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UPREGULATION OF HEPATIC MC4R DURING RAT LIVER REGENERATION

INAUGURAL-DISSERTATION

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ABBREVIATIONS

α-MSH: alpha-Melanocyte-stimulating hormone

ACTH: adrenocorticotropic hormone

BAX: bcl2-associated X protein

Bcl-2: b cell lymphoma 2

BSA: bovine serum albumin

cDNA: complementary deoxyribonucleic acid

c-JNK1/2: c-jun N-terminal kinases 1/2

CK-19: cytokeratin 19

CNS: central nervous system

CT: cycle threshold

dd H_2O: double distilled water

DEPC: diethylpyrocarbonate

DMSO: dimethylsulfoxide

DNA: deoxyribonucleic acid

dNTP: deoxyribonucleoside triphosphate

DRG: dorsal root ganglion

EC_{50}: half maximal effective concentration

ED-1 (CD-68): cluster of Differentiation 68

EDTA: ethylendiaminetetraacetic acid

ELISA: enzyme-linked immunosorbent assay

FCS: fetal calf serum

GAPDH: glyceraldehyde-3-phosphate dehydrogenase

gp: glycoprotein

HC: hepatocyte

HNF-4 α: hepatocyte Nuclear Factor 4 alpha

HO-1: hemoxygenase-1

IF: immunofluorescence

IL-6: interleukin-6

IP: intraperitoneal

IRI: ischemia reperfusion injury

LPS: lipopolysaccharides

Kb: kilobase

kDa: kilodalton

MC4R: melanocortin 4 receptor

mRNA: messenger ribonucleic acid

NFkB: nuclear factor kappa-light-chain-enhancer of activated B cells

NIH: national institute of health

OD: optical density

PBS: phosphate buffered saline

PCNA: proliferating cell nuclear antigen

PCR: polymerase chain reaction

pERK 1/2: phosphorylated extracellular signal-regulated kinase 1/2

PHx: partial hepatectomy

PMSF: phenylmethyl sulfonylfluoride

pSTAT3: phosphorylated signal transducer and activator of transcription 3

RNase: ribonuclease

ROS: reactive oxygen species

rpm: rounds per minute

RT: room temperature

RT-PCR: reverse transcription polymerase chain reaction

SDS: sodium dodecylsulfate

SDS-PAGE: SDS-polyacrylamide gel electrophoresis

SEM: standard error of the mean

SH: sham operation

tERK: total ERK

TNF- α: tumor necrosis factor- alpha

Tris: tris-(hydroxymethyl)-aminomethane

tSTAT3: total STAT3

U: unit

Ubc: ubiquitin C

UV: ultraviolet

WB: western blot

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ABSTRACT

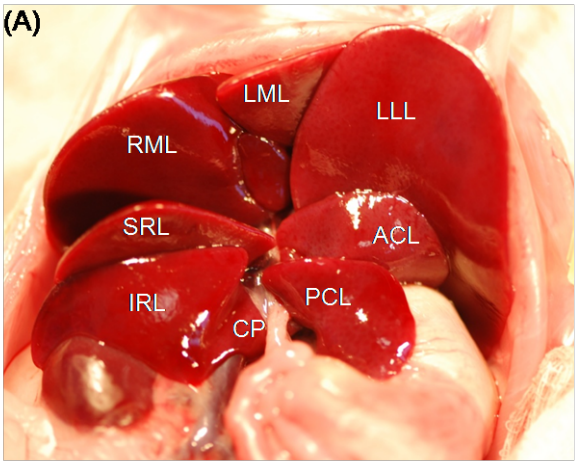
BACKGROUND: Melanocortin 4 receptor (MC4R) is predominantly recognized to mediate energy metabolism and anti-inflammation through the central nervous system. However, the expression of MC4R has recently been identified in rat liver and was shown to be upregulated during acute phase response. This study aims to investigate potential roles of MC4R in liver regeneration. **MATERIALS AND METHODS:** Rat partial hepatectomy (PHx) 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 8h after PHx, hepatic messenger RNA of proinflammatory cytokines interleukin 6 and tumor necrosis factor α reached peak values. Between 8 and 72h after PHx, 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 $4\alpha^+$ cells (HCs) and upregulated during rat liver regeneration. Concurrently, the expression of hepatic MC4R protein was significantly higher in PHx than in SH animals, and phosphorylated extracellular signal-regulated kinase 1/2 was remarkably increased in PHx 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.

1. INTRODUCTION

1.1 Liver regeneration and partial hepatectomy (PHx)

The liver possesses extraordinary capabilities of regeneration, a phenomenon has been observed for several centuries (Michalopoulos and DeFrances 1997). The legend of Prometheus' regenerating liver after being eaten by an eagle indicates that people noted hepatic regeneration even more longer time ago. In the present, PHx has been widely performed as the therapy for both of benign and malignant liver diseases, for instance hemangioma, focal nodular hyperplasia, hepatocellular carcinoma, and cholangiocarcinoma etc. Meanwhile, PHx in rodent animals is one of the most studied experimental models, and reveals a lot of molecular and cellular mechanisms lying in liver regeneration (Michalopoulos 2010) (The anatomy and lobe-specific volume of rat liver are illustrated as in Figure 1). PHx is highly reproducible and can initiate a proliferative signal to the remnant liver mass; this is mediated by tissue specific pathways without significant cell death. The application of genetically modified mice has facilitated the study of signaling pathways participating in liver regeneration (Riehle et al. 2011). Although massive liver resections have been increasingly low risky because of the improvements of understanding of the anatomy and refinement of operative techniques, liver failure following PHx still occurs from time to time and remains incompletely understood (Helling 2006). Liver failure can be regarded as a net loss of functioning hepatic mass to the point that remnant hepatocytes cannot maintain necessary metabolic functions. This means that either a failure to regenerate after partial hepatectomy/liver resection or accelerated destruction of liver cells from necrosis or apoptosis can result in liver failure after operation as well (Helling 2006).

(A)



(B)

Rat liver lobe	V_L/V_T(%)
Left lateral lobe (LLL)	~30%
Left medial lobe (LML)	~38%
Right medial lobe (LML)	
Superior right lobe (SRL)	~12%
Inferior right lobe (IRL)	~10%
Anterior caudate lobe (ACL)	~4%
Posterior caudate lobe (PCL)	~4%
Caudate process (CP)	~2%

Figure 1. Anatomy of rat liver: Rat liver consists of left lateral lobe (LLL), left median lobe (LML), right median lobe (RML), superior right lobe (SRL), inferior right lobe (IRL), anterior caudate lobe (ACL), posterior caudate lobe (PCL), and caudate process (CP) (**A**: Laparotomy was performed and photographed by Xu et al. 2010, permitted for use). **B**: The percentage of rat liver lobe volume (lobe volume (V_L)/total liver volume (V_T) ×%) was tabled as previously reported (Michalopoulos 2010).

In recent years, profiles of global gene-expression have been extensively investigated to explore the complex cascade of molecular events regulating liver regeneration. Liver regeneration following PHx is mostly dependent on the proliferation of hepatocytes (HCs) which are full differentiated and usually quiescent cells, and does not rely on the regeneration of a compartment of hepatic stem cells (Riehle et al. 2008). A great number of genes were found to be involved in hepatocytes replication, and the necessary circuitry required for the regenerating process could be divided into three kinds: metabolic related factors (e.g. Heparin-binding EGF-like growth factor, and Amphiregulin), cytokines (e.g. TNF-α, IL-4, IL-6 and MCP-1) and growth factors (e.g. hepatocyte growth factor, and epidermal growth factor) (Fausto et al. 2006).

It has been extensively known that the early regenerating liver temporally accumulated hepatocellular fat after PHx (Delahunty and Rubinstein 1970; Girard et al. 1971; Glende and Morgan 1968; Gove and Hems 1978). Other works have also showed that fat accumulation was associated with cellular

proliferation in cultured primary hepatocytes (Michalopoulos et al. 1982), raising the probability that fat deposition might in turn control liver cells' proliferation. Furthermore, the patterns of hepatic mRNA induction during early PHx-stimulated liver regeneration suggested the presence of a conserved transcriptional program causing the regulation of transient "steatosis" during the regenerating process (Shteyer et al. 2004; Yu et al. 2003). The effect of endogenous hepatic lipogenesis in controlling liver regeneration is not completely clear. Increased *de novo* hepatic fatty acid production has been reported in regenerating liver (Gove and Hems 1978), but mice with liver specific disruption of the expression of fatty acid synthase (Chakravarthy et al. 2005) displayed relative hepatic fat deposition and liver regeneration after partial hepatectomy compared to that in the wild-type animals, strongly indicating that *de novo* liver lipogenesis was not essential for the development of such a hepatic steatosis during the regeneration (Newberry et al. 2008). These together with other data pointed to systemic fatty tissue as the principal source of the adipose that accumulated in regenerating liver (Glende and Morgan 1968; Klingensmith and Mehendale 1982). However, up to date the mechanisms of this kind of steatosis are still poor documented.

1.2 Melanocortin receptors (MCRs) family

To the current knowledge, the melanocortin network consists of several receptors, agonists, and antagonists. The agonists are all generated from tissue-specific posttranslational process of propiomelanocortin (POMC), which are including α-melanocyte stimulating hormone (α-MSH), β-MSH, γ-MSH, and adrenocorticotropic hormone (ACTH) (Butler 2006). Diverse actions are mediated by the five melanocortin receptors, which are termed from MC1R to MC5R based on the sequence of their cloning (Mountjoy et al. 1992).

The half maximal effective concentration (EC_{50}) of α-MSH is 16 nM

(MW=949.01; 16 nM=15.18 μg) (Benoit et al. 2000). Delivery of 50 μg α-MSH reduced the hepatic inflammation induced by Lipopolysaccharides (LPS) (Chiao et al. 1996) and kidney ischemia-reperfusion injury in rats (Chiao et al. 1997). The dose of 340 μg/kg was also previously used to protect against testicular ischemia reperfusion injury by activating the cholinergic anti-inflammatory pathway, and antagonist (HS024) abolished the effect of α-MSH (Minutoli et al. 2011). α-MSH also prevented LPS-induced liver inflammation by preventing the expression of chemoattractant chemokines that in turn modulated the infiltration of inflammatory cells. The MC1R mRNA was detected in liver tissue (Chiao et al. 1996). However, the precise location was not identified, and it was supposed that α-MSH acted via macrophage since both human and murine macrophages express MC1R (Rajora et al. 1996; Star et al. 1995).

Both MC3R and MC4R were involved in regulating energy homeostasis. However, the role of the MC3R in regulating food intake is controversial. Data from knockout-animals revealed that *Mc3r* gene knockout resulted in increased feed efficiency and adiposity (Butler et al. 2000; Chen et al. 2000). Meanwhile, it was shown that the MC3R is required for entrainment to meal intake (Sutton et al. 2008). The MC4R may regulate both food intake and energy expenditure and is the focus of this study. In addition, the MC5R is expressed widely and especially in exocrine glands. Knockout experiments showed that the MC5R is involved in regulating exocrine gland secretions (Chen et al. 1997).

1.3 Melanocortin 4 receptor (MC4R)

The MC4R is a member of family A of G protein coupled receptors (GPCRs) with the seven-transmembrane structure linked by changeable extracellular loops and intracellular loops; the N terminus is extracellular, while the C terminus is intracellular located (Tao 2010). MC4R played a critical role in

the controlling of energy homeostasis and anti-inflammation (Tao 2010). MC4R function is tightly regulated by MC4R agonist (alpha-melanocyte stimulating hormone, α-MSH) and MC4R inverse agonist (agouti related peptide, AgRP), and both of α-MSH and AgRP are generated from proopiomelanocortin (POMC) peptide (Tao 2010). MC4Rs are generally considered being expressed in the central nervous system (CNS) and exert their biological effects to peripheral organs (Gantz and Fong 2003). However, recent studies identified the findings of MC4R in rat liver and adipose tissues as well (Barb et al. 2010; Malik et al. 2011). Furthermore biological effects of MC4R will be reviewed as the following.

1.4 MC4R and inflammation

According to previous studies, MC4R, the fourth known receptor subtype, a G protein coupled seven-transmembrane receptor, is commonly expressed in the central nervous system (CNS). MC4R predominantly mediated metabolic and anti-inflammatory roles in the CNS (Gantz and Fong 2003; Mountjoy et al.1992). MC4Rs also can employ their special effects on other tissues outside the brain via a central nerve dependent way (Toda et al. 2009), such as the liver (Bitto et al. 2011). It was reported that α-MSH prevented LPS-induced hepatic inflammatory reaction by inhibiting the expression of chemoattractant chemokines that then modulated the infiltration of inflammation-related cells (Chiao et al. 1996). Previous studies indicated that activation of MCRs protected organs against ischemia reperfusion injury (IRI). In a global brain ischemia model of occluding both sides of the common carotid arteries, MC4R agonists counteracted late inflammation and apoptosis responses via regulating tumor necrosis factor-α (TNF-α), Bcl2-associated X protein (BAX), extracellular signal-regulated kinases (ERK1/2), c-jun N-terminal kinases (c-JNK1/2) and caspase-3, down-regulation of B cell lymphoma 2 (Bcl-2), consequently triggering cerebral repair pathway (Spaccapelo et al. 2011).

MC4R agonist induced noticeable neurogenesis and long term functional recovery of memory and learning (Giuliani et al. 2011), which were related with overexpression of the action-dependent gene Zif268 (Giuliani et al. 2009). Similarly, in a rat myocardial IRI model, activation of MCRs modulated the cascades of inflammation and apoptosis caused by prolonged myocardial ischemia and reperfusion, and reduced infarct size, and interestingly, bilateral cervical vagotomy blunted the protective effects of NDP-alpha-MSH (Ottani et al. 2010). In a rat model, MC4R activation protected against testicular ischemia reperfusion injury via activating the cholinergic antiinflammation pathway, in the same study, IL-6 and TNF-α expression were down regulated by MC4R while all positive NDP-α-MSH properties were abolished by chlorisondamine, vagotomy, or a selective MC4R agonist (HS024) (Minutoli et al. 2011). By using a rat kidney IRI model, it was reported that alpha-MSH may significantly attenuate the renal I/R injury by specific kidney-targeted effects via MCRs as well as by systemic cytokine effects (Lee et al. 2008). On the other hand, a striking downregulation of the cellular iron exporter ferroportin-1 has been observed in non-alcoholic steatohepatitis (NASH), however hepcidin was physiologically augmented in dysmetabolic iron overload syndrome (Aigner et al. 2008; Pigeon et al. 2001). However, the precise mechanism of the anti-inflammation function of α-MSH and the interaction with other biochemical process are still not well addressed. Although some progresses have been obtained in protecting fatty donor against IRI, obviously there is still a lot of work to be done to solve this issue.

1.5 MC4R and energy metabolism

Many genes that related to direct energy generating processes were upregulated in liver together with in fatty tissue of α-MSH treated pigs homozygous with a missense mutation in MC4R, for instance electron transport, oxidative phosphorylation, and ATP synthesis (Barb et al. 2010). Targeting

disruption of the MC4R resulted an obese phonotype in mice, indicating this signaling pathway is physiologically crucial in the lipid and energy metabolism (Huszar et al. 1997). Interestingly in mice, administration of the MC4R antagonist (SHU9119) or AgRP in the CNS highly enhanced the ingestion activities, suggesting that antagonizing the MC4R could be an critical orexigenic signal (Wirth and Giraudo 2000). Knockout of MC4R and POMC (Butler and Cone 2001) or over-expression of AgRP (Graham et al. 1997) led to the same obesity in mice, a similar genetic pattern was also indicated in patients with mutations of MC4R (Feng et al. 2006). These reports demonstrated that MC4R played an important role in energy metabolism.

1.6 MCRs and cells proliferation

MCRs mediate the various effects of in response to the stimulations of MCRs agonists. MC2R was the classical adrenocorticotropic hormone (ACTH) receptor expressed in the adrenal cortex that regulates adrenal steroidogenesis and cells proliferation. Bothe of MC3R and MC4R were expressed mainly in central nervous system and were therefore referred to as the neural MCRs. MC5R is expressed widely, especially in the exocrine glands. Studies in the Knockout animal models showed that the MC5R is involved in regulating exocrine gland secretions (Chen et al. 1997).

It was concluded that alpha-MSH can encourage neurite elongation via MC4R locating in dorsal root ganglia neurons as well as the initiation of MC4R was important for the regeneration motor and sensory neurons after axonal injury (Tanabe et al. 2007). In addition to neurons, astrocytes also expressed the MC4R (Caruso et al. 2007; Selkirk et al. 2007). Both synthetic and endogenous peptide ligands as well as small molecular agonists could increase cAMP expression in the astrocytes of rat, this was consistent with previous studies showing that ACTH and α-MSH increased cAMP levels in

astroglial cells (Evans et al. 1984; van Calker et al. 1983) and melanocortins stimulated proliferation and induced morphological changes in cultured rat astrocytes (Zohar and Salomon 1992). On the other hand, $ACTH_{1-24}$ was indicated to decrease the production of ciliary neurotrophic factor mRNA in rodent astrocytes growing *in vitro* as well (Kokubo et al. 2002). Recently, it was found that functional MC4R was also located in human epidermal melanocytes which could promote melanogenesis (Spencer and Schallreuter 2009). Take all together, MC4R may play a significant role in cells proliferation.

1.7 Hypothesis and aims of the analysis

Based on the best of current knowledge, MC4R is extensively involved in metabolic and inflammatory processes. Therefore it is supposed that MC4R could also play a critical role in liver regeneration after PHx. The current study is to explore the importance of MC4R expression in the liver and its regulation during regeneration using an *in vivo* rat PHx model and *in vitro* cultured primary HCs cells. The potential mechanisms were extraordinarily explored.

2. MATERIALS AND METHODS

2.1 Materials and Instruments

2.1.1 Materials and chemicals

Sources	Chemicals
Amersham Biosciences /Buckinghamshire, UK	Hybond-ECL nitrocellulose membranes
Amersham Biosciences /Freiburg, Germany	X-ray films Hyperfilm TM
Applied Biosystems /Darmstadt, Germany	SYBRGreen master mix and stepOne software
Becton Dickinson /Franklin Lakes, NJ, USA	Syringes BD Discardit 2 mL, 5 mL, and 20 mL
	Culture dishes (60 mm) Falcon
Biochrom /Berlin, Germany	M199
	FCS (fetal calf serum)
	Trypan blue
Bioline /Luckenwalde, Germany	dNTP mastermix
Biometra /Goettingen, Germany	Hybond N nylon membrane, disposable NICK columns prepacked with SephadexR Hybridization glass tubes Carbon tetra chloride Ethanol 37% formaldehyde Formamide Glucose Glycerol Kaiser´ s gelatine Meyer´ hemalaun Methanol β- mercaptoethanol Penicillin G Streptomycin TEMED
Bio-Rad /Munich, Germany	Tween 20
Biozym /Oldendorf, Germany	Sterile filter pipette tips
Bohringer /Mannheim, Germany	Ampicillin
Braun /Melsungen, Germany	Braunules 2G14

Sources	Chemicals
Eppendorf /Hamburg, Germany	Serological pipettes (2, 5, 10, and 25 mL), transfer pipettes, plastic tubes (15 and 50 mL)
	Safe-Lock tubes (0.2, 0.5, 1,5 and 2 mL)
Essex Pharma GmbH /Munich, Germany	Temgesic®
Molecular Probes Europe BV /Leiden, The Netherlands	4, 6-diamidino-2-phenylindole (DAPI)
Nicholas Piramal (I) Limited Honeypot Lane/ London, UK	Isoflurane
PAA /Linz, Austria	L-Glutamine
Paesel and Lorei /Frankfurt, Germany	Cesium chloride
Promega/ Mannheim, Germany	Moloney murine leukemia virus reverse transcriptase (M-MLV RT)
Roche /Mannheim, Germany	Complete Protease Inhibitor Cocktail Tablets
Roth /Karlsruhe, Germany	Glycine
	Sodium dodecyl sulfate (SDS)
Sartorius /Göttingen, Germany	Sterile filter Nalgene, 0.2 µm
Serva /Heidelberg, Germany	Tris HCl
Sigma-Aldrich Chemie /Munich, Germany	Ammonium persulfate
	Citric acid
	Dexamethasone
	DMSO
Tocris Bioscience /Ellisville, MO, USA	alpha-Melanocyte-stimulating hormone (α-MSH)

Table 1. Materials and chemicals: The sources and details of materials and chemicals used in this study are shown as above.

2.1.2 Instruments

Sources	Instruments
Applied Biosystems /Foster City, CA, USA	Step One plus Thermal cycler
Bachofer /Reutlingen, Germany	Ultraviolet emitter, 312 nm
Bechman /Munich, Germany	Bench-top, high speed and ultracentrifuges
	Beckman model J2-21 centrifuge and Backman rotor JE-6B
Bender and Hobein /Zurich, Switzerland	Vortex, Genie 2TM
Bio-Rad /Munich, Germany	Power supply, Power Pac 300
Eppendorf /Hamburg, Germany	Thermomixer 5436
GFL /Burgwedel, Germany	Water bath 1083
Heraeus /Hanau, Germany	Thermostat
Heraeus /Hanau, Germany	Sterile bench, type Lamin Air, TL 2472
Hettich Rotaxia /Tuttlingen, Germany	Hettich Mikro Rapid/K centrifuge, 3850 centrifuge, 48RS centrifuge
Jank & Kunkel /Staufen, Germany	Ultra-Turrax TP 18/10 homogenizer
Knick /Berlin, Germany	pH-Meter 761 Calimatic
Konica Europe /Hohenbrunn, Germany	X-ray film developing machine SRX-101A
Kontron instruments /Neufahrn, Germany	Centricon T-2070 ultracentrifuge and Centricon rotor TST55.5-55000rpm
New Brunswick Scientific Co., Inc /Edison, NJ, USA	Incubator with shaker for cell culture, model 3-25
Pharmacia Biotech /Freiburg, Germany	UV spectrophotometer, RNA/DNA Calculator GeneQuant II

Sources	Instruments
Prettl Laminarflow and Prozesstechnik /Bempflingen, Germany	Sterile bench, type MRF 0.612-GS
Schutt Lambortechnic /Goettingen, Germany	Vortex with platform
Siemens /Kassel, Germany	Microwave oven
Siemens /Kassel, Germany	X-ray film cassettes 10x18
Siemens Medical Solutions /Erlangen, Germany	Somatom Balance
ThermoLife Sciences /Egelsbach, Germany	Savant Speed VacR concentrator
Geratechnik /Gera, Germany	Magnetic mixer with warming
Varian /Palo Alto, CA, USA	Clinac 600C
Zeiss /Oberkochen, Germany	Microscope Axioscop with photo camera MC 100 Spot
Zeiss /Oberkochen, Germany	Microscope Axiovert 25
Ziegra /Isernhagen, Germany	Ice machine

Table 2. Instruments: the instruments used in the current study are displayed in the above table.

2.2 Animals and experimental design

2.2.1 Ethics and policy

The animal study was funded by Deutsche Forschungsgemeinschaft SFB 402, project D3. This study was also approved by the Animal Ethics and Research Committee of University Medical Center Goettingen. All experiments were conducted consistent with the laws and regulations of the state of Lower Saxony, Germany. All animals were treated humanely.

2.2.2 Animals

For the experimental studies, male Wistar rats weighed from 180 grams and 220 grams, purchased from Harlan-Winkelmann in Germany, were allowed to be acclimatized for one week before the start of experiments. Food and water were available ad libitum.

2.2.3 Experiments design and surgical operations

The rats PHx were performed by Dr. DS Batusic et al. during 2005-2006, that was under the supervision of Prof. G Ramadori. Rats were sacrificed at 0 h, 2 h, 4 h, 8 h, 16 h, 24 h, 48 h, 72 h, and one week after PHx operation or SH (three rats per time point). Liver samples were snap-frozen in liquid nitrogen and quickly stored at -80 °C until use. Operative procedures: Rat 70% partial hepatectomy (PHx) or sham (SH) operation was performed under isoflurane (Concentration: 1.8-2.5%; Oxygen flow: 1.5 L/min) anesthesia by ventral laparotomy. The median anterior and left lateral hepatic lobes were separately ligated with sutures, and completely excised. SH operations were consisted of a ventral laparotomy of similar extent. The rat liver was gentle manipulated, followed by surgical closure of the abdominal wall similar to PHx-operated rats. To relief pain after operation, a dose of temgesic (0.03 mg/kg) every 6-12 h was

intra muscularly administrated. The technique of rat PHx is also illustrated in Figure 2.

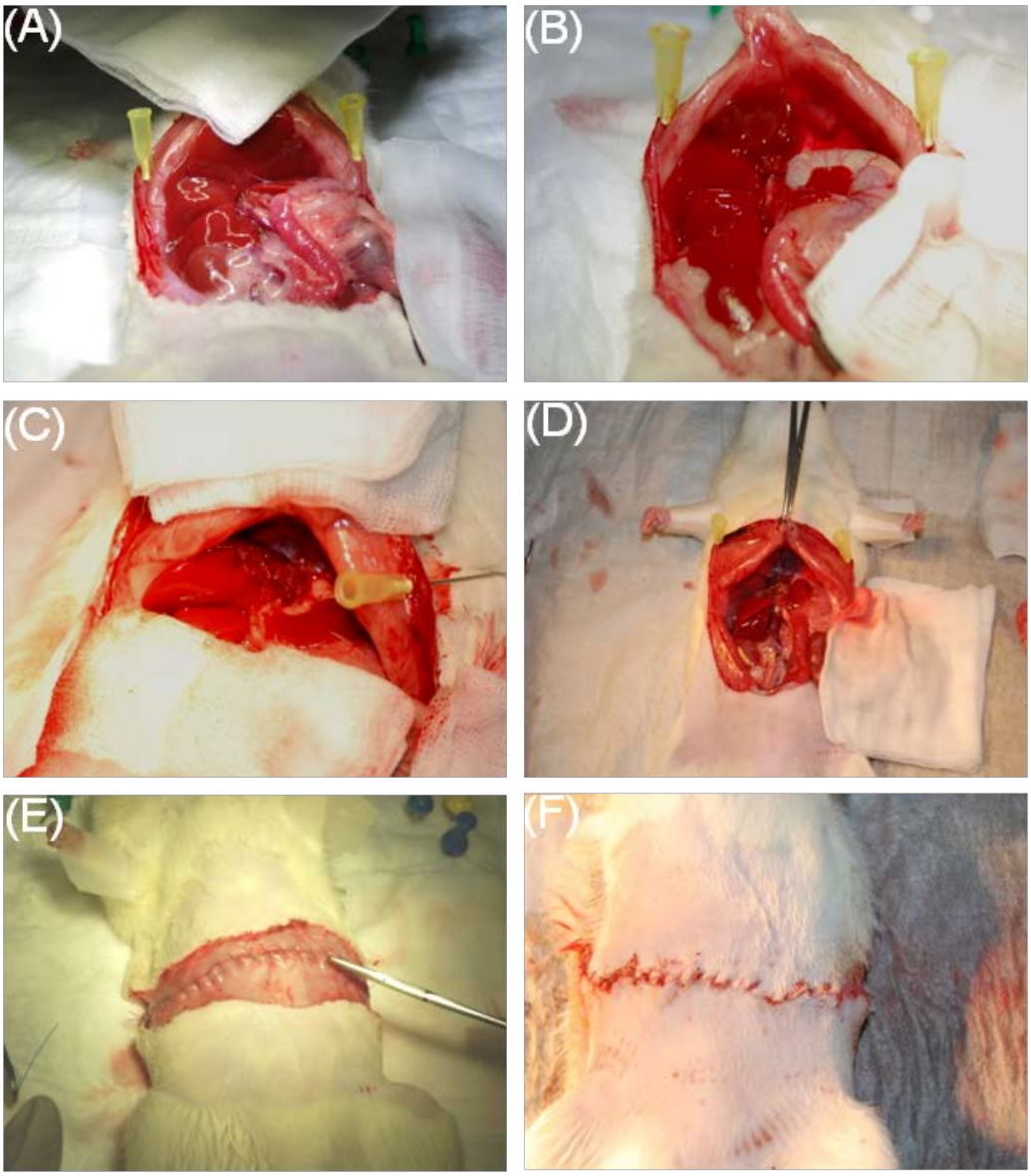


Figure 2. Procedure of rat 70% PHx: Surgical procedure of 70% PHx in rat (Performed and photographed by Xu et al 2010, permitted for use): Laparotomy and exposure of rat liver (A); Resection of left lateral lobe (B); Resection of left median lobe (C); Resection of right median lobe (D); Closure abdominal wall (E,F).

2.3 mRNA extraction, cDNA synthesis and real-time polymerase chain reaction (RT-PCR)

2.3.1 Procedure of mRNA extraction

In short, for each sample 100 mg rat livers was homogenized in 1 mL TRIzol reagent using a power homogenizer, subsequently incubated 5 minutes at room temperature, then 0.2 mL chloroform was added to every sample, which was vigorously shaken and incubated at room temperature for 3 minutes. All samples were then centrifuged at 12000 rpm for 30 minutes, and the aqueous phase obtained in the supernatants was carefully collected. Successively, a volume of 0.5 mL of isopropyl alcohol was added into the collected phase and each sample was incubated for 10 minutes at room temperature. The RNA precipitation was centrifuged again at 12000 rmp for 10 minutes. After a quick washing and vortexing in 75% ethanol, total RNA was shortly air-dried, suspended, and dissolved in RNase and DNase free water with an approximately volume of 50 µL. The concentration of RNA was measured by the absorbance at 260 nm/ 280 nm.

2.3.2 cDNA synthesis

The complementary deoxyribonucleic acid (cDNA) was created from reverse transcription of 3.0 µg of total RNA extract with 50 pM of primer oligo (dT)15, 100 nM of dNTPs, 16 U of protector RNase inhibitor, 1× RT buffer, and 2.5 mL of 0.1 M DTT, 200 U of moloney murine leukemia virus reverse transcriptase (M-MLV RT) for one hour at 40°C.

2.3.3 Primers designing and sequences of primers were as the following

Primers for different genes were designed using the gene bank data (http://www.ncbi.nlm.nih.gov) and the program “Primer 3 v.4.0” (http://bioinfo.ut.ee/primer3-0.4.0/), successively checked by Blast NCBI (http://blast.ncbi.nlm.nih.gov/). All the primer sets used for real-time PCR are

listed as the following Table 3.

Gene	5'-3' Forward	5'-3' Reverse
Actb	ACC ACC ATG TAC CCA GGC ATT	CCA CAC AGA GTA CTT GCG CTC A
β actin	ACC ACC ATG TAC CCA GGC ATT	CCA CAC AGA GTA CTT GCG CTC A
Il-6	GTC AAC TCC ATC TGC CCT TCA G	GGC AGT GGC TGT CAA CAA CAT
Mc4r	CAC AGT ATC GGG CGT TCT TT	GTA ATT GCG CCC TTC ATG TT
Mcp1	AGG CAG ATG CAG TTA ATG CCC	ACA CCT GCT GCT GGT GAT TCT C
Tnf-α	ACA AGG CTG CCC CGA CTA T	CTC CTG GTA TGA AGT GGC AAA TC

Table 3. Primers used in this study: The 5'-3' forward and reverse primers used in this study are shown in above table.

2.3.4 RT- PCR

2.3.4.1 The cDNA samples were analyzed by the RT-PCR using the following ingredients for each PCR reaction: Primer-forward (5 mM) 1.5 µL, primer-reverse (5 mM) 1.5 µL, H_2O 6.5 µL, SYBR Green Master Mix Taq Polymerase 13.0 µL, 1 µl of the cDNA sample or 1 µL of H_2O for the negative control was added to each PCR reaction. Beta actin was used as housekeeping gene. All samples were examined in duplicate.

2.3.4.2. The PCR amplification was performed initially at 95 °C for 20 seconds, then 95°C for 3 seconds, 60°C for 30 seconds for 40 cycles in an ABI prism 7000 sequence detection system, all sample were duplicated analyzed. The differences of genes expression were analyzed using Platinum SYBR Green qPCR mix uracil-DNA glycosylase. The specificity of PCR amplification program was controlled by dissociation curve, and the specific temperature of dissociation of the PCR product was calculated by the primer express software. In the linear range of the amplification, amplification curves were analyzed to obtain the cycle threshold (Ct) value for furthermore analysis. All genes expression were normalized by the house keeping gene and fold change of

expressions were calculated using delta (delta Ct) value method by excel and Prism Graph Pad 4 software.

2.3.4.3. Standard Curve: the genes of interest were amplified by RT-PCR, and the relevant Ct values were recorded. Subsequently, the standard curves were obtained under the setting of following parameters: threshold value=0.2, and the logarithm of the number of copies. Standard curves of the genes of interest were also calculated, and for the housekeeping genes as well. Numbers of copies of genes in the sample were determined by extrapolation of the measured cycle threshold value referred to the relative standard curve. Gene expression in each sample was normalized by calculating the ratio of a number of copies of the gene respected to the number of copies of the housekeeping genes.

2.4 Frozen sections and immunofluorescence (IF) staining

Immunohistochemical analysis was performed on 5 μm thick cryostat sections that were fixed in methanol/acetone (-20°C, 10 minutes) to localize the antigens on the tissues. Antigens were blocked to determine the antibody specificity using the blocking peptide (e.g. goat serum) provided by the manufacturer, blocking was performed according to standard protocol provided by the manufacturer as well. In short, firstly the optimal concentrations of antibodies that consistently gave positive results were determined. Subsequently, the slides were blocked with goat serum and were subjected to the following primary antibodies overnight at 4°C (the detailed information were showed in table 4): rabbit anti-MC4R, mouse anti-ED-1, mouse anti-CK19, rabbit anti-HNF-4α, mouse anti-Ki-67, and mouse anti desmin. After incubated with secondary antibodies, the slides were counterstained with 4, 6-diamidino-2-phenylindole (DAPI) and analyzed under the fluorescence microscope. Side by side, negative controls were established by replacing the primary antibodies with PBS. Randomly, five fields were captured within each

liver section to calculate the average number of Ki-67 positive cells and hepatocytes in rat liver sections. The regeneration indices were defined as number of Ki-67 positive cells divided by total HCs (by percentage).

2.5 Hepatocytes (HCs) isolation and stimulation

Rat primary hepatocytes (HCs) were isolated by Dr. IA Malik et al. (Malik et al. 2010) briefly described as following: laparotomy was done by a middle ventral incision, subsequently the portal vein was cannulated, superior hepatic vena cava was ligated above the diaphragm to close the flow of the perfusion media into a whole circulation. Later, the inferior hepatic vena cava was cut under the liver and cannulated, and perfused in non-recirculative mode through the portal vein with 15 to 200 mL CO_2 enriched medium at a flow rate of 30 mL/minute until the hepatic blood flushed out. To break down components of extracellular matrix, the liver was perfused in recirculative mode with collagenase perfusion medium until it was soften (about 7-11 minutes). Once perfusion was finished, rat livers were immediately excised and transferred into a sterile glass beaker filling with culture medium M 199 and other components. The hepatic capsule (collagen tissue around the liver) was carefully removed and discarded. Then, rat liver tissues were mechanically broken down by sterile forceps in order to collect cell suspension. The hepatic connective tissue and remain as well as large cell masses were filtrated by a nylon mesh (pore-size 79 µm). Non-parenchymal cells and cell debris in wash medium were filtrated by numerous selective sedimentations (20 grams, 2 minutes at 4°C). After the last centrifugation rat hepatocytes were collected and suspended in medium M199 with additives. Then 50 mL of M199 was added to each 1 gram of wet weight of the suspended cells; finally, the cell suspension generally with a density of about 106/2.5 mL.

The isolated hepatocytes were exposed to 10 µg/mL α-MSH in M199 culture medium with 1.5% antibiotics. Then, the *in vitro* stimulated HCs were

harvested at 1 h, 6 h, and 24 h after treatment for protein extraction. Normal saline-treated HCs at all time points served as controls.

2.6 Protein extraction and Western blot analysis

2.6.1 Preparation of tissue homogenates

The entire procedure was performed at 4°C to prevent proteolytic degradation of the total proteins. Approximately, 100 mg of frozen tissue was homogenized by Ultra-Turrax TP 18/10 model homogenizer 10 seconds for 3 times in 10 volumes of 50 mM Tris-HCl buffer (pH=7.4), containing 0.50 M Tris-HCl (pH 7.6), 150 mM NaCl, 10 mM EDTA, 1% (v/v) Triton X-100, 1 mM PMSF, and 10 µL/mL proteases cocktail inhibitors. In order to pellet the nuclei and particular matter, the homogenates were centrifuged for 5 minutes at 10000 rpm under 4°C. Supernatants were collected as cytosolic extraction of protein and the pellets were resuspended in lysis buffer and stored at -20°C for further use. Protein concentrations of supernatants were measured using the bicinchoninic acid (BCA) method (Smith et al. 1985) by the BCA protein assay reagent kit (Pierce, Bonn, Germany). Prepared homogenates were distributed in aliquots and stored at -80°C until use.

2.6.2 Preparation of cell lysate

The HCs frozen on the culture dishes were thawed on ice. The 4°C lysis buffer were including 150 mM NaCl, 1mM EDTA, 1% Triton X-100, 50 mM Tris-HCl, pH 7.4 and supplemented with protease inhibitors. A volume of 500 µL buffer was added to 6 cm dish of the cells, followed by incubation on ice for 10 minutes. Later on, the HCs were scraped with a disposable scraper, transferred to new tubes and went 5 times through a 22 G administration cannula connecting to a syringe. In order to pellet the nuclei and particular matter, the lysates were centrifuged for 5 minutes at 10000 rpm at 4°C and the protein concentrations of supernatants were measured by BCA method using the kit from Pierce. Finally, the lysates were aliquoted and stored at -80°C

condition until use.

2.6.3 Western blot

For each sample, fifty micrograms lysate of the total protein were loaded in a well of 4-12% Nu-PAGE Bis-Tris (Invitrogen) gel and subjected to 2 h electrophoresis at 80 V. Then, the proteins on the gels were transferred to membranes in a semidry apparatus at 30 V for 1.5 h. The membranes were blocked in 5% milk, and incubated with primary antibodies overnight at 4°C. The secondary antibodies were horse reddish peroxidase 29 conjugated goat anti-rabbit and goat anti-mouse immunoglobulins (DAKO) diluted at 1:1000. Membranes were developed with ECL chemiluminescence Kit (Amersham) at different time point. The details of antibodies are listed as in table 4 and table 5.

Primary antibodies

Target	Host	Clonalitiy	Applications	Dilution	Company	Cat. #
β-actin	mouse	monoclonal	WB	1:5000	Sigma-Aldrich	A5441
CK19	mouse	monoclonal	IHC	1:50	Novacastra	B170
ED-1	mouse	monoclonal	IHC	1:100	Serotec	MCA341
ERK	rabbit	polyclonal	WB	1:1000	Cell signaling	CST9102
HNF-4α	rabbit	polyclonal	IHC	1:50	Santa Cruz	SC8987
MC4R	rabbit	polyclonal	IHC	1:200	Abcam	ab24233
			WB	1:500		
Ki-67	mouse	monoclonal	IHC	1:50	Dako	M7248
PCNA	mouse	monoclonal	WB	1:500	DAKO	M0879
phospho-ERK	rabbit	polyclonal	WB	1:1000	Cell signaling	CST9101
phospho-STAT3	rabbit	polyclonal	WB	1:1000	Cell signaling	CST 9131
STAT3	rabbit	polyclonal	WB	1:1000	Cell signaling	CST 9132

Table 4. Primary antibodies: The primary antibodies used for IHC and WB in this study are exhibited.

Secondary Antibody						
Target	Host	Clonalitiy	Applications	Dilution	Company	**Cat. #**
mouse IgG	rabbit	polyclonal/ HRP	WB	1:2000	DAKO	P0161
mouse IgG	goat	alexa fluor-488 -conjugated	IHC	1:200	Invitrogen	A11029
rabbit IgG	swine	polyclonal/ HRP	WB	1:2000	DAKO	
rabbit IgG	goat	alexa fluor -555 -conjugated	IHC	1:400	Invitrogen	A21424

Table 5. Secondary antibodies: The secondary antibodies used for IHC and WB in this study are shown.

2.7 Statistical analysis

All experimental data were shown as mean ± SEM. Student's t-Test and other appropriate methods were used to compare between groups. The RT-PCR and western blot data were statistically analyzed using Graph Pad Prism 4 software (San Diego, USA). Significant differences were accepted at $p < 0.05$.

3. RESULTS

3.1 Hepatic expression of *Mc4r*, *Tnf-α*, *Il-6* and *Mcp-1* mRNA after rat PHx

Using RT-PCR, the mRNA levels of *Mc4r*, *Tnf-α*, *Il-6*, and monocyte chemotactic protein-1 *(Mcp-1)* were analyzed. Compared with the control group, mRNA expression of *Mc4r* was significantly increased in rat livers 8 h and 16 h after PHx (Figure 3A, *p<0.05, respectively). Hepatic mRNA of *Il-6* and *Tnf-α* were significantly up-regulated 4 h and 8 h after PHx compared to control (Figure 3B, 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 16 h and 24 h after PHx compared to control. (Figure 3D; *p<0.05, respectively)

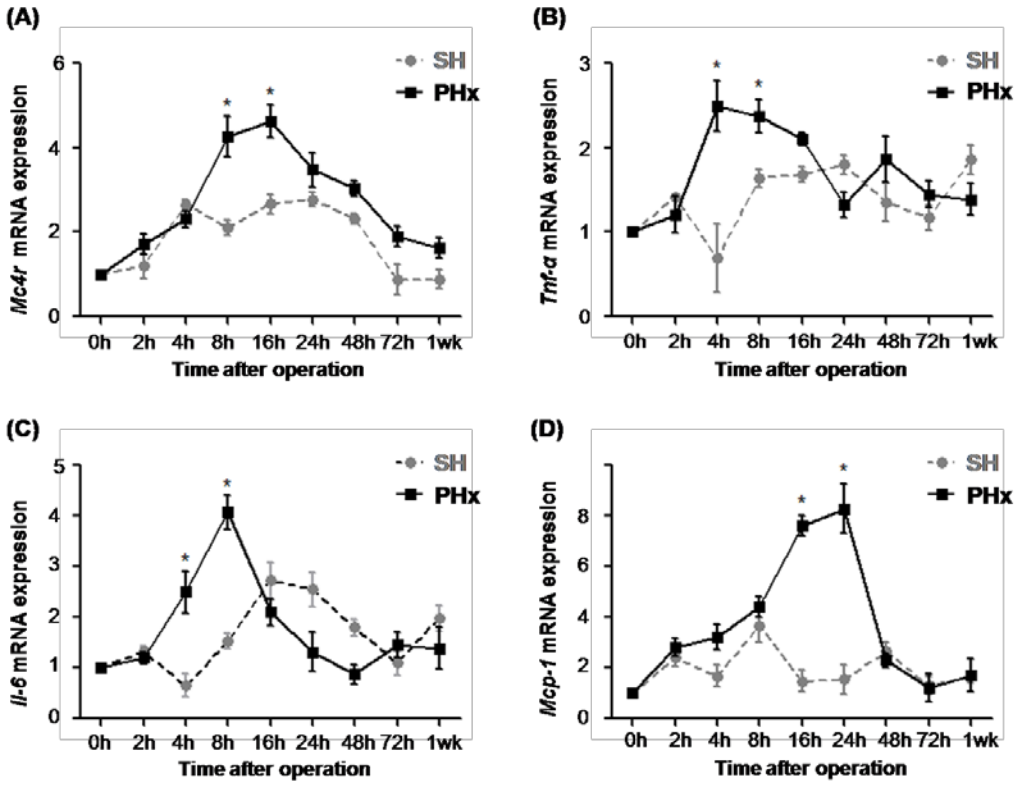


Figure 3. Total RNA from rat liver after PHx were analyzed by RT-PCR: (**A)** The mRNA level of hepatic *Mc4r* in PHx group were significantly higher than in SH group at 8 h and 16 h after operation (*p<0.05). *Tnf-α*, *Il-6,* and *Mcp-1* mRNA were up-regulated in PHx group (**B**, **C** and **D**; *p<0.05, respectively). Beta actin was served as a housekeeping gene.

3.2 Expression of MC4R after PHx in rat liver

3.2.1 Double IF staining of MC4R and HNF-4α in rat liver

It has been reported that hepatocyte nuclear factor-4 alpha (HNF-4α) is stably expressed in the HCs without significant change during rat liver regeneration. Therefore HNF-4α was used as a marker for HCs (Flodby et al. 1993). In order 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 4, most expression of the MC4R protein was overlapped with HNF-4 proteins. Additionally, in comparison with SH or control group, MC4R level was obviously increased at 24 h and 48 h after PHx.

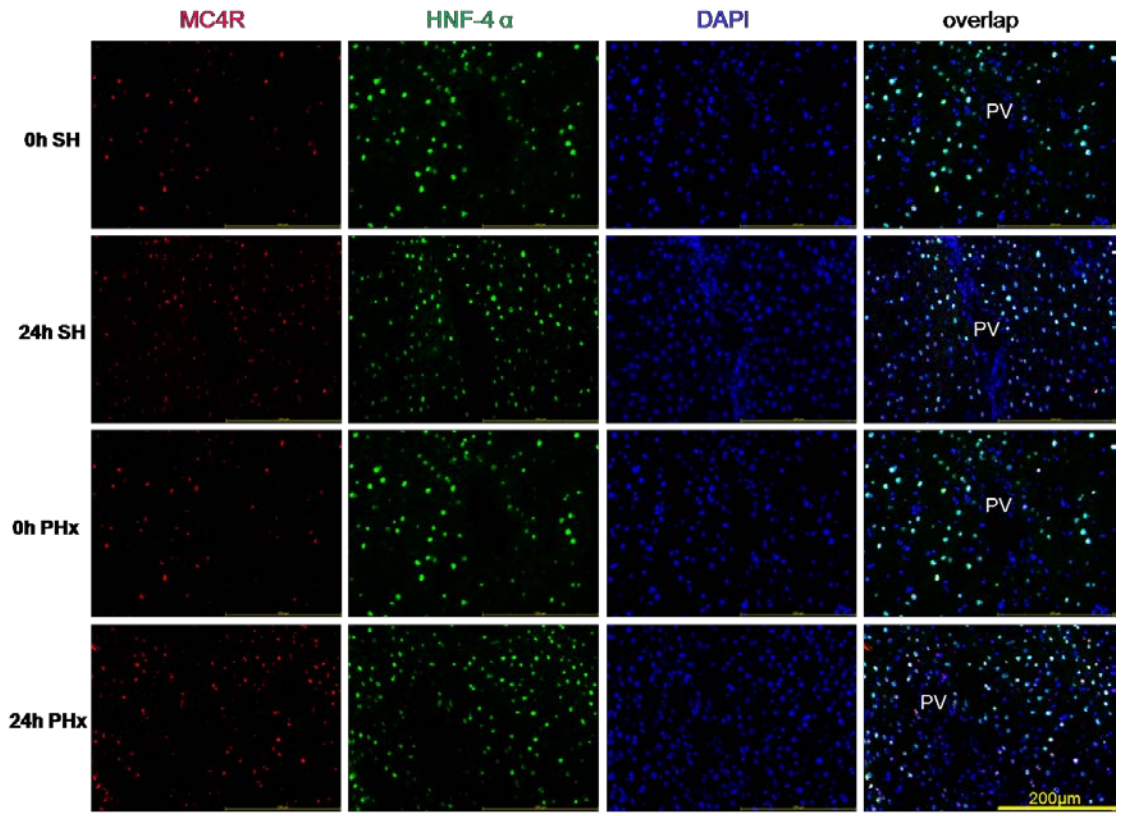


Figure 4. Rat liver double IF staining of MC4R and HNF- 4 α: Liver sections were co-stained with MC4R (red) and HNF- 4 α (green) and PHx groups. Merged pictures 0 h and 24 h after operation indicated that most of the MC4R positive cells were stained by HNF-4 α as well. (Nuclei were stained by DAPI (×200); PV=portal vein)

3.2.2 Double IF staining of MC4R and CK-19 in rat liver

CK-19, which was found in a large number of epithelial cell types (Bartek et al. 1986), has been used as a marker for biliary epithelial cells (Terada and Nakanuma 1993). Double IF staining of MC4R and CK-19 showed that expression of MC4R was rarely in the CK-19 positive cells during liver regeneration (Figure 5).

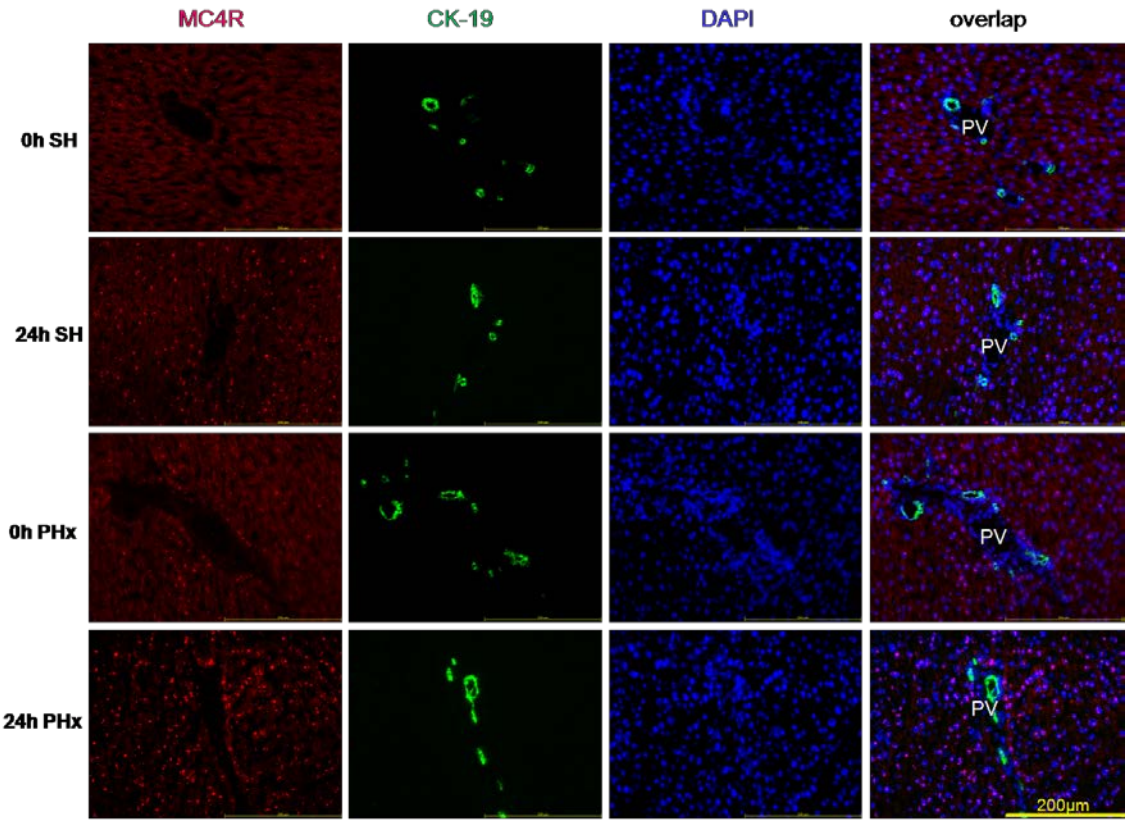


Figure 5. Rat liver double IF staining of MC4R and CK19: Liver tissues were double-stained for MC4R (red) and CK19 (green) in SH and PHx group. Microphotographs 0 h and 24 h after operation showed little MC4R positive cells were found in CK-19 positive cells. (Nuclei were stained by DAPI (×200); PV= portal vein)

3.2.3 Double IF staining of MC4R and ED-1 in rat liver

Double IF staining of MC4R and ED1, a marker for macrophage (Yin et al. 1999), showed little co-expression of MC4R in ED-1 positive cells (Figure 6).

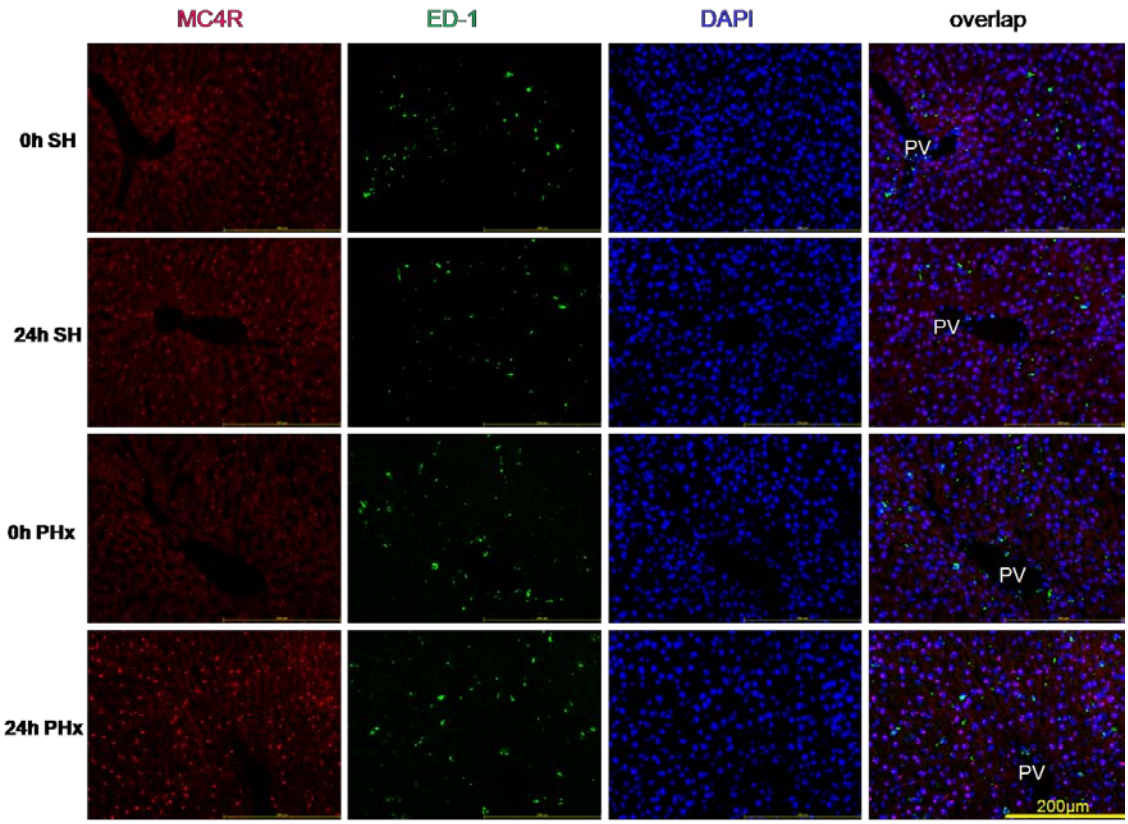


Figure 6. Rat livers double IF staining of MC4R and ED-1: Double IF staining of MC4R (red) and ED1 (green) were performed in SH and PHx rat liver. Pictures 0 h and 24 h after operation showed little co-expression of MC4R positive cells and ED-1 positive cells during rat liver regeneration. (Nuclei were stained by DAPI (×200); PV=portal vein)

3.2.4 Double IF staining of MC4R and Desmin in rat liver

Overlap of MC4R and desmin protein expression had not been observed (Figure 7). Shortly, the expression of MC4R after PHx was mainly found in HCs.

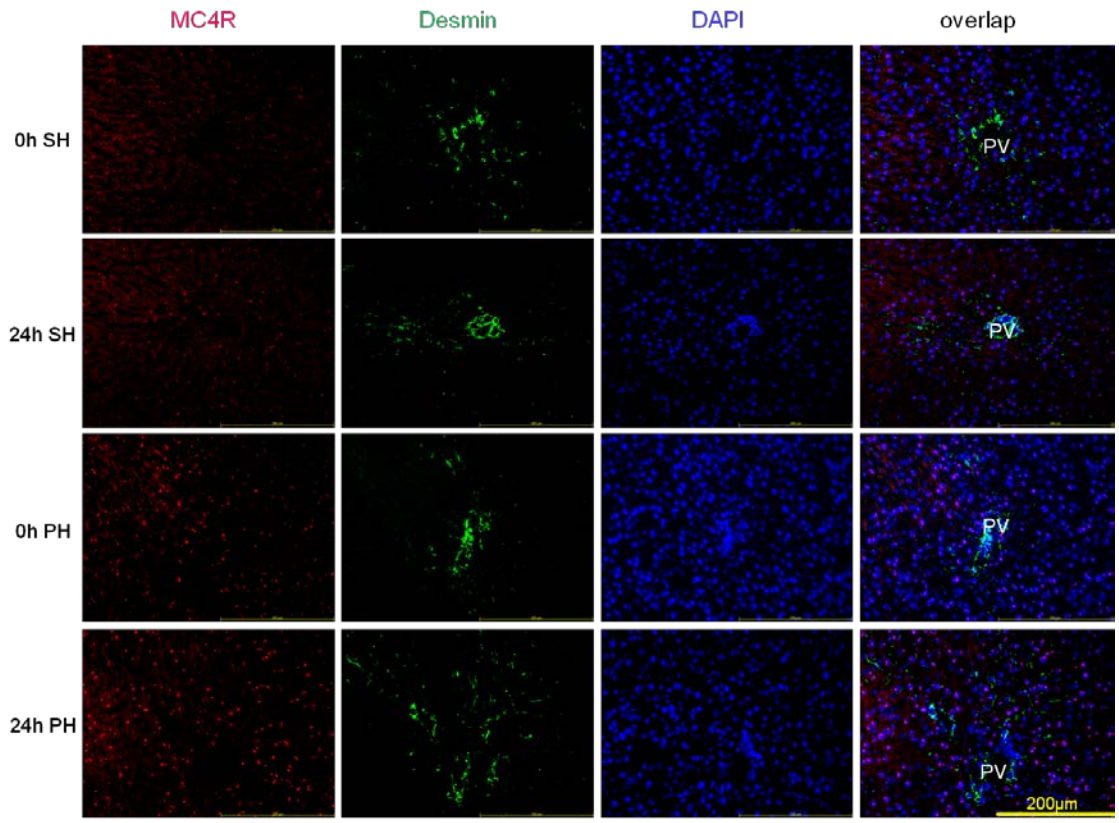


Figure 7. Rat liver double IF staining of MC4R and desmin: Double IF staining of MC4R (red) and desmin (green) were performed in SH and PHx rat liver. Pictures 0 h and 24 h after operation were showed. Little MC4R positive cells were Desmin positive during rat liver regeneration. (Nuclei were stained by DAPI (×200); PV= portal vein)

3.3 MC4R and regeneration after PHx

The expression of Ki-67 protein was mostly found in MC4R positive cells as shown in Figure 8A. However, not all MC4R positive cells were also positive for Ki-67. Regeneration indices (RI) of time points between 16 h and 1 wk were significantly higher after PHx than in control group (Figure 8B, $^{*}p<0.05$, respectively). Western blot analysis for proliferating cell nuclear antigen (PCNA) confirmed the results of RI calculated by Ki-67 positive cells as well (Figure 8C).

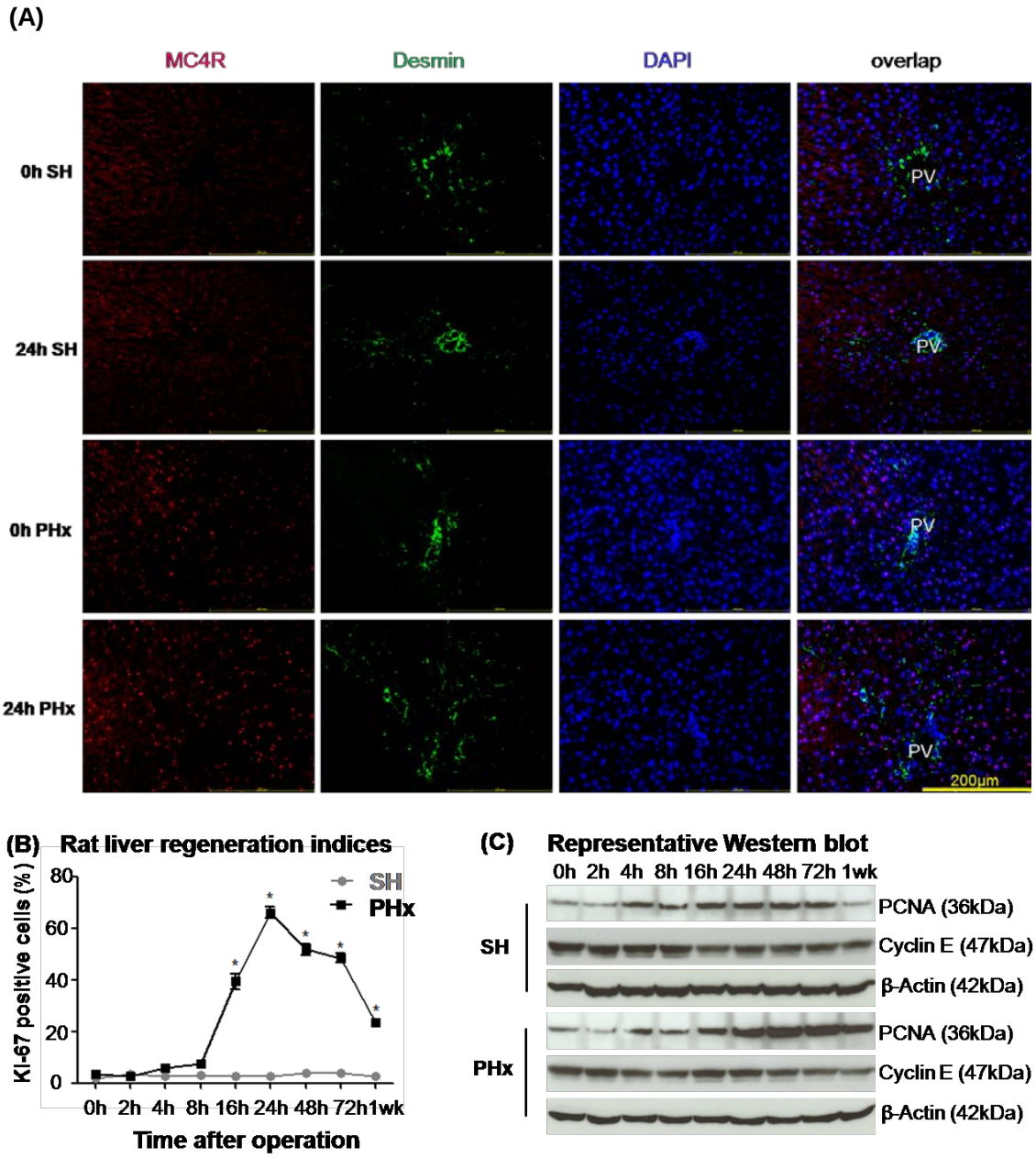


Figure 8. Expression of MC4R and rat liver regeneration: **(A)** Localization of MC4R and Ki-67 in HCs at representative points (0 h and 24 h; Nuclei were stained by DAPI (×200); PHx: partial hepatectomy; SH: Sham; PV: portal vein). **(B)** Regeneration indices were calculated by the percentage of Ki-67 positive HCs. **(C)** Expression of PCNA protein in rat liver, detected by Western blot.

3.4 Hepatic MC4R expression after rat PHx

The relative protein levels of MC4R were quantitatively evaluated by

western blot. As shown in Figure 9, protein levels of MC4R were significantly increased after PHx between 8 h and 48 h compared to the SH group (*p<0.05, respectively).

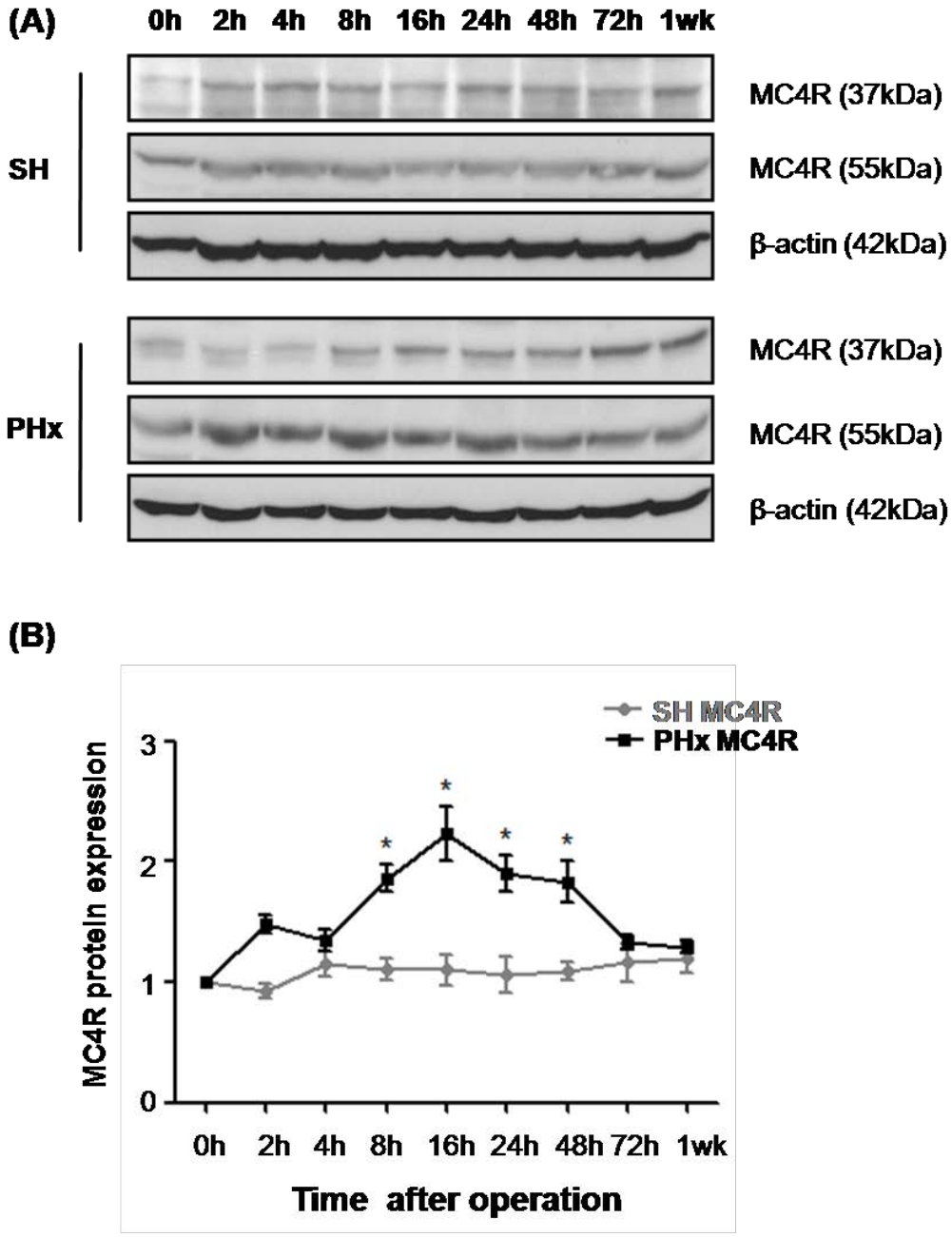


Figure 9. Western blot of MC4R protein in rat livers: **(A)** Representative bands for MC4R protein (normalized by β-actin). **(B)** Densitometric analysis of the relative MC4R protein: Between 8 h and 48 h after the operation, MC4R was significantly upregulated after PHx compared to the corresponding time point of SH group. (*p<0.05, respectively)

3.5 Phosphorylation of ERK in rat liver regeneration

In accordance to the dynamic MC4R changes, the levels of pERK were significantly elevated after PHx 8 h compared to SH groups (Figure 10, **p*<0.05, respectively). Indeed, as reported, targeting MC4R led to activation of the ERK pathway, subsequently triggered several transcriptional events, such as increasing of energy consuming and cell proliferation (Damm et al. 2012). Shortly, the data exhibited a correlation of MC4R and the phosphorylation of ERK during rat liver regeneration.

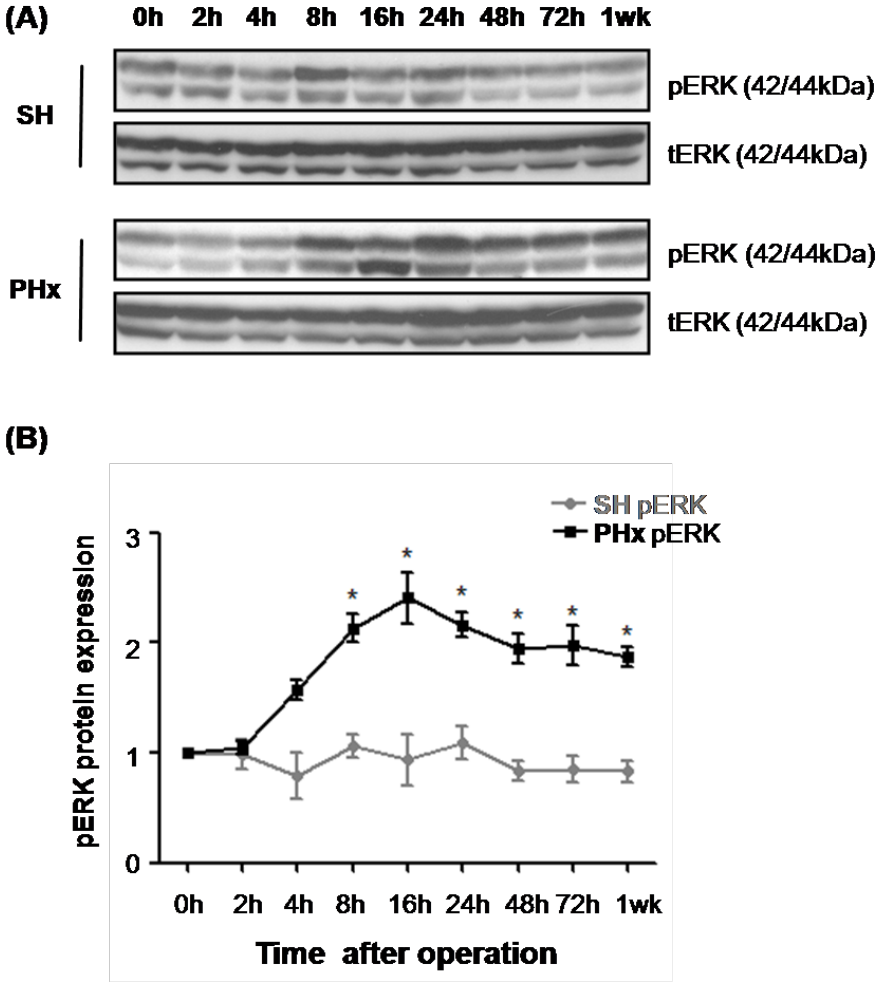


Figure 10. Western blot analysis of pERK in rat livers: **(A)** Representative bands for pERK protein (normalized by total ERK). **(B)** Densitometric analysis of the relative pERK: 8 h after operation, expression of pERK were significantly higher after PHx than in the corresponding SH group. (**p*<0.05, respectively)

3.6 PCNA expression of HCs after *in vitro* stimulation with α-MSH

As MC4Rs were predominantly expressed in HCs and upregulated after PHx, the isolated primary rat HCs were stimulated with α-MSH to examine its regenerative effect and potential mechanism. As shown in Figure 11, no significant changes of the PCNA level in HCs was found after the stimulation with MC4R agonist one hour compared with controls. The amount of PCNA protein were significantly increased 6 h and 24 h after α-MSH treatment compared to the controls (Figure 11.B; *p<0.05, respectively), exhibiting that the MC4R agonist promoted HCs proliferation.

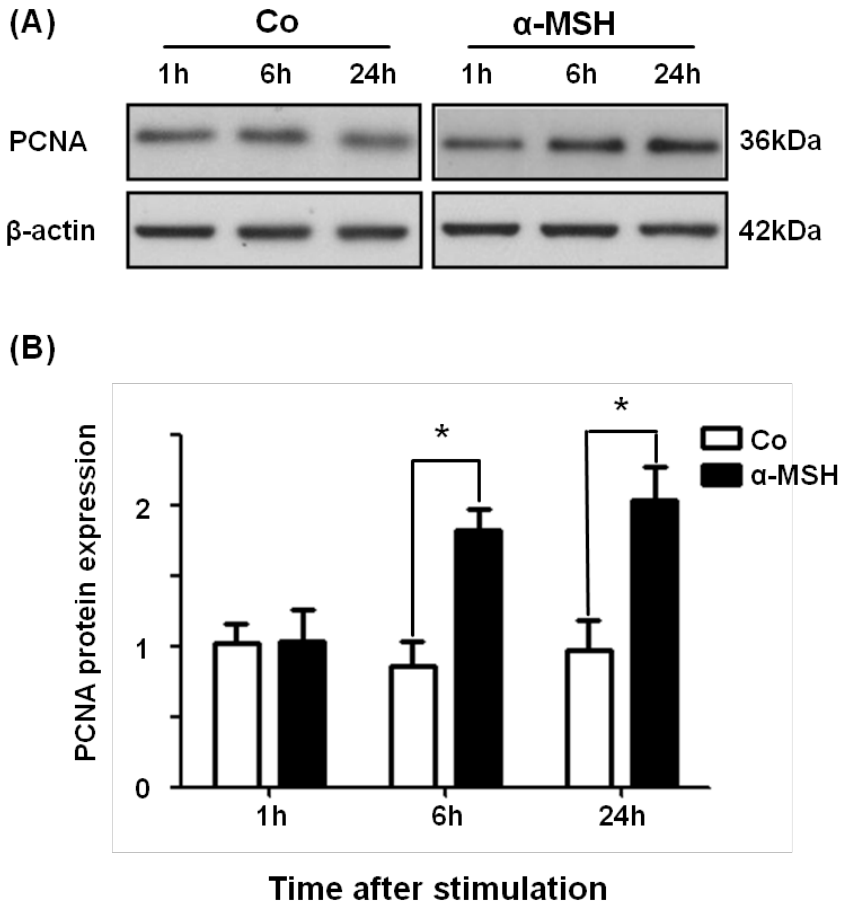


Figure 11. Immunoblotting analysis of PCNA in HCs after *in vitro* treated with α-MSH: **(A)** Representative bands for PCNA protein (normalized by β-actin). **(B)** Densitometric analysis of the relative PCNA showed: The PCNA expression were significantly increased after stimulation with α-MSH 6 h and 24 h compared to control groups. (*p<0.05, respectively)

3.7 pERK expression of HCs after *in vitro* stimulation with α-MSH

Correspondingly, the expression of phosphorylation of ERK was significantly elevated 6 h and 24 h after treatment of α-MSH compared to control groups (Figure 12.A and B, *p<0.05).

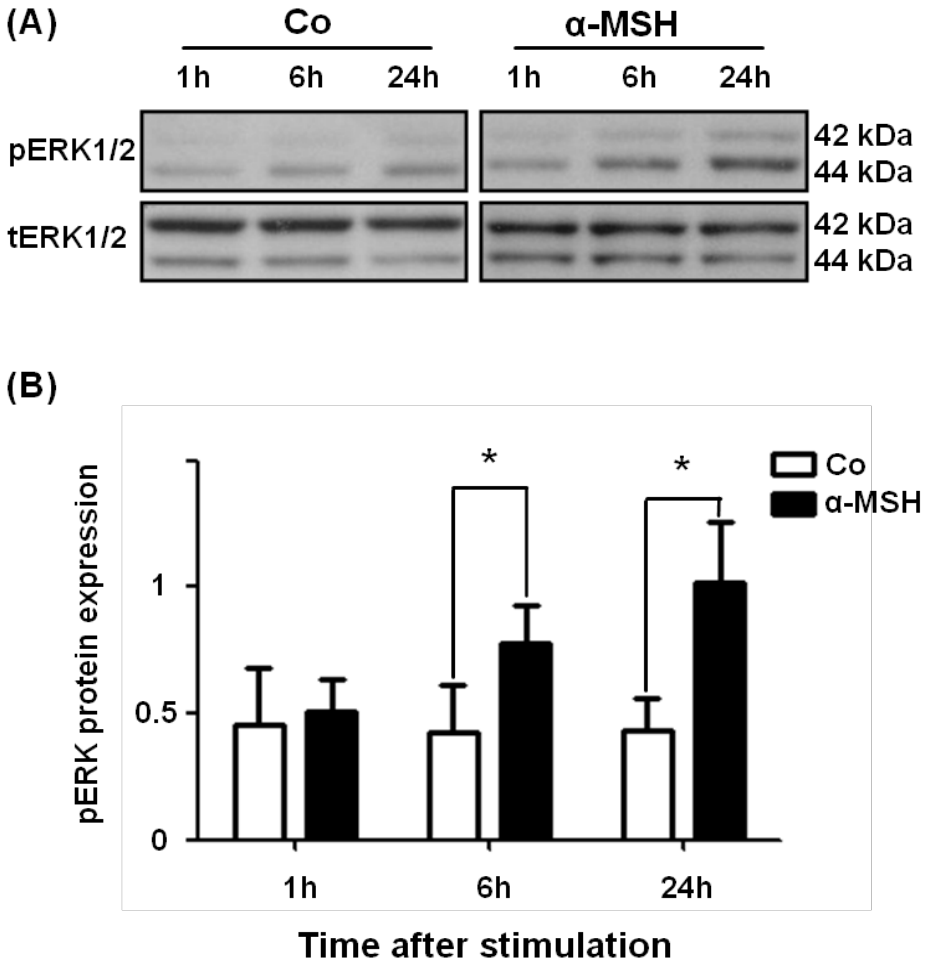


Figure 12. Western blot analysis of pERK in HCs: **(A)** Representative bands for pERK protein (normalized by tERK). **(B)** Densitometric analysis showed that 6 h or 24 h after treatment with α-MSH, expression of pERK was significantly higher than compared to controls. (*p<0.05, respectively)

3.8 pSTAT3 expression of HCs after *in vitro* stimulation with α-MSH

Inversely, the expression levels of pSTAT3 were significantly lower within the HCs 6 h and 24 h after α-MSH treatment compared to control groups

(Figure 13. A and B; *p<0.05). Indeed as reported previously, ERKs could contribute a rapid and inducible inhibition to the IL-6-induced STAT3 activation (Sengupta et al. 1998). The data from this study and literature indicated that activation of MC4R could promote the proliferation of HCs by increasing the level of pERK, and additionally may provide an inhibitory effect to pSTAT3.

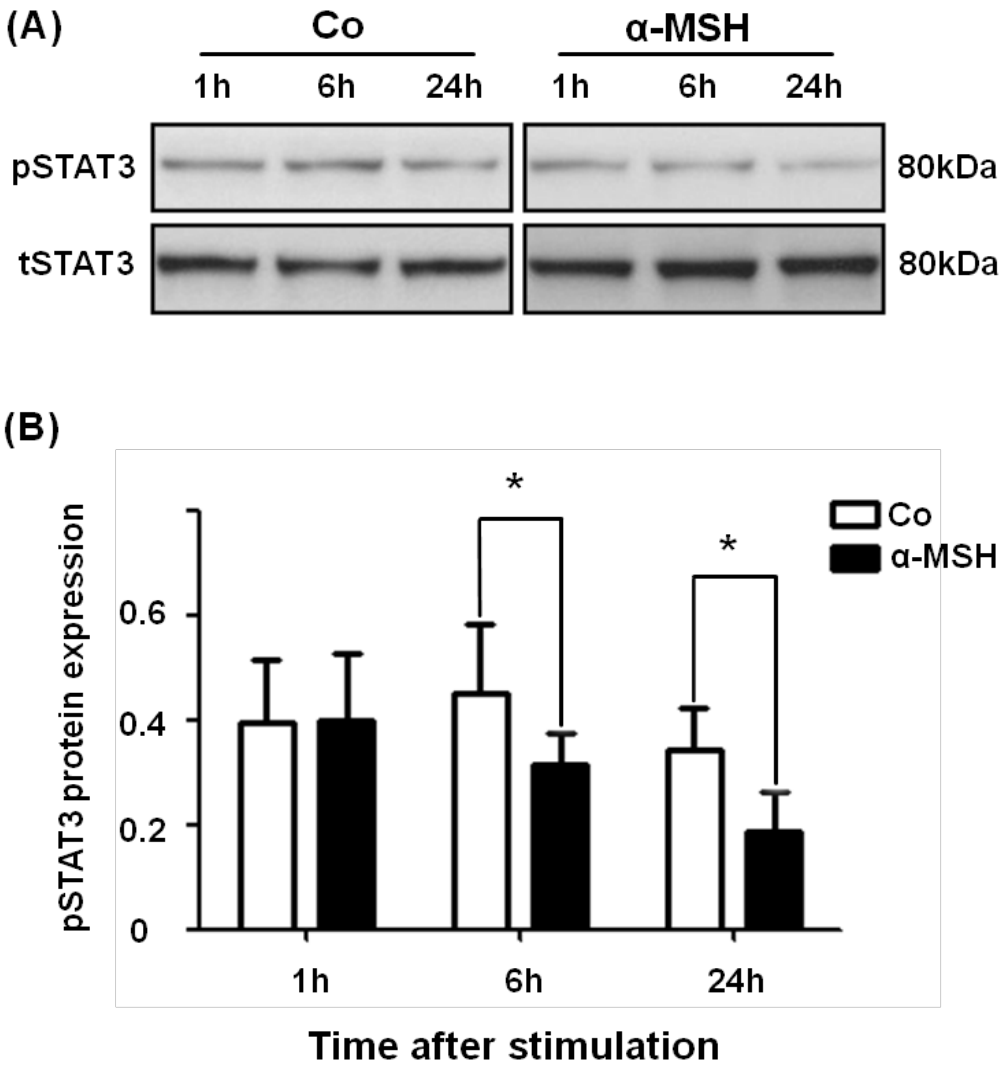


Figure 13. Western blot analysis of pSTAT3 in HCs: **(A)** Representative bands for the pSTAT3 protein (normalized by tSTAT3). **(B)** Densitometric analysis showed that: Expression of pSTAT3 was significantly lower 6 h and 24 h after treatment with α-MSH than compared to control group. (*p<0.05, respectively)

4. DISCUSSION

The current analysis showed that MC4R is principally localized in HCs and upregulated after PHx in the regenerating rat liver, which was correlated to increasing hepatic pERK expression. Moreover, *in vitro* study showed that primary HCs proliferation (PCNA) after stimulated with α-MSH. Furthermore, α-MSH controlled the regenerative signals via regulation of the ERK and STAT3 pathways, indicating that increased hepatic MC4R expression may apply an important role in rat liver regeneration.

4.1 Expression of MC4R in rat liver tissue

It was reported that, northern blotting indicated MC4R was primarily expressed in the brain (Gantz et al. 1993). Furthermore, extensive labeling study suggested that MC4R was also found in cornu ammonis 1 (CA 1) and CA 2 but not in CA 3 and CA 4 regions of the hippocampus. The *Mc4r* mRNA was found in the dentate gyrus, cortex, and amygdala as well (Gantz et al. 1993). Several studies of the MC4R location in the rat brain demonstrated that the *Mc4r* mRNA was widely expressed in thalamus, hypothalamus, cortex, brainstem, and spinal cord tissues (Kishi et al. 2003; Mountjoy et al. 1994; van der Kraan et al. 1999). In the hypothalamus, MC4R was reported to be expressed greatly in paraventricular nucleus (PVN), including both of magnocellular and parvicellular neurons. In a mouse study, it also could be shown that the expression of green fluorescent protein with the regulation of MC4R promoter had a comparable localization of green fluorescent protein as seen in the study using the technique of *in situ* hybridization (Kishi et al. 2003).

In addition to neurons, MC4Rs were also observed to be expressed in astrocytes (Caruso et al. 2007; Selkirk et al. 2007). Synthetic, endogenous peptide ligands as well as small molecular agonists of MC4R could increase cAMP level in rat astrocytes (Selkirk et al. 2007). These finding were consistent with prior studies demonstrating that α-MSH and ACTH increased cAMP levels

in astroglial cells (Evans et al. 1984; van Calker et al. 1983) and melanocortins stimulated proliferation, and induced morphological changes in cultured rat astrocytes (Zohar and Salomon 1992). $ACTH_{1-24}$ was also indicated to downregulate the production of ciliary neurotrophic factor mRNA in rat astrocytes of *in vitro* cultured (Kokubo et al. 2002). Interestingly, it was reported that functional MC4R was also seen in human epidermal melanocytes that could contribute to the process of melanogenesis (Spencer and Schallreuter 2009). It was suggested that the *Mc4r* mRNA was first expressed at embryonic day 14 in lamina terminalis, diencephalon, telencephalon, and spinal trigeminal nucleus of the rat; and up to embryonic day 19, the MC4R was widely transcripted in many other areas of the rat brain (Mountjoy and Wild 1998). The highest level of expression was in the autonomic nervous system. Similar data were confirmed when radiolabeled [Nle4, D-Phe7]- MSH (NDP-MSH) were applied as the probe administrated *in vivo* (Lichtensteiger et al. 1996). Although the NDP-MSH also binded to the MC3R, the binding sites detected with radiolabeled NDP-MSH were most likely MC4Rs because the MC3R genes were only expressed postnatally, but during the fetal period (Kistler-Heer et al. 1998).

Moreover, MC4R gene was also expressed in several peripheral tissues during the fetal period, for instance the developing heart (embryonic day 14), lung (embryonic day 16), muscles involved in respiration such as diaphragm and intercostal muscle (embryonic day 14) (Mountjoy et al. 2003). It has been reported that MC4R was identified not only in HCs but also in non-parenchymal cells (e.g. macrophages) in a systemic inflammatory rat model (Malik et al. 2011). In the present rat model of liver regeneration after PHx, expression of MC4R was mainly found in HCs, but little was in others cell types, such as macrophages, biliary cells and stellate cells. These data indicate that regulation of MC4R in different hepatic cell types may depend on the exact biological process. Therefore, it is understandable that MC4R has mainly been found in

HCs after PHx since hepatocyte proliferation is the major event during rat liver regeneration.

4.2 MC4R and cell proliferation

4.2.1 Source of endogenous alpha-MSH

The source of the endogenous α-MSH in the peripheral nervous system is still not clarified. The most possible spring of α-MSH could be the pituitary gland; indeed, it was suggested that the synthesis and release of melanocortins were initiated in the pituitary gland responsing to stress stimulation (Carr et al. 1990; Lindley et al. 1990; Lookingland et al. 1991). Therefore, α-MSH could be secreted into the blood when stress stimuli was triggered by nerve injury. Some reports also confirmed the possible presence of α-MSH-like peptides in the dorsal root ganglion (DRG) and degenerating nerves (Plantinga et al. 1992; Plantinga et al. 1995), whereas other study indicated that rat peripheral nerves contained no detectable α-MSH (Verhaagen et al. 1988).

4.2.2 MC4R and cells proliferation of CNS

α-MSH was described to stimulate nerve regeneration. In the culture of neuronal cells, alpha-MSH promoted the outgrowth of Neuro 2A cells (neuron cell line), fetal spinal cord cells, neonatal neurons dorsal root ganglion, and neonatal corticospinal nerve cells (Joosten et al. 1996; Peulve et al. 1994; Van der Neut et al. 1988; van der Neut et al. 1992). Van der Neut et al. measured neuritogenesis by ELISA to test neurofilament protein in dorsal root ganglion neurons of neonatal rat and stated that α-MSH stimulated neurite proliferation in a bell shaped and dose responsive manner with the maximal effect of 100 nmol/L (van der Neut et al. 1992). In the Neuro 2A cell line, the neurite proliferation effects of α-MSH was repressed by the MC4R antagonist, indicating that MC4R facilitated neurite elongation because of the stimulation of alpha-MSH (Adan et al. 1996). In the injury of spinal cord, α-MSH promoted

spinal neurite proliferation and improved functional recovery (Lankhorst et al. 1999). In animal experiments, administration of α-MSH rapidly increased the amount of myelinating cells and reduced the recovery duration of sensory and motor function following sciatic nerve injury (Bijlsma et al. 1983; Dekker 1988; Verhaagen et al. 1986). In contrast, treatment of an α-MSH antagonist decreased the number of functional recovery of animals (Plantinga et al. 1995).

Mechanically, it was suggested that MC4R agonists encouraged significant neurogenesis and long term functional recovery of memory and learning by using a gerbils model (Giuliani et al. 2011). As a possible mechanism treating of MC4R agonist affected the Wnt-3A signaling pathway (Giuliani et al. 2011). Consequently the ERK pathway was activated, both of which were involved in cell proliferation (Yun et al. 2005). Independently, another mouse study also showed that α-MSH also promoted neurite elongation via MC4R (Tanabe et al. 2007).

4.2.3 MC4R and cells proliferation of hepatocyte

In the current analysis, it could be shown *in vivo* that upregulation of MC4R correlated with the protein level of pERK during rat liver regeneration. Moreover in the current *in vitro* analysis*,* stimulation of MC4R significantly increased the expression of PCNA and pERK in isolated primary HCs. These findings suggested that targeting MC4R could lead to activation of the ERK pathway during rat liver regeneration and subsequently trigger many transcriptional events, such as increasing of energy consuming and cell proliferation. Interestingly, although activation of the ERK pathway showed a proliferative effect in normal cell types such as neurons and HCs, strong activation of ERK inhibited proliferation of the tumor cells (HepG2 cell line), while this inhibitory effect was completely restored in conditions of reduced expression of pERK induced by hepatocyte growth factor (Tsukada et al. 2001). It could be referred

that the ERK pathway bi-directionally regulated cell proliferation relying in the intensity of ERK phosphorylation, but the exact mechanism remains obscure.

4.3 MC4R and inflammation

In this study, a significant increase of IL-6 and TNF-α mRNA level could be observed in the remnant rat liver after PHx. This observation is comparable to the results in the literature (Trautwein et al. 1996). Primarily, the effect of IL-6 in hepatic regeneration was originally regarded to be proliferative, since *IL-6* KO mice showed a striking deficit in DNA synthesis after PHx (Yamada et al. 1997). Moreover, treatment with anti-TNF-α antibodies inhibited hepatic DNA replication (Akerman et al. 1992), and liver regeneration was dramatically blocked in mice of *Il-6* and *TNF receptor type I* (*Tnfr1*) KO (Cressman et al. 1996; Yamada et al. 1997). However, impaired liver regeneration in *Tnfr1* KO mice was corrected by IL-6 injection (Yamada et al. 1997). After the binding of IL-6 and TNF-α to their receptors (e.g. glycoprotein 80 and glycoprotein 130) on HCs, the proliferative signals were initiated. Subsequently, the STAT3 pathway was activated, and several genes were targeted. One important target gene of STAT3 was *Socs3*, which acted in the feedback loop to reduce ongoing activation of IL-6 signaling by decreasing the phosphorylation of STAT3 (Campbell et al. 2001).

4.4 STAT3 and ERK pathways in rat liver regeneration

Within this study, a significant downregulation of pSTAT3 could be observed in parallel with upregulation of pERK after stimulation with MC4R agonist. These findings supported the hypothesis that pERK could inhibit the ongoing activation of pSTAT3 (Campbell et al. 2001). Furthermore, activation of the ERK pathway may offer a positive signal for cell proliferation, while inhibited the regenerative signal induced by STAT3 pathway as well as *Socs3* did, because of the proliferation of cells was definitely not only controlled by a single signaling pathway. These findings could be a possible explanation to the

bi-directionally regulation of the ERK pathway in cell proliferation, depending on the intensity of the regenerative signal and the signals from other regulators, e.g. STAT3 pathway. Additionally, this interaction between ERK and STAT3 pathways also could be an alternative mechanism for the anti-inflammatory effect of MC4R agonists. MC4R, which was mainly functional in the energy homeostasis as above mentioned, could provide proliferative and anti-inflammation effects. This unique modulation could be considerable to be applied in some complicated pathophysiological processes, e.g. hepatocelluar cancer after surgery with the issues of inflammation, HCs regeneration and tumor recurrence. Clearly, further *in vivo* studies are required to explore this topic.

4.5 Conclusion

In conclusion, expression of MC4R was predominantly localized and up-regulated in HCs during rat liver regeneration. Activation of hepatic MC4R could promote proliferation of rat HCs associated to the ERK signaling pathway. Additionally, these findings indicated that activation of MC4R could modify the regenerative signal of STAT3 pathway as well (Figure 14).

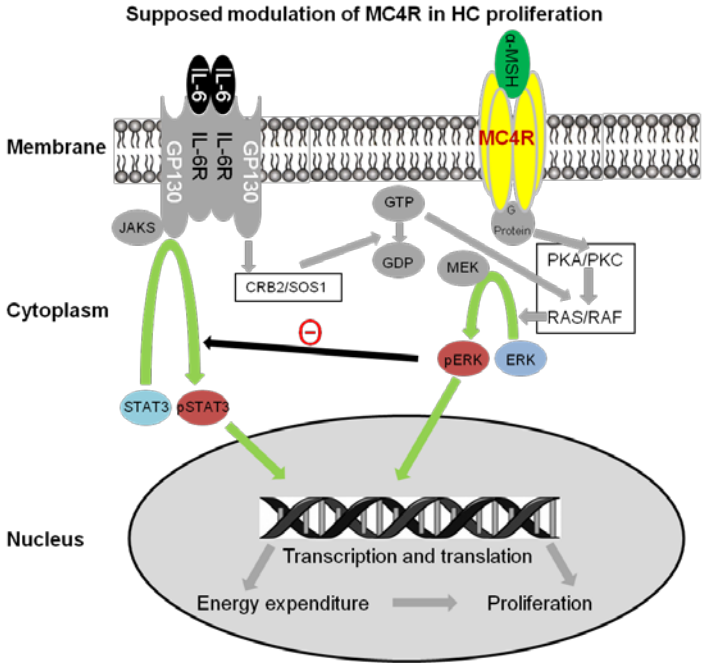


Figure 14. MC4R in HCs proliferation: As MC4R was targeted by α-MSH, phosphorylation of ERK was increased and subsequently provided an inhibitory effect to the ongoing activation of STAT3. Consequently, regenerative signals from these two pathways were modulated. (illustrated by Xu)

4.6 Limitations of this experimental study

In the present study, the expression and upregulation of MC4R was analyzed in rat livers after PHx. Moreover, the present data indicated that the possible mechanism was related to the ERK and STAT3 pathway and the interaction between both of them. However, the furthermore *in vivo* data are still needed to validate this hypothesis. For example, administration of MC4R agonist after PHx may provide stronger and direct evidences to the proliferative actions of MC4R in rat liver regeneration. Treatment with inhibitors of the ERK and/or STAT3 pathway could help to understand role of both pathway in liver regeneration. Furthermore, mice PHx model with the genetically modified MC4R, ERK and/or STAT3 pathways could provide significant understand to this topic.

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