Research Article

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# Loss of $G_s\alpha$ impairs liver regeneration through a defect in the crosstalk between cAMP and growth factor signaling

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**Background & Aims**: The stimulatory G protein α subunit ($G_s\alpha$) activates the cAMP-dependent pathway by stimulating the production of cAMP and participates in diverse cell processes. Aberrant expression of $G_s\alpha$ results in various pathophysiological disorders, including tumorigenesis, but little is known about its role in liver regeneration.

**Methods**: We generated a hepatocyte-specific $G_s\alpha$ gene knockout mouse to demonstrate the essential role of $G_s\alpha$ in liver regeneration using a mouse model with 70% partial hepatectomy (PH) or an intraperitoneal injection of carbon tetrachloride ($CCl_4$).

**Results**: $G_s\alpha$ inactivation dramatically impaired liver regeneration and blocked proliferating hepatocytes in G1/S transition due to the simultaneous depression of cyclin-dependent kinase 2 (CDK2) and cyclin E1. Loss of $G_s\alpha$ led to a fundamental alteration in gene profiles. Among the altered signaling cascades, the MAPK/Erk pathway, which is downstream of growth factor signaling, was disrupted secondary to a defect in phosphorylated Raf1 (pRaf1), resulting in a deficiency in phosphorylated CREB (pCREB) and CDK2 ablation. The lack of pRaf1 also resulted in a failure to phosphorylate retinoblastoma, which releases and activates E2F1, and a decrease in cyclin E1. Although these factors could be phosphorylated through both $G_s\alpha$ and growth factor signaling, the unique function of Raf1 in the growth factor cascade collapsed in response to the lack of $G_s\alpha$.

**Conclusion**: The growth factor signaling pathway that promotes hepatocyte proliferation is dependent on $G_s\alpha$ signaling. Loss of $G_s\alpha$ leads to a breakdown of the crosstalk between cAMP and growth factor signaling and dramatically impairs liver regeneration.



## Introduction

The liver has a unique capacity to rapidly and completely regenerate after a partial hepatectomy (PH) or chemical injury [1]. Following a two-thirds (or 70%) PH in rodents, quiescent hepatocytes synchronously re-enter the cell cycle and divide until they restore the original liver mass over a period of approximately 7 days. Three types of transmembrane receptors transmit extracellular mitotic signals, including ion channel-linked receptors, enzyme-linked receptors, and G protein-coupled receptors (GPCRs). Ion channel-linked receptors are mainly involved in rapid signaling events in electrically excitable cells, such as neurons. The roles of enzyme-linked receptor (including growth factor receptors and cytokine receptors) cascades that regulate liver regeneration have been comprehensively explored; however, little is known about the function of GPCRs in the regenerating liver.

Guanine nucleotide-binding proteins (G proteins) comprise a family of proteins that are involved in the transmission of GPCR-related signals from a variety of external stimuli to the interior of the cell [2]. There are two classes of G proteins: the first class functions as monomeric small GTPases, whereas the second class participates in a heterotrimeric G protein complex of α, β, and γ subunits. This protein complex functions as a molecular switch, in that when the heterotrimeric complex combines with a ligand, GDP is replaced by GTP and is released from the Gα subunit. This process is followed by the dissociation of Gα from Gβγ [3]. The stimulatory G protein α subunit ($G_s\alpha$) activates the cAMP-dependent pathway via the stimulation of the production of cAMP from ATP [4]. cAMP then acts as a second messenger that interacts with and activates protein kinase A

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*Abbreviations:* cAMP, cyclic adenosine monophosphate; $CCl_4$, carbon tetrachloride; CDK, cyclin-dependent kinase; CREB, cAMP-responsive element-binding protein; Erk, extracellular signal-regulated kinase; GPCR, G-protein-coupled receptor; $G_s\alpha$, the stimulatory G protein α subunit; JNK, c-Jun N-terminal kinases; MAPK, mitogen-activated protein kinases; PH, partial hepatectomy; PKA, protein kinase A; Rb, retinoblastoma protein.

(PKA) [5]. PKA can phosphorylate countless downstream targets that are involved in a number of pathways.

The $G_s\alpha$ gene (*GNAS* in humans and *Gnas* in mice) is a complex imprinted gene that encodes multiple gene products through the use of alternative promoters and its first exon. Accumulating evidence has demonstrated that aberrant expression of $G_s\alpha$ leads to various dysfunctions in cell growth, proliferation, apoptosis, differentiation, and metabolism. Liver-specific disruption of $G_s\alpha$ increases hepatic glycogen synthesis and reduces the expression of enzymes involved in gluconeogenesis [6]. Mice with β-cell-specific $G_s\alpha$ deficiency develop severe early-onset, insulin-deficient diabetes accompanied by a severe defect in β-cell proliferation [7]. Recent studies have indicated that mutations in *GNAS* or G protein dysfunction are related to many diseases [8]. *GNAS* mutations are involved in the tumorigenesis of hepatobiliary and pancreatic tissues originating from the foregut endoderm [9]. Activating point mutations at codon 201 of *GNAS* have been detected in approximately two-thirds of intraductal papillary mucinous neoplasms and in half of intraductal papillary neoplasms of the bile duct [10,11]. Frequent *GNAS* mutations have also been identified in intrahepatic cholangiocarcinomas and are associated with poor overall survival [11]. Although often absent in hepatocellular carcinoma, somatic *GNAS*-activating mutations are involved in the molecular pathway of hepatocellular adenomas by activating the IL6/STAT3 cascade [12]. $G_s\alpha$ seems to be closely involved in the regulation of cell proliferation, but the signaling pathway remains unclear. In the present study, we used a liver-specific $G_s\alpha$ knockout mouse model to define the biological function of $G_s\alpha$ in liver regeneration.

## Materials and methods

### *Mice*

These experiments were approved by the Animal Care and Use Committee of Sichuan University. $G_s\alpha^{loxP/loxP}$ mice were kindly provided by Dr. HS Li, West China Second University Hospital, Sichuan University. Albumin-Cre transgenic mice were purchased from Cyagen Biosciences Inc., Guangzhou, China. Hepatic-$G_s\alpha^{-/-}$ mice were generated by crossing $G_s\alpha^{loxP/loxP}$ mice to albumin-Cre mice, and their genotypes were determined using PCR amplification of tail DNA (the primers for PCR are listed in Supplementary Table 1). Eight-week-old male mice were challenged by classic 70% PH surgery or an intraperitoneal injection of 10 ml/kg body weight of a 10% solution of carbon tetrachloride ($CCl_4$) in olive oil as previously described [13].

### *Histology and immunohistochemistry*

Liver specimens for histology and immunohistochemistry were fixed in 10% buffered formalin for 48 h. Paraffin sections (4 μm thick) were prepared for hematoxylin and eosin (H&E) staining. DNA synthesis and cell proliferation were measured by BrdU incorporation assays and immunohistochemical staining for Ki67.

### *Western blotting*

The liver tissues were homogenized for protein extraction. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were performed, and an ECL reagent was used for chemiluminescence detection. The antibodies and reagents used in this study are listed in Supplementary Table 2.

### *mRNA isolation and real-time RT-PCR*

Total mRNA was purified from 50 mg of liver tissue preserved in RNAlater with the RNeasy Mini kit (QIAGEN). mRNA was reverse transcribed to cDNA using an iScriptcDNA Synthesis kit (Bio-Rad). A CFX Connect Real-Time System (Bio-Rad) was used for all real-time PCR. cDNA template was diluted 1:10 and amplified using SoAdvanced Universal SYBR Green Supermix (Bio-Rad) under standard conditions. Gene expression levels were normalized to GAPDH using the comparative CT method. The sequences of the indicated primers are presented in Supplementary Table 3.

### *Immunoprecipitation*

Cell lysates were prepared using RIPA buffer containing a phosphatase inhibitor and protease inhibitor cocktail. A Reversible Immunoprecipitation System kit (Millipore) was used according to the manufacturer's instructions. The antibodies used in this study are listed in Supplementary Table 2.

### *Chromatin immunoprecipitation assay*

A chromatin immunoprecipitation assay (ChIP) was performed using a Magna ChIP™ G Chromatin Immunoprecipitation Kit from Millipore according to the manufacturer's instructions. The sequences of primers for the *cdk2* and *cyclin E1* genes were presented in Supplementary Table 4.

### *Cell culture and siRNA transfection*

The mouse liver cancer cell line Hepa1-6 was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were maintained at 37 °C in a 5% (v/v) $CO_2$ atmosphere and subcultured every 3 days. Transfection with siRNAs against the *Gnas* gene (5′GCAGCAAGCUCAUCGACAAT T3′) was performed using Lipofectamine2000. Scrambled siRNA was used as a control. Before transfection, the cells were synchronized with 100 ng/ml nocodazole for 16 h.

### *Immunofluorescence*

The cells were seeded onto coverslips that were pre-coated with fibronectin. At 48 h after transfection, the cells were permeabilized in 0.1% Triton X-100 for 10 min. Immunofluorescence analysis was performed, and the immunostained cells were visualized with a Leica DM4000B Fluorescence microscope.

### *Statistical analysis*

The results are expressed as the mean ± standard deviation (SD). The differences between groups were tested for significance using an unpaired, 1-tailed Student's *t* test with Welch correction. $p < 0.05$ was considered as significant.

For more details see Supplementary material.

## Results

### *Impaired liver regeneration in $G_s\alpha^{-/-}$ mice following 70% PH or $CCl_4$ challenge*

The hepatic-$G_s\alpha^{-/-}$ mice presented normal survival, body weight, food intake, and metabolic rates, consistent with a previous work by Chen M and colleagues [6]. The mutant livers were much larger in size and displayed greater glycogen deposition than their wild-type (WT) littermates (Supplementary Fig. 1).

To investigate the role of $G_s\alpha$ in liver regeneration, we performed a 70% PH on $G_s\alpha^{-/-}$ and WT mice. To our surprise, approximately 65% of the $G_s\alpha^{-/-}$ mice died within 30 h of surgery (Fig. 1A), and the surviving mice exhibited much slower liver recover rates (Fig. 1B, C). Although hepatic glycogen was much higher in mutant livers before surgery, it was remarkably decreased after PH, while in the WT regenerating livers, the glycogen remained at a relative stable level (Supplementary Fig. 2). As indicated by BrdU and Ki67 immunoreactivity and mitotic figures, active proliferation was observed in the