanocortin [6] or overexpression of AgRP [7] led to the same obese phenotype in mice. A similar genetic pattern was also observed in humans with mutations in MC4R [8]. These reports highlighted the crucial effects of MC4R in energy metabolism.

It was reported that α-MSH prevented lipopolysaccharide-induced hepatic inflammation by inhibiting production of chemokines, which then modulated the infiltration of inflammatory cells [9]. Moreover, activation of MC4R attenuated cerebral, myocardial, testicular and renal inflammation and ischemia–reperfusion injury through regulating extracellular signal–related kinase (ERK) 1/2, tumor necrosis factor α (TNF-α) and so forth, consequently trigging repair pathway [10–13]. Interestingly, gene expression of all MCR subtypes was recently discovered in rat liver, and the level of MC4R was most dramatically increased during acute phase response [3]. However, the precise mechanism of how MC4R exerts anti-inflammatory effects and interacts with other biochemical processes is not well defined yet.

Liver regeneration after partial hepatectomy (PH) is mostly dependent on the replication of hepatocytes (HCs), which are completely differentiated and normally quiescent cells, and do not rely on the activation of a compartment of hepatic stem cells [14]. Tremendous genes are involved in liver regeneration, and the essential circuitry required for the process could be categorized into three networks: metabolic (e.g., Heparin-binding epidermal growth factor and amphiregulin), cytokines (e.g., interleukin 6 and TNF-α), and growth factors (e.g., hepatocyte growth factor, and epidermal growth factor) [15].

Based on the best of present knowledge, MC4R is involved in metabolic and inflammatory processes, so we hypothesized that MC4R could play an important role in the proliferation of liver cells after PH. The present study investigates the MC4R expression pattern in regenerating rat liver after 2/3 PH and its interaction with other signaling pathways being involved in regulation of liver regeneration.

Materials and methods

Chemical reagents

The following materials were used in this study: Moloney murine leukemia virus reverse transcriptase, Promega, Mannheim, Germany; SYBRGreen master mix and stepOne software, Applied Biosystems, Darmstadt, Germany; Complete Protease Inhibitor Cocktail Tablets, Roche, Mannheim, Germany; Hybond enhanced chemiluminescent (ECL) nitrocellulose membranes; Amersham Biosciences, Buckinghamshire, UK. ECL solutions A and B Western blotting protocol, GE Healthcare (Braunschweig, Germany); Film processor machine, Konica SRX-101A, medical film processor; 4, 6-diamidino-2-phenylindole was from Molecular Probes Europe BV, Leiden, The Netherlands; Goat and rabbit serum, Dako, Glostrup, Denmark, and α-MSH, Tocris Bioscience, MO.

Animals and experimental design

Male Wistar rats, purchased from Harlan-Winkelmann, Germany (body weight 180–200 g), were used for the experiments and were housed at a room temperature of 22°C–24°C and a relative humidity of ~65% with a 12-h light–dark cycle for 1 wk for allowance of acclimatization before the start of the experiments. The animals were kept on standard laboratory chow until 12 h before surgery and fresh tap water *ad libitum* till the beginning of the experiments. All experiments were conducted in accordance with the German legislation on protection of animals and the NIH Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council) and were approved by the local governmental ethical committee.

2/3 PH or sham (SH) operation was performed under ether anesthesia by midventral laparotomy, ligation of the median anterior and left lateral hepatic lobes separately with a silk suture, and complete excision of ligated lobes. SH operations consisted of a midventral laparotomy of similar extent, gentle manipulation of the liver, followed by surgical closure of the abdominal wall similar to PH-operated rats.

Rats were sacrificed at 0, 2, 4, 8, 16, 24, 48, 72 h and 1 wk after PH or SH (three rats per time point). Livers were snap frozen in liquid nitrogen and stored at −80°C until use.

Isolation of HCs and treatment with α-MSH

Rat primary HCs were isolated from healthy, non-hepatectomized rats according to the method previously described [16]. The isolated cells were exposed to 10 μg/mL α-MSH in M199 culture medium with 1.5% antibiotics. HCs were then harvested in all *in vitro* experiments at 1, 6, and 24 h after treatment for protein study. Saline-treated cultured HCs served as negative controls at all studied time points. The entire experiment of HCs isolation and Western blots were independently repeated three times.

Isolation of total RNA and real-time polymerase chain reaction

Total RNA was isolated from rat livers and converted into complementary DNA by reverse transcriptase for real-time

polymerase chain reaction (RT-PCR). β-actin was used as housekeeping gene. All samples were assayed in duplicate. Sequences of the used primers were as follows: **β-actin** 5′-3′ Forward ACC ACC ATG TAC CCA GGC ATT, 5′-3′ Reverse CCA CAC AGA GTA CTT GCG CTC A; **MC4R** 5′-3′ Forward CAC AGT ATC GGG CGT TCT TT, 5′-3′ Reverse GTA ATT GCG CCC TTC ATG TT; monocyte chemotactic protein 1 (**MCP-1**) 5′-3′ Forward AGG CAG ATG CAG TTA ATG CCC, 5′-3′ Reverse ACA CCT GCT GCT GGT GAT TCT C; **TNF-α** 5′-3′ Forward ACA AGG CTG CCC CGA CTA T, 5′-3′ Reverse CTC CTG GTA TGA AGT GGC AAA TC; **IL-6** 5′-3′ Forward GTC AAC TCC ATC TGC CCT TCA G, 5′-3′ Reverse GGC AGT GGC TGT CAA CAA CAT.

Protein extraction and Western blot analysis

Stored liver tissue samples and primary cultured HCs were lysed in lysis buffer containing 0.50 M Tris-HCl pH 7.6, 150 mM NaCl, 10 mM EDTA, 1% (vol/vol) Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 10 μL/mL cocktail proteases inhibitors. After subsequent processing, the protein concentration was determined using the Coomassie method. Fifty microgram protein of total hepatic or HCs lysate was loaded in wells of 4%–12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and separated by electrophoresis followed by protein transfer onto a nitrocellulose membrane according to the manufacturer's instructions. Immunodetection was performed according to the ECL-Western blotting protocol. Primary antibodies against MC4R (Abcam, ab24233), proliferating cell nuclear antigen (PCNA; Dako, M0879), phosphorylated ERK (pERK; Cell Signaling (Frankfurt, Germany), CST9101), ERK (Cell Signaling, CST9102), phosphorylated signal transducer and activator of transcription 3 (pSTAT3; Cell signaling, CST 9131), STAT3, (Cell signaling, CST 9132), and β-actin (Sigma-Aldrich (Steinheim, Germany), A5441) were used in this study.

Immunofluorescence staining and cell counting

Five-micrometer cryostat sections were prepared, air-dried, fixed with methanol and acetone (−20°C, 10 min). Subsequently, the slides were blocked with goat serum and were subjected to the following primary antibodies overnight at 4°C: rabbit anti-MC4R (Abcam, ab24233), mouse anti-ED-1/CD68 (Serotec (Puchheim, Germany), MCA341), mouse anti-CK19 (Novacastra (Wetzlar, Germany), B170), rabbit antihepatocyte nuclear factor(HNF) 4α (Santa Cruz (Heidelberg, Germany), SC8987), mouse anti-Ki-67 (Dako, M7248), and mouse anti Desmin (Dako, M0760). After immerse with secondary antibodies, the slides were counterstained with 4, 6-diamidino-2-phenylindole and analyzed using an epifluorescence microscope (Zeiss, Goettingen, Germany). Alongside, negative controls were established by replacing the primary antibodies with phosphate buffered saline. Using an epifluorescence microscope, five random high-power fields (200-fold magnification) were captured per section. The numbers of all HCs and Ki-67-positive cells per high-power field were counted, and the regeneration indices, defined as the percentage of Ki-67-positive HCs, were calculated.

Statistical analyses

The RT-PCR and Western blot data were statistically analyzed using Graph Pad Prism 4 software (San Diego; CA). Data are shown as means ± standard error of mean. Student t-test was used to compare between groups, significant difference was accepted at $P < 0.05$.

Results

Increased expression of MC4R, TNF-α, IL-6, and MCP-1 messenger RNA in remnant rat liver

By using RT-PCR analysis, the messenger RNA (mRNA) levels of MC4R, TNF-α, IL-6, and MCP-1 were studied. Compared with SH group, hepatic MC4R mRNA expression was significantly increased at 8 and 16 h after PH (Fig. 1A, $^*P < 0.05$). Hepatic mRNA levels of TNF-α and IL-6 were similarly upregulated at 4 and 8 h in PH group compared with SH group (Fig. 1, B and C; $^*P < 0.05$, respectively). In addition, the hepatic mRNA level of MCP-1 (a small cytokine secreted by monocytes, macrophages, and dendritic cells) was significantly elevated at 16 and 24 h after PH compared with SH-operated animals (Fig. 1D; $^*P < 0.05$, respectively).

MC4R expression pattern and dynamics after PH

It was reported that HNF-4α was stably expressed in HCs, and there was no significant change during rat liver regeneration. Therefore, HNF-4α was used as a marker for HCs [17]. To explore the localization of MC4R in rat liver, HNF-4α and MC4R antibodies were used to apply double IF staining. As shown in Figure 2A, MC4R protein was mostly expressed in HNF-4 positive cells. When comparing SH-operated and partially hepatectomized animals, we found that MC4R expression was obviously increased at 24 h after PH. Cytokeratin 19 (CK19), which was found in a large number of epithelial cell types [18], has been used as a marker for biliary epithelial cells [19]. Double IF staining of MC4R and CK19 showed that the expression of MC4R was rarely found in CK19 positive cells during rat liver regeneration (Fig. 2B). Double IF staining of MC4R and ED-1 (marker for macrophages) [20] exhibited a low expression of MC4R in ED-1 positive cells (Fig. 2C). MC4R expression was also not seen in desmin positive cells (Fig. 2D). In short, here we demonstrated that the expression of MC4R after PH was mainly in the HCs.

Coexpression of Ki-67 and MC4R after PH

As shown in Figure 3A, expression of Ki-67 was mostly found in MC4R positive cells. However, not all MC4R positive cells expressed Ki-67. Quantitative analysis of double-stained immunofluorescent tissue sections revealed that regeneration index was significantly increased in partially hepatectomized animals after 16 h until 1 wk after surgery compared with SH-operated animals (Fig. 3B, $^*P < 0.05$, respectively). Western blotting analysis for PCNA confirmed the results of regeneration index (data not shown).

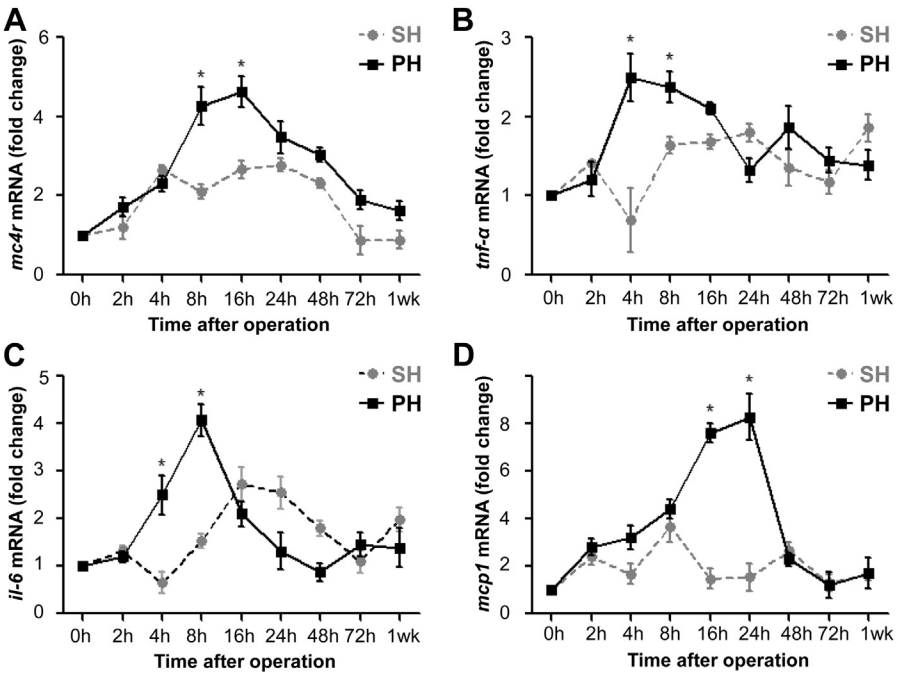

Fig. 1 – Hepatic mRNA content of (A) MC4R, (B) TNF-α, (C) IL-6, and (D) MCP-1 at different time points after PH (black squares) or SH operation (gray circles). $n = 3$ for each time point. Data are presented as mean ± standard error of mean, fold change was calculated from the normalized mean value of 0 h. *$P < 0.05$ *versus* SH.

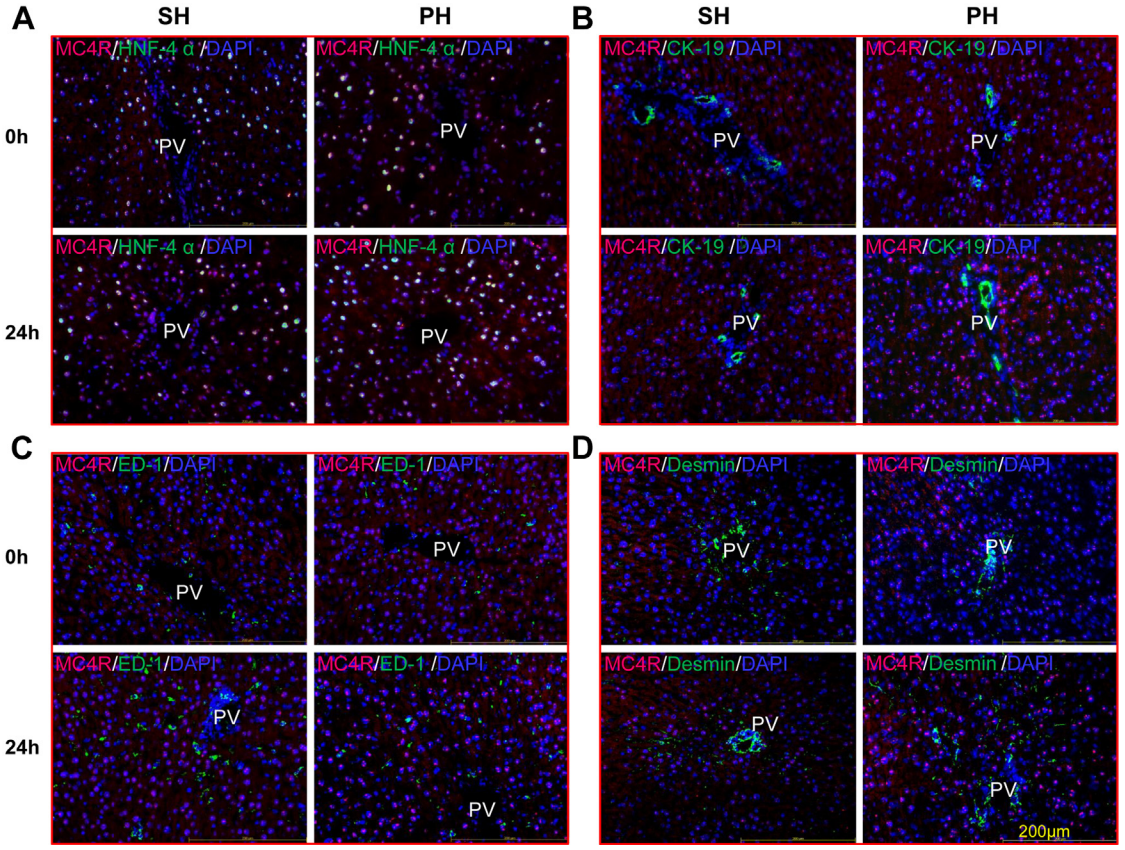

Fig. 2 – Representative microphotographs of merged pictures for MC4R (red) with (A) HNF-4α, (B) CK19, (C) ED-1 and (D) desmin (green) in SH and PH rat livers at 0 (upper panel) and 24 h (lower panel) after surgery, respectively. (Nuclei were stained by 6-diamidino-2-phenylindole [×200]. PV: portal vein).

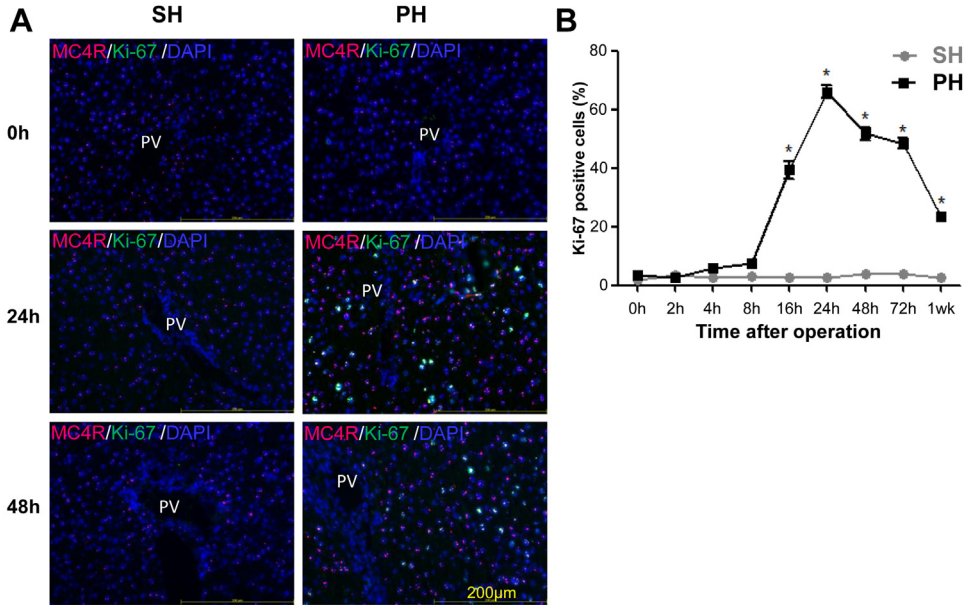

Fig. 3 – (A) Representative microphotographs of merged pictures for MC4R (red) and Ki-67 (green) in rat livers at 0, 24, and 48 h after surgery. (Nuclei were stained by 6-diamidino-2-phenylindole [×200]. PV: portal vein). (B) Quantitative analysis of regeneration indices in SH and PH rat livers as calculated by the percentage of Ki-67-positive HCs. $n = 3$ for each time point. Data are presented as mean ± standard error of mean, *$P < 0.05$ *versus* SH.

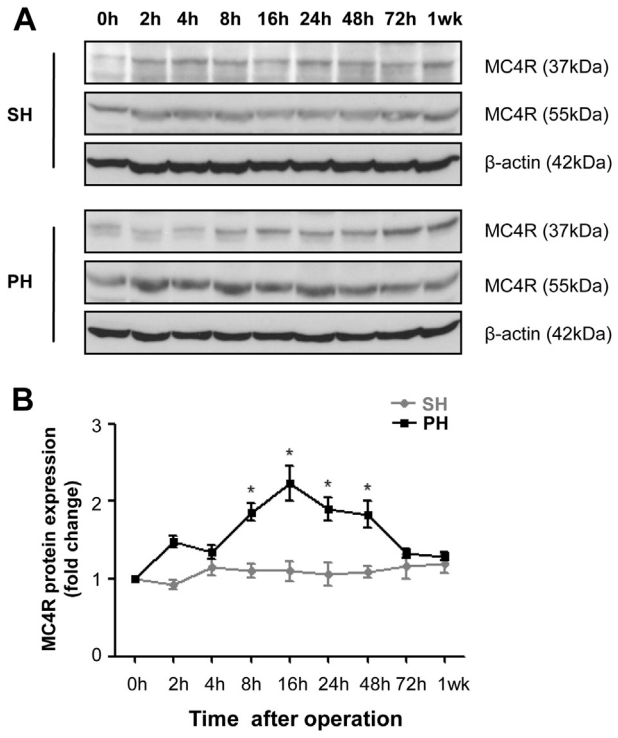

Fig. 4 – (A) Representative Western blots for MC4R protein expression in rat livers after SH (upper panel) and PH (lower panel), normalized to β-actin. (B) Quantitative densitometric analysis of the MC4R protein expression. Data are presented as mean ± standard error of mean. *$P < 0.05$ *versus* SH.

Correlation between hepatic MC4R and pERK protein levels in regenerating rat liver

Relative protein levels of MC4R and pERK were analyzed by Western blotting. As shown in Figure 4, levels of MC4R were significantly increased in PH group between 8 and 48 h after surgery compared with SH group (*$P < 0.05$, respectively). Basically in accordance with the changes of MC4R expression, levels of pERK were significantly elevated in PH group 8 h after PH compared with SH group (Fig. 5, *$P < 0.05$, respectively) underlining other's findings on a MC4R-dependent ERK pathway activation [21].

In vitro *effects of α-MSH stimulation on PCNA, pERK, and pSTAT3 protein expression in isolated primary HCs*

Since in normal and regenerating rat liver, MC4R was predominantly expressed in HCs, isolated primary rat HCs were stimulated with α-MSH to examine potential effects on signaling pathways involved in liver regeneration.

As shown in Figure 6, no significant change of PCNA level in HCs was found at 1 h after stimulation with the MC4R agonist. However, the amount of PCNA protein was found significantly increased at 6 and 24 h after α-MSH stimulation compared with control group (Fig. 6B; *$P < 0.05$, respectively), exhibiting that the MC4R agonist provided a proliferative effect on HCs. Correspondingly, expression of pERK was significantly elevated at 6 and 24 h after treatment with the MC4R agonist compared with control groups (Fig. 7A and B, *$P < 0.05$). Inversely, the expression of pSTAT3 was significantly lower at

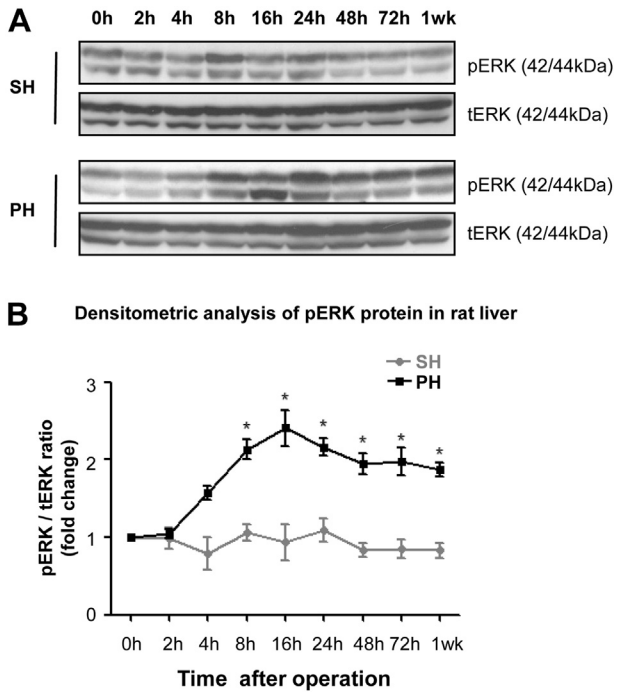

Fig. 5 – (A) Representative Western blots for pERK protein expression in rat livers after SH (upper panel) and PH (lower panel), normalized to total ERK. (B) Quantitative densitometric analysis of the pERK protein expression. Data are presented as mean ± standard error of mean. *$P < 0.05$ *versus* SH.

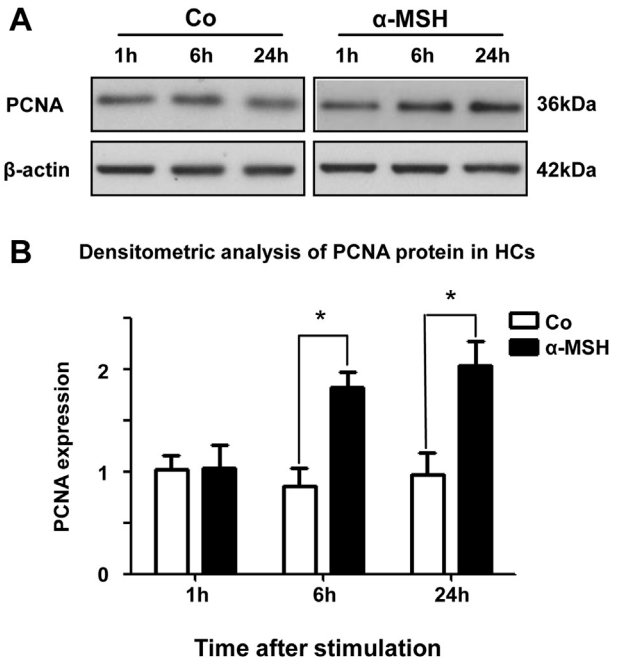

Fig. 6 – (A) Representative Western blots for PCNA protein expression in isolated primary HCs, normalized to β-actin. HCs were coincubated with either saline (control, co; left panel) or α-MSH (right panel) for 1, 6, and 24 h. (B) Quantitative densitometric analysis of PCNA protein expression in control (white bars) and α-MSH-stimulated (black bars) primary HCs. Data are presented as mean ± standard error of mean. *$P < 0.05$ *versus* control.

6 and 24 h in α-MSH-treated groups than in control groups (Fig. 7C and D, *$P < 0.05$), which is in line with previous reports on ERK-dependent inhibition of IL-6-induced STAT3 activation [22].

Discussion

The present study confirms previous reports that MC4R is expressed in HCs. However, we show herein for the first time that MC4R is upregulated in the process of liver regeneration as early as 8 h after partial liver resection. Furthermore, we herein demonstrate that the time course of MC4R expression correlates to the course of hepatic pERK expression. Moreover, *in vitro* stimulation of MC4R by administration of α-MSH promoted primary HC proliferation and modulated the regenerative signals *via* influencing ERK and STAT3 pathways (Fig. 8). Thus, one could suggest that MC4R may exert an important role in the modulation of rat liver regeneration.

Apart from its expression in neurons and other parenchymal cells, MC4R was also identified in nonparenchymal cells, such as macrophages [3]. In our rat model of liver regeneration expression of MC4Rs was mainly found in HCs after PH, whereas other cell types, such as macrophages, biliary cells, and stellate cells displayed no MC4R expression. These data suggest that regulation of regeneration may differ in different hepatic cell populations and that MC4R may not be ubiquitously involved in the process of cell proliferation.

It was reported that MC4R agonists induced remarkable neurogenesis and long-lasting functional recovery of learning and memory by using a gerbil model, and the possible mechanism was that treating with MC4R agonist affected the Wnt-3a signaling pathway [23]. Consequently, the ERK pathway was activated, both of which are involved in cell proliferation [24]. Independently, another study showed that α-MSH promoted neurite elongation *via* MC4R in mice [25]. In the present study, we found *in vivo* that upregulation of MC4R was correlated to the protein level of pERK during rat liver regeneration. Moreover *in vitro* stimulation with MC4R agonist significantly increased the expression of PCNA and pERK protein levels in isolated primary HCs. These findings suggest that modulation of MC4R during rat liver regeneration could lead to an activation of ERK pathway, which in turn triggers several transcriptional events, such as increase of energy expenditure and cell proliferation. Activation of ERK pathway exerts a proliferative effect in normal cell types, such as neurons and HCs. However, hepatocyte growth factor induced overactivation of ERK has been demonstrated to cause an inhibition of cell proliferation in the human hepatocellular carcinoma cell line HepG2. [26]. It could be deduced that ERK pathway bidirectionally regulates cell proliferation depending on the intensity of ERK phosphorylation and thus the level of ERK activity.

In our study, significant increases of hepatic IL-6 and TNF-α mRNA levels after PH were observed [27]. The primary

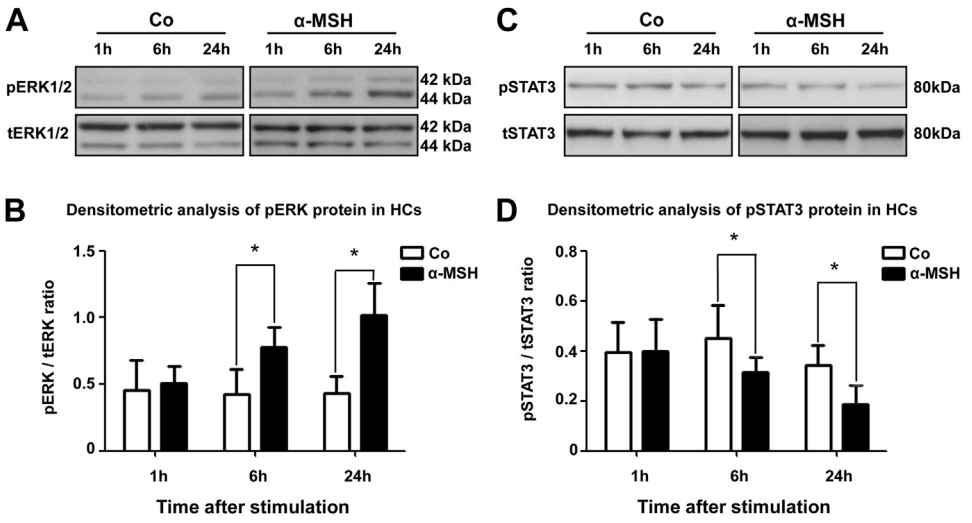

Fig. 7 – (A) Representative Western blots for pERK protein expression in isolated primary HCs, normalized to total ERK. HCs were coincubated with either saline (control, co; left panel) or α-MSH (right panel) for 1, 6, and 24 h. (B) Quantitative densitometric analysis of pERK protein expression in control (white bars) and α-MSH-stimulated (black bars) primary HCs. (C) Representative Western blots for pSTAT3 protein expression in isolated primary HCs, normalized to tSTAT3. Hepatocytes (HCs) were coincubated with either saline (control, co; left panel) or α-MSH (right panel) for 1, 6, and 24 h. (D) Quantitative densitometric analysis of pSTAT3 protein expression in control (white bars) and α-MSH-stimulated (black bars) primary HCs. Data are presented as mean ± standard error of mean. *P < 0.05 *versus* control, respectively.

function of IL-6 in liver regeneration was originally shown to be proliferative, as IL-6 knockout (KO) mice had a striking deficit in DNA replication after PH [28]. Moreover, treatment with anti-TNF-α antibody inhibited the hepatic DNA replication [29], and liver regeneration was dramatically blocked in IL-6 and TNF receptor type I KO mice [28,30]. However, impaired liver regeneration of TNF receptor type I KO mice was corrected by IL-6 administration [28]. After binding of IL-6 and TNF-α to their respective receptors (e.g., gp80 and gp130)

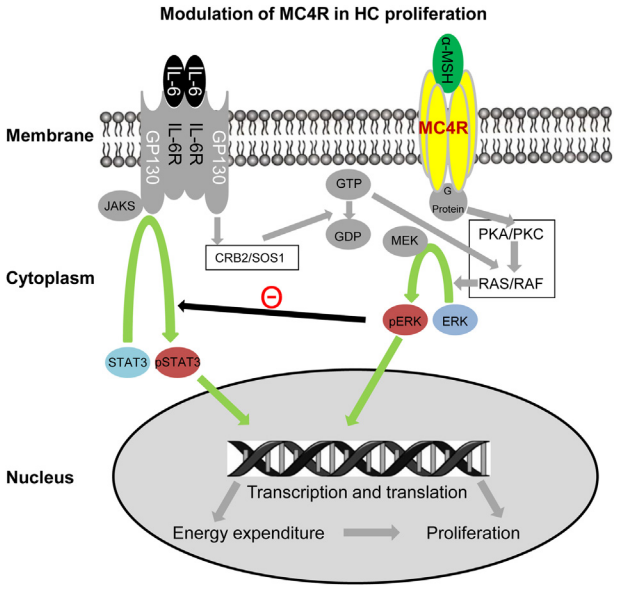

Fig. 8 – Schematic diagram for MC4R-mediated crosstalk of ERK and STAT3 pathways in proliferating hepatocytes during liver regeneration.

on HCs, the proliferative signals are initiated. Subsequently, STAT3 is activated and several genes are targeted. One important target gene of STAT3 is *SOCS3*, which acts in a feedback loop to prevent ongoing activation of IL-6 signaling by inhibiting STAT3 phosphorylation [31].

We also observed a significant downregulation of pSTAT3 protein level in parallel with the upregulation of the protein level of pERK after the stimulation of MC4R agonist, supporting the concept that pERK could inhibit the ongoing activation of pSTAT3 [31]. In other words, activation of ERK pathway may offer a positive signal for cell proliferation, although it could supply an inhibition to the regenerative signal induced by STAT3 pathway (Fig. 8). This might be a possible explanation of the bidirectional regulation of ERK pathway in cell proliferation: depending on the intensity of regenerative signal and the signals from other regulators, for example, STAT3 pathway. In addition, this interaction between ERK and STAT3 pathways could also be an alternative mechanism for the anti-inflammatory effects of MC4R agonists. MC4R, which is mainly functional in the energy homeostasis as mentioned previously, could provide proliferative and anti-inflammatory effects. This unique modulation is considerable to be applied in complicated pathophysiological processes, for example, development of hepatocelluar carcinoma after surgery with the issues of inflammation, HC regeneration, and tumor recurrence. Clearly, further studies are demanded to explore this topic in detail.

Because the presented results are derived from *in vitro* experiments, conclusions whether the observed changes in MC4R expression are biologically relevant are limited. So far it is not known yet, whether and to what extent MC4R is involved in the process of liver regeneration in general. Furthermore it is unclear, whether the potential involvement of MC4R in liver

regeneration is biologically relevant, in thus that liver regeneration is accelerated on MC4R stimulation. So, additional *in vivo* experiments have to be performed to investigate the effects of MC4R stimulation or blockade on regenerating livers.

Conclusions

From our results, we conclude that MC4R is predominantly expressed in HCs and is relevantly upregulated during rat liver regeneration. Expression dynamics of MC4R expression follow the expression of pERK, suggesting that activation of hepatic MC4R could promote hepatocellular proliferation correlated to ERK signaling pathway. In addition, it may modulate the regenerative signal of STAT3 pathway.

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Author contributions:

Xu, M was involved in cell culture, PCR, Western blot immunofluorescence, data acquisition, and manuscript draft writing.

Alwahsh, SM was involved in cell culture, PCR, Western blot immunofluorescence, and data acquisition.

Ramadori, G was responsible for financial support and provision of tissue samples.

Kollmar O was involved in experimental design, data analysis, critical discussion, and proof reading.

Slotta JE was involved in experimental design, data analysis, critical discussion, and manuscript preparation.

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