Deceleration of liver regeneration by knockdown of augmenter of liver regeneration gene is associated with impairment of mitochondrial DNA synthesis in mice

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Han L, Dong L, Yu H, Sun G, Wu Y, Gao J, Thasler W, An W. Deceleration of liver regeneration by knockdown of augmenter of liver regeneration gene is associated with impairment of mitochondrial DNA synthesis in mice. Am J Physiol Gastrointest Liver Physiol 309: G112–G122, 2015. First published May 14, 2015; doi:10.1152/ajpgi.00435.2014. — Hepatitic stimulator substance, also known as augmenter of liver regeneration (ALR), is a novel hepatic mitogen that stimulates liver regeneration after partial hepatectomy (PH). Recent works have indicated that a lack of ALR expression inhibits liver regeneration in rats, and the mechanism seems to be related to increased cell apoptosis. The mitochondria play an important role during liver regeneration. Adequate ATP supply, which is largely dependent on effective mitochondrial biogenesis, is essential for progress of liver regeneration. However, ALR gene expression during liver regeneration, particularly its function with mitochondrial DNA synthesis, remains poorly understood. In this study, ALR expression in hepatocytes of mice was suppressed with ALR short-hairpin RNA interference or ALR deletion (knockout, KO). The ALR-defective mice underwent PH, and the liver was allowed to regenerate for 1 wk. Analysis of liver growth and its correlation with mitochondrial biogenesis showed that both ALR mRNA and protein levels increased robustly in control mice with a maximum at days 3 and 4 post-PH. However, ALR knockdown inhibited hepatic DNA synthesis and decelerated liver regeneration after PH. Furthermore, both in the ALR-knockdown and ALR-KO mice, expression of mitochondrial transcription factor A and peroxisome proliferator-activated receptor-γ coactivator-1α were reduced, resulting in impaired mitochondrial biogenesis. In conclusion, ALR is apparently required to ensure appropriate liver regeneration following PH in mice, and deletion of the ALR gene may delay liver regeneration in part due to impaired mitochondrial biogenesis.

hepatic stimulator substance; augmenter of liver regeneration; mitochondrial biogenesis; liver regeneration

THE LIVER IS WELL-KNOWN FOR ITS EXTRAORDINARY ABILITY TO REGENERATE FOLLOWING 70% PARTIAL HEPATECTOMY (PH) OR INJURY (9). PH was first described by Higgins and Anderson in 1931 (17) in a model that clearly demonstrated the regenerative capacity of the liver. The entire process of liver regeneration following PH takes up to 5–7 days in rodents, during which time the remnant liver lobes grow via extensive proliferation of all hepatic cell types. Although numerous biochemical factors are involved in liver regeneration, only a few have been found to be capable of inducing hepatocyte proliferation in vitro (5). In 1975, La-
also triggered mitochondrion-associated apoptosis, implying that ALR is an essential survival factor in hepatocytes (40).

Mitochondrial function is a known prerequisite for the progression of liver regeneration. During PH-induced liver regeneration in rats, mitochondrial oxidative phosphorylation in the remaining hepatocytes is extensively enhanced to maximize the production of ATP to ensure an adequate energy supply for cellular DNA synthesis and hepatocyte proliferation (4, 20). Failure to mobilize mitochondrial activities could lead to a marked delay in liver regeneration (36). In regard to the association of ALR with liver regeneration, as previously reported, ALR is quickly released into the serum within 24 h after PH, whereas the hepatic level of ALR remained decreased for 12 h post-PH in adult rats (14). The results of a later study reported that ALR treatment inhibited the expression of apoptotic genes for 24 and 48 h post-PH in rats with a parallel increase in Bcl-2 expression (34). Very recently, Francavilla et al. reported that ALR knockdown led to a delay in liver regeneration, which was possibly due to an increase in cell apoptosis (12). Undoubtedly, oxidative stress and reactive oxygen species (ROS) are often accompanied with PH-induced injury and are believed to be one of the most common causes of cell death, which can result in poor progression of liver regeneration. However, on the other hand, mitochondrial biogenesis plays a pivotal role during liver regeneration. It is widely accepted that sufficient energy production is exclusively dependent on oxidative phosphorylation in the mitochondrial membrane. Yeast cells that lack functional Erv1 have an incomplete mitochondrial genome, loss in membrane potential, disordered respiration capacity, and abnormal mitochondrial morphology/distribution, resulting in significantly reduced cell growth. Mammalian cells with mutant ALR have thus far been only reported in patients with myopathy manifesting deficiencies in the mitochondrial respiratory chain. However, the impact of ALR defects with mitochondrial biogenesis and its relation to liver regeneration await to be elucidated. Therefore, we thought that it would be very interesting to investigate whether the downregulation of ALR expression might impair mitochondrial biogenesis, thereby possibly contributing to another potential mechanism impeding liver regrowth after PH.

In the present study, ALR expression during liver regeneration was investigated. Inhibition of ALR was achieved by gene silencing with short-hairpin RNA (shRNA) or knockout (KO) in an animal model. Our results clearly exhibited that impairment of mitochondrial DNA (mtDNA) biogenesis might underlie the deceleration of PH-induced liver regeneration.

**MATERIALS AND METHODS**

**Animals and treatments.** C57BL/6j mice weighing 18–20 g were obtained from the Academy of Military Medical Sciences (Beijing, China). PH was performed, and liver regeneration was recorded as described by Higgins and Anderson (17). All animals were treated humanely, and the experimental protocols were approved by the Human and Animal Ethics Committee of Capital Medical University (Beijing, China). The resected liver tissues were weighed to ensure an equal extent of PH between animals. Sham-operated mice underwent laparotomy and liver manipulation rather than PH. A total of 50 mice were randomly assigned to one of two groups. In the ALR-silent mice, 100 μg of scramble-control plasmid for ALR-shRNA was administered via portal vein injection. In the acute liver injury and regeneration model, C57BL/6j and ALR-KO mice were administered CCl4 (Sigma-Aldrich, St. Louis, MO) dissolved in olive oil (30%, vol/vol) via a single intraperitoneal injection (1 μl/g body wt). Mice in the control group received the same volume of vehicle (olive oil) intraperitoneally.

**Calculation of liver weight and recovery.** The mouse liver weight recovery (LMR) was estimated according to the following formula: 

\[
\text{LMR} = 100\% \times \frac{Ma}{Mb}
\]

where Ma is the weight of the liver at death and Mb is the total liver weight before PH, which is estimated by dividing the weight of the resected segment by 0.7 (e.g., 70% resection in this experiment).

**Magnetic resonance imaging.** The experimental mice were anesthetized (4% chloral hydrate administrated ip at 10 ml/kg body wt) and examined using a horizontal 7-T Biospec spectrometer (Bruker Medical, Ettlingen, Germany) with a 3.5-cm birdcage coil. Coronal T2-weighted spin-echo images of the entire liver were acquired for alignment and to determine liver volume. Zero filling of k-space was applied, resulting in a matrix of 256 × 256 pixels.

**Generation of ALR-KO mice.** Embryonic stem cell (ESC) clone DEPD00501_2_D08 from the International Mouse Phenotyping Consortium was used to produce Gfeptm1/KOMP KO mice using standard blastocyst injection and chimera breeding techniques. Blastocyst injections were performed by Cyagen Biosciences (Santa Clara, CA). Highly chimeric males (by coat color) were bred with C57BL/6j mice to obtain homogeneous homozygous offspring. The resultant offspring were randomly assigned to one of two groups. In the ALR-shRNA group, male mice were also triggered mitochondrion-associated apoptosis, implying that ALR is an essential survival factor in hepatocytes (40).

**ALR-shRNA.** Target sites in the mouse ALR mRNA (accession no.: NM_023040), which are shown in Table 1, were selected using an RNAi design site (Shanghai GenePharma, Shanghai, China). Four shRNAs (ALR shRNA-1 to ALR shRNA-4) targeting different regions of ALR mRNA and a scrambled control shRNA that does not correspond to any mammalian mRNA were generated. All ALR shRNA plasmids constructs were verified by DNA sequencing. To minimize off-target effects, two ALR shRNA constructs (shRNA-2 and shRNA-4) were applied in the knockdown assay.

**Generation of ALR-KO mice.** Embryonic stem cell (ESC) clone DEPD00501_2_D08 from the International Mouse Phenotyping Consortium was used to produce Gfeptm1/KOMP KO mice using standard blastocyst injection and chimera breeding techniques. Blastocyst injections were performed by Cyagen Biosciences (Santa Clara, CA). Highly chimeric males (by coat color) were bred with C57BL/6j mice to obtain homogeneous homozygous offspring. The resultant offspring were randomly assigned to one of two groups. In the ALR-shRNA group, male mice were

### Table 1. **HSS shRNA, nonsilencing sequences, and PCR primers used in the experiment**

<table>
<thead>
<tr>
<th>shRNA</th>
<th>Sequences</th>
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<tbody>
<tr>
<td>HSS shRNA-1</td>
<td>GCCGTGCACAAATGAGGTGAATC</td>
</tr>
<tr>
<td>HSS shRNA-2</td>
<td>GATTCACCCTCAATGGGAGGCG</td>
</tr>
<tr>
<td>HSS shRNA-3</td>
<td>GCCAGAAGCCAACACACAGGATAT</td>
</tr>
<tr>
<td>HSS shRNA-4</td>
<td>GTCAGAGTATAGTACGAGGTTCG</td>
</tr>
<tr>
<td>NC shRNA</td>
<td>AAGCTCTCATCTGAGGACCCG</td>
</tr>
<tr>
<td>PC shRNA</td>
<td>ATGCAGAGAAGAGAAGAAGTAAGCAGCTCAAG</td>
</tr>
<tr>
<td>MTRNR1</td>
<td>CTTGTGCGTGTGGGCTCACATAC</td>
</tr>
<tr>
<td>RMB15</td>
<td>GAGGAGCCGTTTTAATTACGTTGAAA-3'</td>
</tr>
<tr>
<td>18S RNA</td>
<td>GAGAAGGCAAGGCCGAGGAGGCGA-3'</td>
</tr>
<tr>
<td>EF1 alpha</td>
<td>GAAAGAAGGCCCAGTGGTGTA-3'</td>
</tr>
<tr>
<td>PGC-1a</td>
<td>GAGGAGCCAAATCAGGAGGAGG-3'</td>
</tr>
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**HSS, hepatic stimulator substance; NC, negative control; PC, positive control; shRNA, short-hairpin RNA; MTRNR1, mitochondrial encoded 12S RNA; RMB15, RNA-binding motif protein 15; TFAM, mitochondrial transcription factor A; PGC-1α, peroxisome proliferator-activated receptor-γ coactivator-1α.
females, and their offspring were genotyped using a standard PCR assay. G1 heterozygotes (Gfer<sup>+/−</sup>) were backcrossed to C57BL/6J for one generation before heterozygous offspring were intercrossed.

**Genotyping.** Wild-type (WT) and heterozygous mice were genotyped by PCR of tail-tip samples using one forward (F: CCTCTGGCGTGACAACGCAAGC) and two reverse (R: AGTTGCACAGTTAGGAGTGCAGTACGCT; and R2: AAGCAGGCCACCCAACTGACC) primers. The WT allele was targeted by primers F and R and identified as a 1,400-bp amplicon, whereas the mutant allele was targeted by primers F and R2 and identified as a 337-bp amplicon.

**Quantitative real-time reverse transcription PCR and Western blot analyses.** Total RNA from mouse liver samples was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) and then reverse transcribed as described previously (7). ALR mRNA levels were estimated by quantitative real-time reverse transcription (qRT)-PCR with a

**Fig. 1. mRNA and protein expression documenting the increase in ALR after liver regeneration.** A: augmenter of liver regeneration (ALR) mRNA levels increased sharply at days 3–4 post-partial hepatectomy (PH). B: representative immunoblot analyzed for ALR expression at serial times after PH in the liver. C: ALR expression, as revealed by Western blotting (B), increased at days 3–5 post-PH. Data are expressed as means ± SD of 3 animals for each group. *P < 0.05 vs. 0 h.

**Fig. 2. Knockdown of ALR delays liver regeneration in mice.** A: data show the percentage of liver weight regained after PH in the scramble and ALR-shRNA mice (n = 3 mice/time point for each group). B: representative images of the livers of scramble and ALR-shRNA mice at day 5 post-PH, indicating the difference in liver size between the two groups. C: representative T2-weighted axial magnetic resonance imaging (MRI) of the scramble and ALR-shRNA mice at days 4 and 6 post-PH. D: liver volume monitoring after PH in the scramble and ALR-shRNA mice. Percentage of liver volume compared with the volume before surgery was calculated for each animal, and the means ± SD were plotted (*P < 0.05).
Prism 7300 Sequence Detecting System (Applied Biosystems, Carlsbad, CA). 18S rRNA was amplified in parallel as an internal control. For Western blotting, liver tissue was harvested and cell protein was prepared as previously reported (7). Fifty micrograms of protein were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrotransferred on a nitrocellulose membrane, which was then blocked using routine methods and incubated with an anti-ALR antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight. The membranes were stained with goat anti-mouse IgG secondary antibodies (Cell Signaling Technology, Danvers, MA) and then developed with enhanced chemiluminescence reagents (Santa Cruz Biotechnology). The membranes were rehybridized with antibodies against glyceraldehyde 3-phosphate dehydrogenase (Kangcheng Biology Engineering, Shanghai, China), as a loading control.

Immunohistochemical analysis. All mice received an intraperitoneal injection of bromodeoxyuridine (BrdU; Sigma-Aldrich) at 100 mg/kg 2 h before termination of the animal study. BrdU incorporation was detected in tissue sections by immunohistochemical analysis using either anti-BrdU (Cell Signaling Technology) or anti-Ki-67 antibody (Abcam, Cambridge, UK). Hepatocyte nuclear staining was observed using an automated upright microscope system (DM5000B; Leica Microsystems, Wetzlar, Germany). The proliferation indexes, as determined by two blinded investigators, were quantified as the mean number of BrdU- and Ki-67-positive hepatocytes in three fields per section at ×200 magnification.

mtDNA copy number. DNA from each tissue was extracted using a DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer’s instructions. The copy number of MTRNR1 (mitochondrially encoded 12S rRNA) in mtDNA was measured by qRT-PCR. Total DNA (50 ng) was employed as a template, and the expression level was normalized against the nuclear RNA-binding motif protein 15 gene. The qRT-PCR reaction was performed using a Power SYBR Green Master kit (Applied Biosystems) and run on the ABI Prism 7300 Sequence Detection System (Applied Biosystems).

Data analyses. Data are presented as means ± SD unless otherwise noted. Statistical significance was determined using the Student’s t-test or one-way analysis of variance. A probability (P) value of <0.05 was considered statistically significant.

RESULTS

ALR expression was upregulated during liver regeneration after PH. Hepatic ALR mRNA and protein expression were evaluated by qRT-PCR and Western blot analyses. In PH mice, an increase in ALR levels was observed as early as 1 day post-PH compared with the value at time 0. Maximal expression of ALR (∼3-fold higher than that at time 0) was reached by day 4 post-PH, and values subsequently declined to the time 0 level during the remainder of the observational period. No similar ALR profile was registered in the sham-operated mice that did not undergo PH (Fig. 1).

ALR expression was downregulated by shRNA. To exclude potential off-target silencing effects mediated by specific shRNAs, we employed four different shRNA sequences of ALR. Based on their inhibitory effects, the mice were administered ALR shRNA-2 or ALR shRNA-4 immediately after PH. These shRNAs significantly downregulated ALR expression at day 1 post-PH, and the inhibitory effect lasted 7 days (data not shown).

Knockdown of ALR decelerated liver regeneration following PH in mice. To determine whether the ALR gene affected liver regeneration following PH in mice, we analyzed a liver growth curve, which reflects the capacity of the liver to return to its normal mass.
staining for Ki-67. As shown in Fig. 3, observing BrdU incorporation into nuclear DNA (nDNA) and regeneration. Liver proliferation kinetics were analyzed by induced by shRNA-4 treatment (data not shown). which showed a similar trend of decelerated liver regrowth mice, data from ALR-inhibited. To confirm these data from tissues of both groups. However, compared with scramble-D days 2 Ki-67 nuclear staining (indicated by enhanced hepatic BrdU incorporation and positive a marked increase in DNA synthesis in the remaining liver, as indicated by enhanced hepatic BrdU incorporation and positive Ki-67 nuclear staining (days 2 and 3 post-PH) in the liver tissues of both groups. However, compared with scramble-treated mice, tissues from the ALR shRNA mice showed a significant decrease in both BrdU incorporation and Ki-67-positive staining at days 2 and 3 post-PH (Fig. 3, C and D), suggesting a marked reduction in PH-induced DNA synthesis in the hepatocytes of ALR shRNA mice. These findings were confirmed in ALR-KO mice. Although it was not possible to breed homozygous ALR-/- offspring (nominated Gfer-/-) in our laboratory, likely because of embryonic lethality induced by complete ALR KO, ALR protein levels were markedly reduced in the heterozygous ALR-KO mice (Gfer-/-) (see Fig. 6A). As shown in Fig. 4, the capacity for liver regeneration (days 3 and 7 post-PH) and DNA synthesis (day 2 post-PH) was notably inhibited in ALR-KO mice; thus, liver mass was regained more slowly than in WT animals (Fig. 4).

To further confirm these results, an acute liver injury model (CCL4 intoxication) was also constructed to assess the role of ALR in liver regeneration. Three days after injection of CCL4, mice were killed, and liver samples were obtained. Hematoxylin and eosin staining revealed no pathological changes in the livers from Gfer-/+ and ALR-KO (Gfer-/-) mice injected with olive oil only, and their hepatocytes showed no signs of degeneration, necrosis, or fibrosis. Typical pathological characteristics such as ballooning degeneration, steatosis, broad infiltration of inflammatory cells, and spotty necrosis observed in the CCL4-treated mice confirmed the successful establishment of liver injury. Moreover, liver damage was significantly augmented, as indicated by a significant increase in the necrotic area in ALR-KO mice after CCL4 injection (data not shown). Next, liver proliferation in Gfer-/+ and ALR-KO (Gfer-/-) mice was assessed after CCL4 treatment. The results

![Fig. 4. Liver regeneration and hepatic DNA synthesis was inhibited in ALR-KO mice. Percent liver recovery (days 0, 3, and 7 post-PH) based on calculated liver weight (A) and deduced by MRI analysis (B and C). D: immunoassays were performed for BrdU in liver sections from wild-type (WT) and KO mice killed at day 2 post-PH. Quantification of BrdU-positive cells was conducted over 5 randomly selected fields. Similarly, DNA synthesis was also notably inhibited in ALR-KO mice compared with that of WT mice (n = 3 for each group).](http://ajpgi.physiology.org/Downloaded from http://ajpgi.physiology.org/ by 10.220.33.6 on October 21, 2017)
showed that the BrdU incorporation into dividing hepatocytes was significantly reduced in the ALR-KO mice compared with that in Gfer+/+ mice (see Fig. 8, A and B), which was similar to the results after PH.

**ALR downregulation impaired mitochondrial biogenesis in mice.** As a next step, we were interested in determining whether ALR knockdown affected mitochondrial biogenesis, thereby impairing the respiratory electron transfer pathway and ATP production (32). The levels of mitochondrial transcription factor A (TFAM) and peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) were evaluated as markers of mitochondrial biogenesis. As shown in Fig. 5, A and C, mRNA levels of both TFAM and PGC-1α were markedly decreased in ALR shRNA-2 mice compared with scramble mice at day 4 post-PH (*P < 0.05). The corresponding protein levels were found to be similarly reduced (Fig. 5, B and D). Likewise, mRNA levels of PGC-1α and TFAM in ALR shRNA-4 and ALR-KO mice were also greatly reduced (Fig. 6B) at day 4 post-PH.

**Fig. 5. Knockdown of ALR impairs mitochondrial biogenesis.** mRNA (A and C) and protein (B and D) levels of peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) and mitochondrial transcription factor A (TFAM) in hepatic mitochondria were assayed by quantitative real-time reverse transcription (qRT)-PCR and Western blotting, respectively. E: the mitochondrial (mtDNA)-to-nuclear (nDNA) DNA ratio relative copy number was determined by qRT-PCR in hepatic mitochondria from the scramble and ALR-shRNA-2 mice. F: electron microscopic images and mitochondrial nos. in liver tissue sections from ALR-shRNA-2- and scramble-treated mice at day 4 post-PH. Liver samples were prepared from either scramble- or ALR-shRNA-2-treated mice at day 4 post-PH. Hepatocyte mitochondria manifested with mild morphological changes in ALR-shRNA mice, whereas swollen cristae and giant mitochondria were observed in scramble-treated mice. *P < 0.05 compared with the scramble-treated group.
post-PH compared with WT mice. Similarly, protein levels of these proteins were also reduced (Fig. 6C). As shown in Fig. 8D, both TFAM and PGC-1α protein levels were also significantly reduced in ALR-KO mice compared with ALR WT mice following CCl₄-induced acute liver injury.

In addition, the replicative capacity of hepatic mtDNA synthesis was impaired both in ALR shRNA and ALR-KO mice after PH or CCl₄ intoxication. The ratio of mtDNA copy number relative to nDNA was dramatically decreased in ALR shRNA-2-treated livers compared with scramble RNA-treated livers (P < 0.05), by 65 and 55% at days 3 and 4 post-PH, respectively (Fig. 5E). Similarly, the ratio of mtDNA copy number to nDNA in the livers of ALR shRNA-4 and ALR-KO mice was markedly reduced (Fig. 6D), and the reduction of this

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Fig. 6. Confirmation of mtDNA replication deficits in ALR-shRNA and KO mice. A: efficiency of ALR inhibition resulting from shRNA-4 and KO. B: PGC-1α and TFAM mRNA levels were notably decreased in ALR shRNA-4 and KO mice compared with those of scramble-treated and WT mice. C: similarly, inhibition of ALR expression by shRNA or KO strategies decreased PGC-1α and TFAM protein levels. D: mtDNA/nDNA relative copy number was determined by qRT-PCR in hepatic mitochondria from the ALR shRNA-4 and KO mice. E: electron microscopic images and mitochondrial nos. in liver tissue sections from WT and KO mice. Liver samples were prepared from either WT or KO mice on day 3 post-PH.

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ratio in the livers of ALR-KO mice occurred 3 days after CCl₄ injection (Fig. 8E). These findings suggest that hepatic mtDNA replication was, at least to some extent, subject to damage as a result of the downregulation of ALR.

Electron microscopy revealed that the mitochondria of liver cells were regular in size, cristae-rich, and featured a typical, folded intermembrane space and dense matrix in scramble-treated and Gfer⁺/⁺ mice (Figs. 5F and 6E). The ALR knockdown and ALR-KO mice both showed morphological abnormalities in the mitochondrial ultrastructure, which included a decreased number of cristae, whereas those present were observed to be deficient or swollen (Figs. 5F and 6E). Mitochondrial counts further confirmed that the number of hepatic mitochondria was significantly decreased when the expression of ALR decreased (Figs. 5F and 6E).

To analyze the consequence of potential mitochondrial biogenesis, hepatic ATP content was measured. ATP levels in the livers of ALR shRNA-2 and ALR-KO mice were significantly decreased compared with controls at day 3 post-PH (Fig. 7A). As shown in Fig. 8F, mitochondrial energy production was markedly impaired in mice after CCl₄ injection, which was most obvious in ALR-KO mice. Lesions to the mitochondria were also induced by excessive production of ROS, since the hepatic levels of malondialdehyde (MDA), a metabolite product of lipid peroxidation, were largely increased by 148 and 138% in ALR shRNA-2 and ALR-KO mice, respectively, compared with the controls (Fig. 7B). Similarly, MDA levels in the CCl₄-treated mice were also significantly elevated compared with oil control mice (ca. 125%), and this elevation was markedly increased in the ALR-KO mice by 136% compared with ALR WT animals (Fig. 8), suggesting that profound oxidative stress and lipid peroxidation might have occurred in the liver tissue once ALR expression was inhibited. These results indicated that the protective role of ALR on mitochondrial function is related to the promotion of mitochondrial biogenesis.

**DISCUSSION**

The novelty of this study is the demonstration that a deficiency in ALR expression impaired mitochondrial biogenesis, which could be considered as one of the mechanisms causing a delay in liver regeneration in mice after PH or CCl₄ injection. This hypothesis is based upon the findings of an investigation using ALR shRNA mice and an ALR-KO model. We believe that this finding is of vital importance to further elucidate the contribution of ALR to liver regeneration.

In yeast, erv1 deletion is lethal, since the mutants display an aberrant mitochondrial morphology (1). Thus far, no genetically ALR-depleted mice have been created because destroying the ALR gene in the ESCs leads to a loss in mitochondrial membrane potential, excessive mitochondrial fragmentation, elimination of damaged mitochondria via autophagy (mitophagy), and caspase-induced apoptosis (41). We suspect that mitochondrial lesions eventually occur in ALR⁻/⁻ animals and may ultimately prove lethal during the gestational period. Nevertheless, we obtained heterozygous-defective ALR mice, which showed a significant reduction in ALR expression and will provide an alternative model to explore ALR function during the regulation of liver regeneration.

Similarly, Francavilla et al. demonstrated that liver regeneration capacity after PH may be diminished and mitochondria-associated cell apoptosis was increased following ALR knockdown (12). However, the study only examined hepatocyte growth and apoptosis within 48 h after PH, which may not reveal ALR function during the period of liver regeneration. To evaluate apoptosis of hepatocytes after 48 h post-PH, we employed the TUNEL assay and analyzed the activation of caspase-3 at 72 h post-PH, but found no significant increase in the activation of caspase-3 or the TUNEL-positive cells in the livers of ALR shRNA and ALR-KO mice (data not shown), suggesting that apoptosis may not be a primary response to ALR inhibition during liver regeneration at 72 h post-PH. Although enhanced cell apoptosis contributes to some extent to the delayed liver regeneration, the cardinal mechanism that causes the slowdown in liver regeneration after a massive loss of its volume is primarily due to insufficient hepatocyte proliferation in the remnant liver. Considering the important role of ALR during mitochondrial oxidative phosphorylation and mtDNA biogenesis, the fundamental basis of energy production seems to be critical for cell proliferation, although the importance of ALR insufficiency to mitochondrial biogenesis and liver regeneration remains doubtful and requires further investigation. The results of the current study demonstrated that, although there was a difference in liver volume between the ALR-shRNA and control mice by day 7 post-PH, this difference remained significant when ALR-KO and WT mice were compared, indicating that partially defective ALR may nevertheless hamper liver growth. In parallel, mtDNA transcription and mitochondrial biogenesis were all severely affected in ALR shRNA- or ALR-KO mice (Figs. 5 and 6).

The association between impairment of mitochondrial biogenesis and liver regeneration after PH, particularly with respect to ALR, has rarely been investigated. Although an earlier study by Polimeni et al. (33) indicated that intraperitoneal injection of ALR protein in rats increased TFAM expression accompanied by enhanced oxidative phosphorylation capacity, the study nevertheless presented unexpected results. Mitochon-

![Fig. 7 Levels of ATP (A) and malondialdehyde (MDA, B) in the livers of ALR shRNA-2 and ALR-KO mice at 72 h post-PH. A: at 72 h post-PH, the mitochondrial ATP content of livers was analyzed using the CellTiter-Glo luminescent cell viability assay kit (Promega) according to the manufacturer’s instructions. Data are expressed as means ± SD of 4 animals in each group. B: liver tissues were homogenized and centrifuged. The supernatant was collected and used for the MDA assay. Values are presented as means ± SD, n = 4.](http://ajpgi.physiology.org/content/early/2017/09/21/ggi.4810/figure/7)
Fig. 8. Hepatocyte proliferation was inhibited, and mitochondrial biogenesis was impaired in ALR-KO mice after carbon tetrachloride (CCl4) injection. A: immunohistochemical analysis of BrdU incorporation of WT and ALR-KO mice at 72 h after CCl4 injection. B: percentage of BrdU-positive cells in WT and ALR-KO mice at 72 h after CCl4 injection (n = 4). C and D: ALR, PGC-1α, and TFAM expression at 72 h after CCl4 injection. E: mtDNA/nDNA relative copy no. was determined by qRT-PCR in hepatic mitochondria from the WT and ALR-KO mice. F and G: levels of ATP (F) and MDA (G) in the livers of WT and ALR-KO mice. In all panels, data are expressed as means ± SD (n = 4).

Mitochondrial biogenesis requires a complex interplay between the nuclear and mitochondrial genomes (16). PGC-1α is a key transcriptional regulator of cellular energy metabolism to stimulate mitochondrial biogenesis (3). TFAM is capable of binding and wrapping mtDNA, as well as activating and regulating mtDNA transcription and replication (8, 30, 37). TFAM plays a key role in mitochondrial biogenesis, since it is essential to the initiation of both the replication and
expression of mtDNA, which encodes portions of the mitochondrial proteins, such as cytochrome c oxidase II (13). It has been postulated that small amounts of TFAM are necessary to initiate mtDNA replication, whereas mtDNA expression is activated only at high concentrations (2). Our findings showing reduced expression of PGC-1α and TFAM and debilitated replication capacity of hepatic mtDNA at day 4 post-PH in ALR shRNA mice relative to scramble mice support the hypothesis that ALR plays an important role in mitochondrial biogenesis.

The reduced mtDNA copy number may attenuate the activity of mitochondrial respiratory enzyme complexes, which are major source of ROS generation, and result in oxidative impairment. The increase in MDA levels in the livers of ALR shRNA- and ALR-KO mice after PH are shown in Fig. 7. Because mitochondria both generate ROS and its effect-target, oxidative stress is inseparably linked to mitochondrial impairment. Electron microscopy imaging of livers from ALR shRNA- and ALR-KO mice after PH revealed morphological evidence of mitochondrial injury, such as swollen mitochondria with extensive degeneration or even loss of cristae (Figs. 5 and 6). The observed mitochondrial morphological alterations and reduced mtDNA copy number are thought to deplete ATP levels. Emerging evidence suggests that the ALR modulates mitochondrial integrity and function. We observed considerable ATP deficits in the livers of ALR shRNA- and ALR-KO mice after PH, which indicated an alteration in mitochondrial energy metabolism due to the lack of ALR. Depletion of energy in the form of ATP may lead to a cascade of deleterious effects during liver regeneration. Therefore, excessive ROS production, mitochondrial impairment, and reduced ATP levels by the lack of ALR can be speculated as one of the possible mechanisms for the inhibition of liver regeneration.

Although PH is the most common model to study liver regeneration, chemically mediated hepatotoxic injury, such as with CCl₄, is also commonly used as a model of liver injury. To confirm the results from PH, we also investigated whether ALR might be involved in liver regeneration after CCl₄ treatment. In accordance with the report by Hongbo et al. (18), CCl₄ treatment significantly reduced ALR expression in the livers, even in those of ALR-KO mice (Fig. 8). The downregulation of ALR promoted CCl4-induced hepatic necrosis (data not shown) and markedly attenuated the number of BrdU-positive cells at day 3 post-CCl₄ injection, which implicates ALR in the pathogenesis of CCl₄-induced liver injury. In the current study, we also found that expression levels of PGC-1α and TFAM were abated, the copy number of mtDNA was reduced, ATP levels were attenuated, and MDA levels were increased in the livers of ALR-KO mice compared with WT mice after CCl₄ injection. These results strongly indicated that the lack of ALR inhibits the endogenous regeneration after CCl₄-induced liver injury by impairing mitochondrial biogenesis.

In summary, our results showed that ALR is apparently required for the normal restoration of liver mass after PH in mice. Inhibition of ALR expression by shRNA or gene KO notably delayed liver regeneration because of comparatively weakened mtDNA synthesis.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS
Author contributions: L.-h.H., H.Y., G.-y.S., Y.W., and J.G. performed experiments; L.-h.H., L.-y.D., and H.Y. analyzed data; L.-h.D. drafted manuscript; L.-y.D. interpreted results of experiments; L.-y.D. prepared figures; W.T. and W.A. conception and design of research; W.A. edited and revised manuscript; W.A. approved final version of manuscript.

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HSS AND LIVER REGENERATION


