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PHD1 의 억제에 의한 miR-122 의 HIF 유도가 간허혈 관용 향상에 미치는 영향에 관한 연구

HIF-dependent induction of miR-122 enhances hepatic ischemia tolerance via repression of PHD1

울산대학교 대학원
의 학 과
강 우 형

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지도교수 황신

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울산대학교대학원
의 학 과
강 우 형

강우형의 의학박사학위 논문을 인준함

심사위원 안철수 인
심사위원 황 신 인
심사위원 문덕복 인
심사위원 송기원 인
심사위원 주선형 인

울 산 대 학 교 대 학 원
2018년 2월

Abstract

Hepatic ischemia and reperfusion injury contributes significantly to morbidity and mortality of liver transplantation. MicroRNAs constitute a family of noncoding RNAs that regulate gene expression at the post-translational level through mRNA-repression. Here, we hypothesized that microRNAs could be targeted to enhance hepatic ischemia-tolerance. A targeted microRNA screen in a murine model of liver ischemia demonstrated the largest increase for miR-122 – a liver-specific microRNA implicated in hepatitis C virus propagation. Mice with hepatocyte-specific deletion of miR-122 (miR-122loxp/loxp Alb Cre+ mice) experienced profound ischemic liver injury. Transcriptional studies implicated hypoxia-inducible factor HIF1A in the induction of miR-122, and identified the oxygen-sensing prolyl-hydroxylase PHD1 as novel miR-122 target gene. Further studies indicate that HIF1A-dependent induction of miR-122 participates in a feed-forward pathway for liver protection via enhancing hepatic HIF-responses through PHD1 repression. These findings suggest that pharmacologic approaches promoting miR-122 or repressing PHD1 expression can be targeted therapeutically to enhance hepatic ischemia tolerance

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Introduction

Hepatic ischemia and reperfusion injury is an important source of morbidity and mortality during major liver surgery and hepatic transplantation[1]. Early graft failure after liver transplantation is frequently caused by ischemia and reperfusion injury, and is associated with extremely high rates of morbidity and mortality[2]. Due to a growing waiting list of recipients with urgent need for liver transplantation, there is an increasing demand for liver grafts for transplantation. Despite of the fact that they are more prone to ischemia and reperfusion injury, marginal livers are now also being considered for transplantations more frequently[3]. Ischemia and reperfusion injury also has important immunologic consequences, such as affecting the severity of early liver rejection[4] or the subsequent recurrence of viral hepatitis[5,6]. Therefore, studies on finding novel therapeutic interventions to dampen hepatic ischemia and reperfusion injury are areas of intense investigation[1].

MicroRNAs (miRNAs) constitute a family of short noncoding RNA molecules of 20 to 25 nucleotides in length that regulate gene expression at the posttranscriptional level via repression of target genes[1]. They are involved in the control of a wide range of biological functions and processes, such as development, differentiation, metabolism, growth, proliferation, and apoptosis. While previous studies had implicated miRNAs in ischemia and reperfusion injury[1], the contributions of miRNAs to hepatic ischemia and reperfusion injury are largely unknown. In order to identify miRNAs that could be targeted for liver protection during ischemia and reperfusion injury, we performed a targeted miRNA array utilizing a murine model of hepatic ischemia and reperfusion injury. In this model, partial liver ischemia is achieved via a hanging weight system to minimize surgical trauma[7-11]. Surprisingly, these studies pointed us towards miR-122, a highly conserved liver-specific miRNA that constitutes 70% of the cloned hepatic miRNA in the adult mouse[12]. Several key observations underscore the importance of miR-122 in liver biology and disease. Antisense-

mediated inhibition of miR-122 in mice leads to the induction of genes that are normally repressed in adult liver, suggesting that this miRNA is important for the maintenance of the terminally differentiated hepatocyte gene expression program[13]. Furthermore, miR-122 inhibition reduces serum cholesterol by indirectly causing the repression of genes involved in cholesterol biosynthesis, thereby protecting animals from diet-induced hypercholesterolemia[14]. Additionally, miR-122 plays a non-canonical role in the life cycle of HCV. Through interaction with two seed sequence binding sites located at the 5′-end of the HCV genomic RNA, miR-122 performs an incompletely understood function that is essential for replication of the virus[15]. Accordingly, intravenous administration of locked nucleic acid (LNA) antisense miR-122 oligonucleotides has been found therapeutic in the treatment of patients with hepatitis C[16]. In contrast to its detrimental role during hepatitis C, the present studies indicate a protective role for miR-122 in promoting hepatic ischemia tolerance.

Materials and methods

Human liver tissue. Liver samples were obtained from patients undergoing orthotopic liver transplantation (Supplementary Table 1). Liver biopsies (I) were taken at the conclusion of cold ischemia time (CIT) during back table preparation of the cadaveric liver allograft (Figure 6K). A second biopsy (R) was taken immediately prior to closure of the abdomen following drain placement (Figure 6K). Importantly, total reperfusion time is defined as the time from portal vein perfusion to abdominal closure at the conclusion of the procedure.

Mice. All animal protocols were in accordance with Asan Medical Center, guidelines for the use of living animals and were approved by the Institutional Animal Care and Use Committee of the University of Ulsan, School of Medicine guidelines for animal care. All mice were housed in a 12-h-light-dark cycle and were used gender-, age- and weight-matched between 12 and 16 weeks. In transcript and pharmacological studies C57BL/6J

mice obtained from Jackson Laboratories were used. Conditional *Mir122*$^{loxP/loxP}$ *Albumin Cre+ mice* were obtained by crossing *Mir122*$^{loxP/loxP}$ and Albumin Cre+ mice together. Albumin Cre+ mice were obtained from Jackson Laboratories whereas *Mir122*$^{loxP/loxP}$ mice are characterized previously by collaborators [37].

Murine model of partial liver ischemia. A murine model of partial liver ischemia was employed using a hanging-weight system as previously described [38].

Transcriptional analysis. IL-6 and TNF-α transcript levels in livers with and without ischemia and PHD1 and PGK1 transcript levels in cell cultures with and without exposure to hypoxia were measured by (RT)-PCR (iCycler, Bio-Rad Laboratories Inc.) as previously described [38].

Analysis of liver mRNA and miRNA. Total RNA was isolated from Chang cells, murine liver tissue using Qiazol Reagent and separated into mRNA and miRNA components following manufactures instructions (SA-Biosciences, Qiagen). cDNA from miRNA was generated using miScript RT II kits (Qiagen) and transcript levels were determined by real-time RT-PCR (iCycler; Bio-Rad Laboratories Inc.). Primer sets for mmu-miR122, hsa-miR122 and RNU6 (MS00033740) (Qiagen) were used following manufactures instructions. cDNA from mRNA was generated using iScript (Bio-Rad) and transcript levels were determined by real-time RT-PCR (iCycler; Bio-Rad Laboratories Inc.). Primers were QuantiTect from Qiagen (ms Il-6 # QT00098875, ms Tnfa #QT00104006, ms Actin QT01136772, human TNFa # QT01079561, human IL-1b # QT00021385, human Actin #QT01680476).

Chromatin Immuno-Precipitation (ChIP). ChIP was performed to assess in vivo DNA-protein interactions at the promoter sequences using the ChIP assay kit, according to the manufacturer's instructions (Active Motif, Carlsbad, CA). Briefly, cells were harvested and protein-DNA complexes were cross-linked by fixation in 1% (v/v) formaldehyde in minimal culture medium for 10 min. at room temperature, and nuclei were extracted. Chromatin was

enzymatically digested for 11 min. to yield DNA fragments ranging in size from 200 to 1,500 bp. The chromatin solution was reserved as "input" sample. The remaining chromatin was immunoprecipitated overnight at 4^0C with 3ug of antibody specific to Hif-1a or IgG isotype control. The chromatin/antibody complexes captured on the beads were washed several times and then eluted in 50 ul of elution buffer. The immunoprecipitated and input sample cross-links were reversed by incubation for 2.5h at 65^0C. After treatment with proteinase K for 1h at 37^0C the reaction was stopped, and the resulting DNA analyzed using PCR. Conventional PCR signals were stained with ethidium bromide on 1% agarose gels.

In vitro transduction of virus overexpressing GFP-miR-122. Chang cells were transduced with $3x10^6$ ifu of either GFP-control or GFP-has-miR-122 expressing virus and 8 ug/ml of Polybrene (Millipore). After 48 hours, cells were harvested and mRNA was isolated using the RNeasy kit (Qiagen). cDNA was generated using RT^2 First Strand kit (SABioscience).

Binding of miR-122 to 3' UTR of PHD1. ***Overexpression:*** Chang cells were transduced with $3x10^6$ ifu of either GFP-control or GFP-miR-122 virus and 8 ug/ml of Polybrene (Millipore). After 48 hours, cells were transfected with control pMiR Target or with PHD1 3'UTR reporter constructs (Origene). 24 hours later, cells were harvested and luciferase expression was measured using the Dual Luciferase Reporter kit (Promega) per manufactures instructions. ***Hypoxia:*** Chang cells were transfected with control pMiRTarget or PHD1 3'UTR reporter constructs (Origene), incubated for 24 hours and then expose at 1% for 24 hours, cells were harvested and luciferase expression was measured using the Dual Luciferase Reporter kit (Promega) per manufactures instructions.

Immunoblotting experiments. To measure PHD1, HIF1a or HIF2a protein content, cells were harvested in (5 % SDS, 25 % sucrose, 50mM Tris pH 8.0, 0.5mM EDTA, and complete protease inhibitor (Roche)). The protein was re-suspended in reducing Laemmli sample buffer and heated to 90 ^{0}C for 5 min. Samples were resolved on a 10 % polyacrylamide gel

and transferred to nitrocellulose membranes. The membranes were blocked for 1h at room temperature in T-TBS supplemented with 5% Blotting grade Non-Fat Milk (Bio-Rad, 170-6404). The membranes were incubated in primary antibody at concentrations given for h at room temperature, PHD1, HIF1a or HIF2a. After three 10 min washes in T-TBS, the membranes were incubated in a goat anti-mouse secondary antibody at concentration of 1:10,000 (Thermo Scientific, 31460). The wash was repeated and proteins were detected by enhanced chemiluminescence (Pierce Thermo-Fisher). To control for protein loading, blots were stripped and re-probed for □-actin using a mouse monoclonal anti-human □-actin primary antibody (Calbiochem, CP01) and a goat anti- mouse secondary antibody (Calbiochem, 401225).

Liver histology. Liver tissue was harvested following 24h of reperfusion and stained and evaluated as previously described [39].

Isolation of hepatocytes and endothelial cells. Liver preparation was performed as decribed in detail by Wei et al [18]. Endothelial cells were collected as described previously [12].

Hepatocyte cell culture. Human Chang cells were obtained from ATCC and were cultured as previously described [12].

Hsa-miR-122 promoter studies. Chang cells were plated on 6 well plates and grown to 80% confluence, and co-transfected with 3ug each of the hsa-miR-122 promoter full length, truncations, mutation or empty vector (pGL4.17) and 0.5ug of pRL-TK vector expressing the renilla luciferase gene (Promega), using the FuGENE HD transfection reagent (Promega) according to manufacturer's protocol. The cells were transfected overnight and then exposed to hypoxia for 24 hours. Expression of the hsa-miR-122 promoter reporter genes was assayed by lysis of the cells and the Dual Luciferase Assay Kit (Promega) according to the manufacturer's instructions using a Biotek Synergy 2 Multimode Microplate Reader. The luciferase expression in the samples was normalized with respect to transfection efficiency of each sample as determined by the luciferase assay and was measured (mean ± SD; n=4;

*indicates $p<0.05$ over normoxic controls).

PHD1 genetic deletion in cell cultures. Chang cells were transfected with pLKO.1-puro-PHD1 KO (PHD1 KO, 3 μg), or pLKO.1-puro (3 μg) using a FuGENE HD transfection Reagent (Promega). Cells transfected with pLKO.1-puro plasmid alone without the PHD1 KO gene (Con) were used as a control. For stable transfection, cells were cultured in the selective medium with 2 μg/ml puromycin for 1 month. Then, drug resistant individual clones were isolated and incubated for further amplification in the presence of a selective medium.

In vivo treatment with miR-122 mimic. NLE (maxSuppressor in vivo RNALancer II) was purchased from BIOO Scientific (Austin, TX). NLE consists of 1, 2-dioleoyl-sn-glycero-3-phosphocholine, squalene oil, polysorbate 20, and an antioxidant that- in complex with synthetic miRNAs form nanoparticles in the nanometer diameter range. A single dose of 20ug synthetic mouse microRNA-122 (Dharmacon, Lafayette, CO) formulated with NLE according to the manufactures instructions was injected IV 24 hours before liver ischemia experiments.

In vivo treatment with antisense PHD1 Oligo. Mice were treated with a PHD1 specific small molecule inhibitor (576146) or the respective control compound (549144) provided by ISIS Pharmaceuticals, Inc. Mice were treated two times a week (first and fourth day) for two weeks with 50mg/kg IP. Liver ischemia was performed 4 days after the last injection.

Statistical Analysis. Liver injury score data are given as median and range. All other data are presented as mean ± SD from three to eight animals per condition. We performed statistical analysis using the Student's t test. A value of $p < 0.05$ was considered statistically significant. For Western blot analysis 3 repeats were performed. For all statistical analysis GraphPad Prism 5.0 software for Windows XP was used.

Study Approval. All the animal samples were approved by the AMCIRB at Asan Medical Center. Protocols of all animals were conducted in accordance with the Republic of Korea

IACUC Guidelines to the using of the living animals and were also approved by the Institutional Animal Care and Use Committee of the Asan Medical Center guidelines for animal care (AMC-IACUC Protocol no. 2015-04-172).

Results

MiR-122 is induced during hepatic ischemia and reperfusion injury. We hypothesized that miRNAs could play a critical role in mediating hepatic ischemia tolerance via altering the expression of specific target genes. To make progress on this front, we performed a targeted miRNA array during hepatic ischemia and reperfusion injury, focusing on miRNAs that are known to be expressed in the liver[17-22]. For the purpose of these studies, we exposed mice to partial hepatic ischemia and reperfusion injury by applying 45min of hepatic ischemia of the left liver lobe, followed by 6h reperfusion. We compared miRNA expression in the post-ischemic liver with sham controls. Interestingly, we observed the most robust induction of miRNA expression for the hepatic miRNA mmu-miR-122 (**Fig. 1a**). Additional studies with different ischemia and reperfusion time periods confirmed increased mmu-miR-122 expression following ischemia and reperfusion injury of the liver (**Fig. 1b, c**). Moreover, exposure of human cultured hepatocytes (Chang liver cells) to ambient hypoxia (1% of oxygen) was associated with time-dependent increases of hsa-miR-122 expression (**Fig. 1d**). Together, these findings demonstrate induction of miR-122 during hepatic ischemia and reperfusion injury.

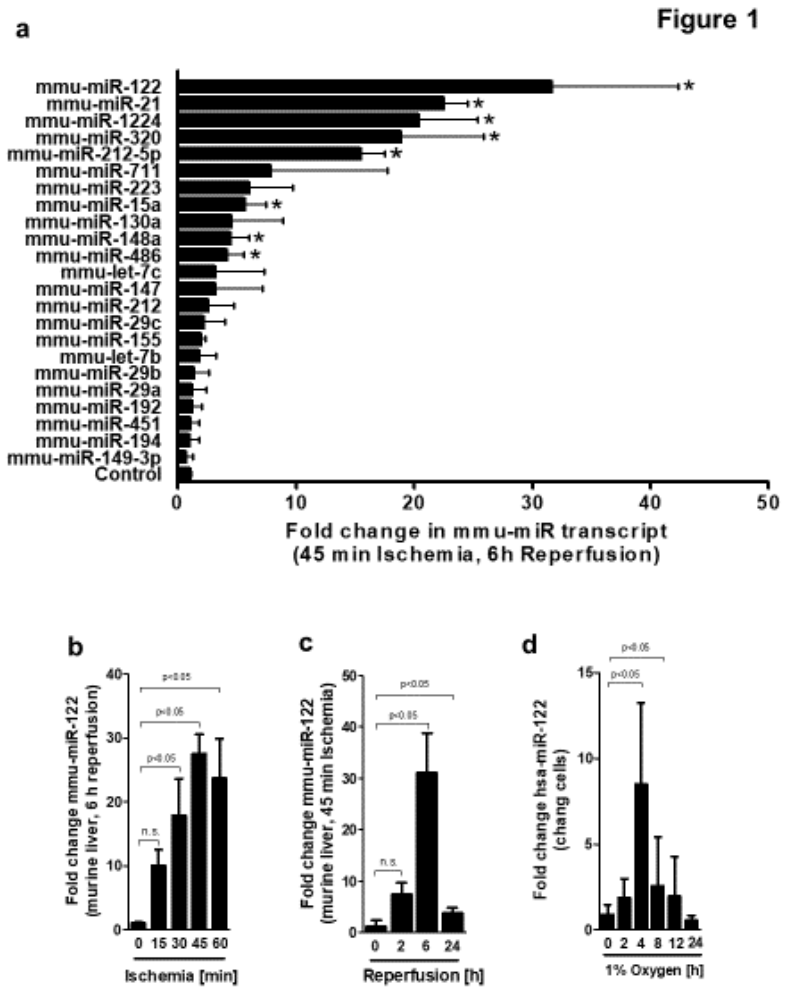

Figure 1: ***Identification of miR-122 as hypoxia-responsive miRNA during hepatic ischemia and reperfusion injury.*** (a) Targeted miRNA array after hepatic ischemia (45 min) and reperfusion (6h; n=3). (b) Hepatic mmu-miR-122 transcript levels following indicated hepatic ischemia times and 6h reperfusion (n=4). (c) Hepatic mmu-miR-122 transcript levels after 45 min of liver ischemia and indicated periods of reperfusion (n=4). (d) Expression of mmu-miR-122 following hepatic ischemia (45min) and 6h of reperfusion in the serum. (e) Hsa-miR-122 transcript levels in cultured hepatocytes (Chang cells) exposed to indicated periods of hypoxia (1% oxygen; n=4)

MiR-122 induction during hepatic ischemia is mediated by hypoxia-inducible factor HIF1A. Based on the above findings showing that miR-122 is induced during murine hepatic ischemia and reperfusion injury, or following hypoxia exposure of human hepatocytes, we next set out to study the transcriptional mechanism governing miR-122 expression. Unlike some miRNAs, which are encoded on transcripts coincident with other miRNAs or protein-coding genes, miR-122 is derived from a transcript that only encodes for miR-122 and previous studies had shown that the miR-122 promoter is prominently regulated by a cluster of liver-specific transcription factors[23, 24]. However, hypoxia control of the miR-122 promoter is currently unknown. Notably, the human miR-122 promoter (-1055 bp of TSS) contains 2 putative HIF binding sites (hypoxia response elements, HREs; **Fig. 2a**). Initial studies with the full-length miR-122 promoter cloned into a PGL4 vector demonstrated robust hypoxia responsiveness. Subsequent truncations or site-directed mutagenesis of the full-length promoter identified a specific HRE responsible for hypoxia-inducibility of the miR-122 promoter (**Fig. 2b**). Studies with chromatin immune-precipitation (ChIP) assays demonstrate direct binding of HIF1A to the above area within the mir-122 promoter (**Fig. 2c**). Moreover, hypoxia-inducibility of the miR-122 promoter was abolished in Chang cells with lentiviral-mediated siRNA repression of HIF1A (**Fig. 2d-f**), while miR-122 induction remained intact in Chang cells with siRNA-mediated repression of HIF2A (**Fig. 2g-i**). Similarly, miR-122 induction during hepatic ischemia and reperfusion was abolished in mice with hepatocyte-specific deletion of *Hif1a* (*Hif1a*$^{loxp/loxp}$ Alb Cre+ mice; **Supplementary Fig. 1a,b; Fig. 2j**), while hepatocyte-specific deletion of *Hif2a* (*Hif2a*$^{loxp/loxp}$ Alb Cre+ mice; **Supplementary Fig. 1c,d; Fig. 2k**) did not affect miR-122 inducibility. Together, these findings indicate that miR-122 is a HIF1A target gene, and implicate a functional role for Hif1a in miR-122 induction during hepatic ischemia and reperfusion injury.

Figure 2

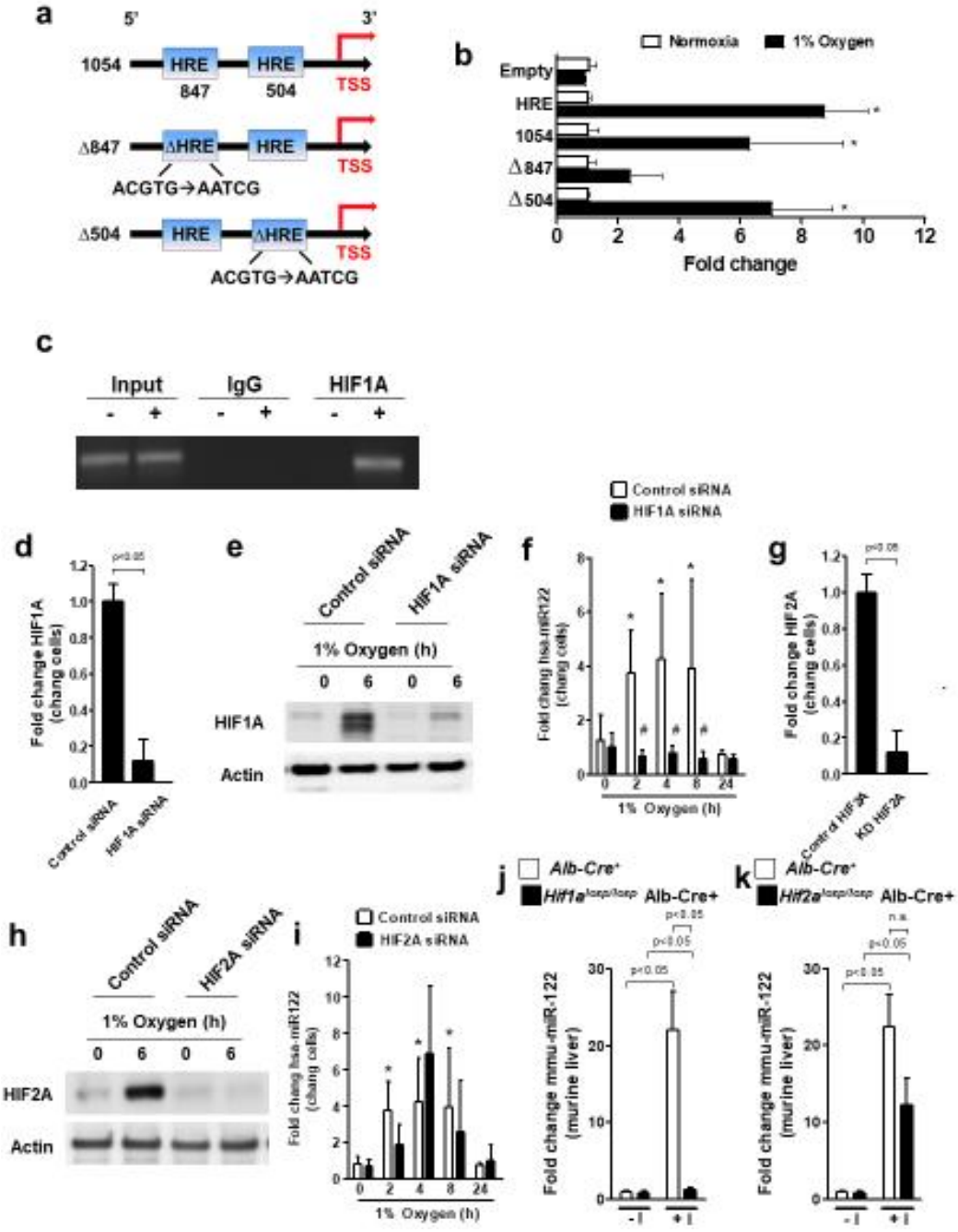

Figure 2: ***Role of HIF in miR-122 induction.*** (a) Luciferase reporter constructs of the miR-122 promoter. (b) Luciferase reporter assay. Chang cells transfected with each luciferase reporter construct shown in (a) were exposed to hypoxia or cotransfected with pcDNA3-hif-1α. Relative luciferase activities were expressed in comparison with the activity of pGL3-Basic. (c) ChIP assay. Input, amplified hif-1α

from a 1:100 dilution of total input chromatin:IgG, immunoprecipitation with non-specific IgG. (d) RT-PCR. Hif-1α-siRNA transfected with normal Chang cells. (e) Western blot. Chang cells were transfected with Hif-1α-siRNA. (f) RT-PCR of miR-122. Chang cells were transfected with Hif-1α-siRNA and exposed to hypoxia time dependent manners. (g) RT-PCR. Chang cells were transfected with hif-2α-siRNA. (h) Western blot for hif-2α. Chang cells were transfected with Hif-2α-siRNA and exposed to hypoxia time dependent manners. (i) RT-PCR of miR-122. (j-k) RT-PCR. Specific deletion of Hif-1 and 2α following ischemia with and without ischemia. Data presented as mean ± SD of three to eight samples per group. $P<0.05$, one-way analysis of variance followed by Bonferroni's multiple comparison test.

Hepatocyte-specific deletion of miR-122 (*miR-122*$^{loxp/loxp}$ Alb Cre+ mice) is associated with increased ischemic liver injury. Based on above studies showing Hif1a-dependent induction of miR-122 during conditions of limited oxygen availability, we next set out to address the functional role of miR-122 during hepatic ischemia and reperfusion injury. Previously describe *miR-122*$^{loxp/loxp}$ Alb Cre+ mice are viable, and bread normally, and do not show obvious abnormalities when kept in a pathogen-free environment[15], while showing efficient deletion of hepatic miR-122 levels (**Fig. 3a**). However, exposure of miR-122$^{loxp/loxp}$ Alb Cre+ mice to hepatic ischemia and reperfusion injury (45min of ischemia, 24h of reperfusion) revealed that *miR-122*$^{loxp/loxp}$ Alb Cre+ mice experience dramatically increased levels of liver injury compared to Alb Cre+ mice matched in age, gender and weight (**Fig. 3b-h**), including increases of AST, ALT, LDH, histologic liver injury, and markers of liver inflammation. As proof of principle for the assertion that miR-122 plays a functional role in regulation of hepatic ischemia tolerance, we next reconstitute *miR-122*$^{loxp/loxp}$ Alb Cre+ mice utilizing treatment with nano-particles containing miR-122 mimetics via neutral lipid emulsion (NLE). We pre-treated *miR-122*$^{loxp/loxp}$ Alb Cre+ mice with a single dose of 20ug of synthetic mouse miRNA-122 mimetic formulated with NLE or with an NLE vector control

24h prior to hepatic ischemia and reperfusion injury (**Fig. 3i**). This protocol was associated with robust increases in hepatic miR-122 levels (**Fig. 3j**), while functional studies demonstrated reconstitution of a wild-type phenotype with regard to ischemia-associated increases of AST, ALT, LDH, histologic liver injury, and markers of liver inflammation (**Fig. 3k-q**). Together, these studies provide genetic evidence for a protective role of miR-122 during hepatic ischemia and reperfusion injury.

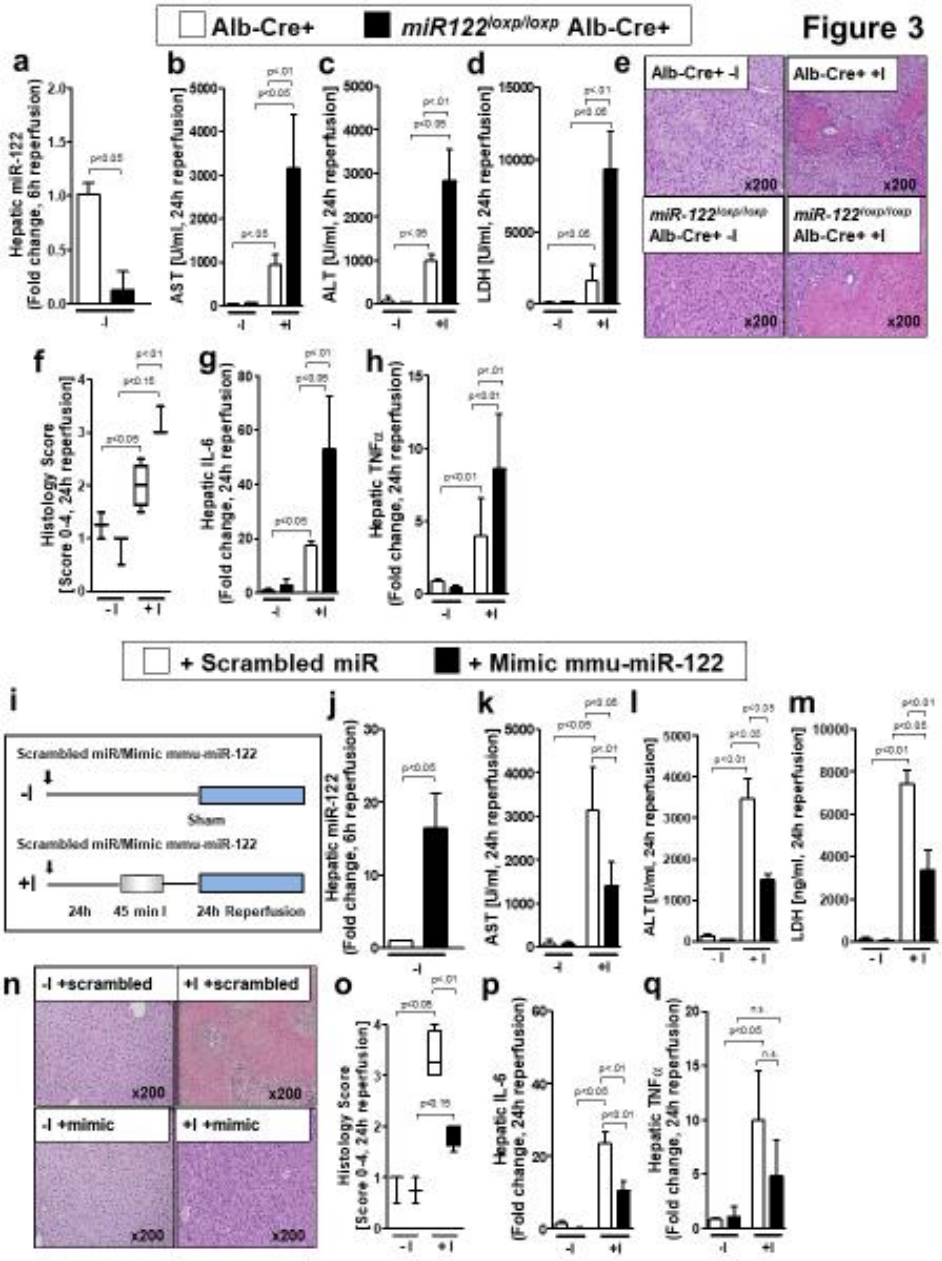

Figure 3: ***Ischemic liver injury in mice with hepatocyte-specific deletion of miR-122.*** MiR-

$122^{loxp/loxp}$ Alb Cre+ mice or littermate controls (Alb Cre+) matched in weight, gender and age were exposed to 45 min of liver ischemia and reperfusion. (a) Hepatic mmu-miR-122 after 45 min of ischemia and 6h of reperfusion. (b-h) Assessment of hepatic injury after 45min of ischemia and 24h of reperfusion (n=4). Reconstitution of miR-$122^{loxp/loxp}$ Alb Cre+ mice. Experimental approach; miR-$122^{loxp/loxp}$ Alb Cre+ mice were reconstituted by intravenous treatment with nano-particles containing a control vector or miR-122 mimetics. (i-j) Hepatic miR-122 levels after 45min of ischemia and 6h of reperfusion. (k-q) Assessment of hepatic injury after 45min of ischemia and 24h of reperfusion (n=4). Data presented as mean ± SD of three to eight samples per group. $P<0.05$, one-way analysis of variance followed by Bonferroni's multiple comparison test.

Identification of PHD1 as miR-122 target gene. Based on the profound increases of liver injury observed in *miR-$122^{loxp/loxp}$* Alb Cre+ mice, we hypothesized that repression of a putative miR-122 target gene could mediated hepatic ischemia tolerance. Utilizing electronic miRNA target gene prediction (PITA, RNA hybrid, miRecords, Target Scan Human), we identified 113 putative miRNA target genes with at least two positive predictions in the above search engines Of these 113 putative miR-122 target genes, only 14 were known to be expressed in the liver To test these putative mir-122 targets in a functional assay, we generated Chang cells with overexpression of hsa-miR-122 (**Fig. 4a**). Indeed, all 14 target genes were repressed in Chang cells with miR-122 overexpression. We observed the most profound repression for the putative miR-122 target gene PHD1 (**Fig. 4b**) – an oxygen sensing prolylhydroxylase which is critical for the regulation of HIF protein stabilization[25]. Indeed, hypoxia-exposure of Chang cells was associated with PHD1 mRNA repression during ambient hypoxia, with time kinetics that provide an almost exact mirror image of hypoxia-elicited induction of miR-122 (**Fig. 4c,d**). We next generated two luciferase reporter plasmids with one containing the wild-type 3'-UTR of PHD1, or a mutated version where specific binding of miR-122 to the 3'-UTR does not occur (**Fig. 4e**). Transfection of these

plasmids into Chang cells confirmed repression of the wild-type plasmid in Chang cells with miR-122 overexpression, or following hypoxia exposure (and concomitant miR-122 induction) – a response which was completely abolished following mutation of the PHD1-3'-UTR reporter plasmid (**Fig. 4f, g**). Together, these studies indicate that miR-122 can function to repress PHD1.

PHD1 repression augments hepatic HIF1A responses. After having identified PHD1 as hepatic miR-122 target, we next performed studies to address the functional role of PHD1 repression. PHD1 is known to hydroxylate HIFs during normoxic conditions, thereby tagging HIF for proteasomal degradation. During limited oxygen availability, HIF-hydroxylation is attenuated, leading to the initiation of hypoxia-elicited gene expression[25]. Therefore, we hypothesized that miR-122 repression of PHD1 could function to enhance hepatic HIF responses. To address this hypothesis, we generated Chang cells with lentiviral-mediated repression of PHD1 (**Fig. 4h, i**). Indeed, hypoxia-induced stabilization of HIF1A was significantly enhanced following PHD1 knockdown (**Fig. 4j**). Consistent with elevated HIF1A signaling, miR-122 expression was elevated in PHD1 knockdown Chang cells (**Fig. 4k**). Similarly, previously described *Phd1*$^{-/-}$ mice (**Fig. 4l**)[26] experience elevated Hif1a protein levels following hepatic ischemia and reperfusion injury (**Fig. 4m**), while expression of mmu-miR-122 are elevated (**Fig. 4n**). Finally, we found that Phd1 levels in mice with hepatocyte-specific miR-122 deletion (*miR-122*$^{loxp/loxp}$ Alb Cre+ mice) experience elevated Phd1 transcript and protein levels, in conjunction with failure to stabilize Hif1a protein levels during hepatic ischemia and reperfusion injury (**Fig. 4o**). In the context of previous studies showing that *Phd1*$^{-/-}$ mice are protected during hepatic ischemia and reperfusion injury[27], the present findings indicate the likelihood that miR-122 elicited repression of PHD1 could functions to provide liver protection via enhancing hepatic HIF responses.

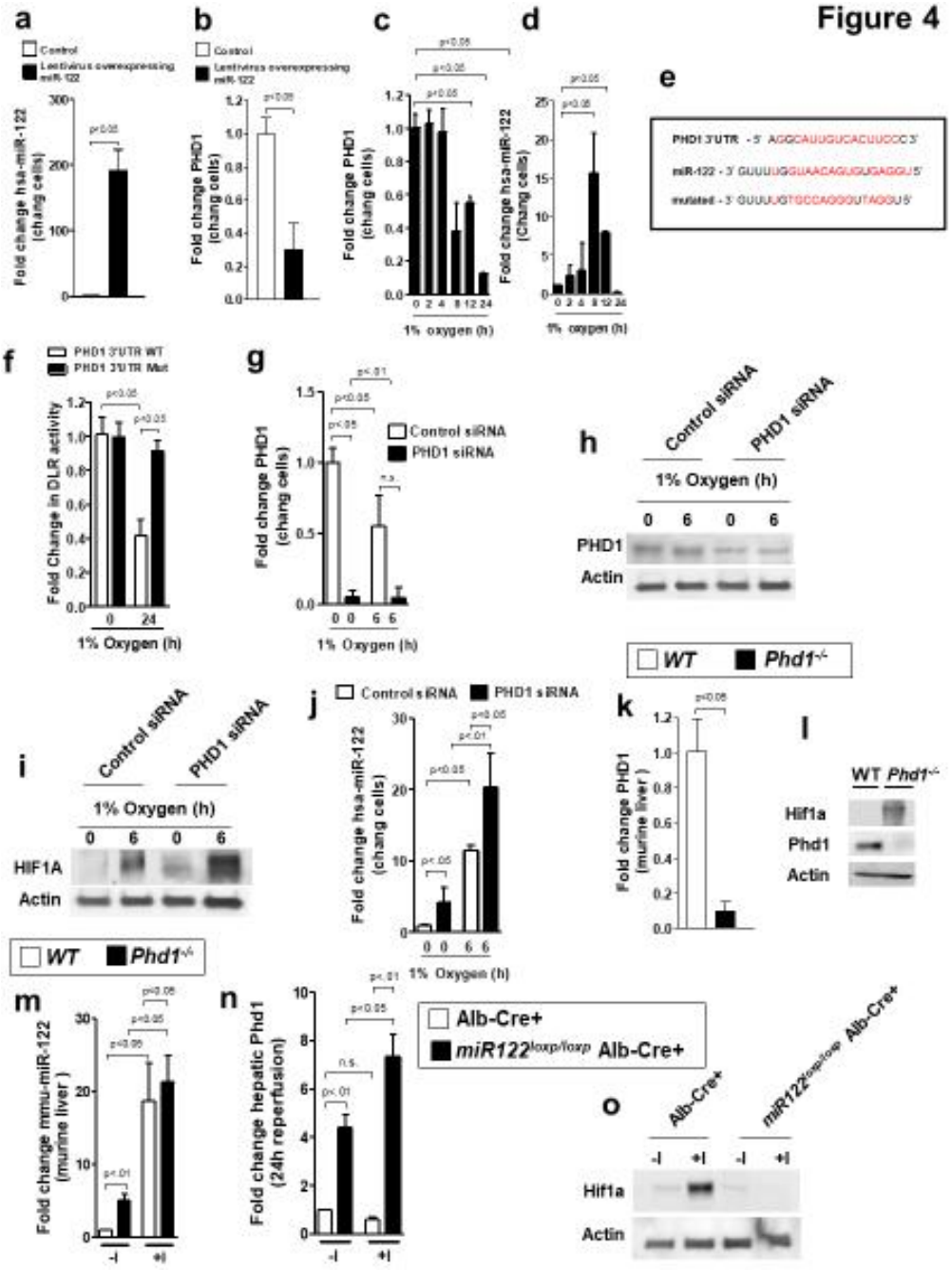

Figure 4: ***PHD1 is a miR-122 gene target in human hepatocyte cells.*** (a) Baseline has-miR-122 in lentiviral-transduced, human hepatocyte Chang cells compared to control Chang (lenti-ctr) cells. (b) Analysis of PHD1 transcripts in lentiviral-transduced human hepatocyte Chang cells compared to control Chang using RT-PCR relative to the housekeeping gene GAPDH. (c) PHD1 mRNA levels and (d) fold change of miR-122 under the hypoxic level in

a time dependent manner. (e) Schematic of luciferase reporter plasmid compared to control 3' UTR plasmids showing the binding sequence between PHD1 3'UTR and has-miR-122. (f) Luciferase activities of PHD1 3' UTR WT plasmid compared to control 3' UTR plasmid after transfection into lentiviral-transduced human hepatocyte cells (lenti-ctr or lenti-122). (g) PHD1 mRNA expression level (h) PHD1, and (i) HIF1 α western blot in PHD1 siRNA treated normal hepatocyte Chang cells. (j) Fold change of miR-122 level in PHD1 siRNA transfected Chang cells under hypoxia. (k) Expression of PHD1mRNA and (i) HIF 1 α level in whole Phd1 -/- mice. (m-n) RT-PCR of miR-122 and Phd1 following ischemia and without ischemia in Phd1 whole body -/- mice. (o) Hif1 α western blot in liver specific miR-122 deletion mice following with ischemia and without ischemia. Data presented as mean ± SD of three to eight samples per group. $P<0.05$, one-way analysis of variance followed by Bonferroni's multiple comparison test.

Reconstitution of miR-122$^{loxp/loxp}$ Alb Cre+ mice via Phd1 repression. To demonstrate that the observed increases of hepatic injury in *miR-122$^{loxp/loxp}$* Alb Cre+ mice are functionally related to elevated hepatic Phd1 levels, we next attempted reconstitution studies with antisense oligonucleotides targeting murine Phd1 (ASO-Phd1). For this purpose we pre-treated *miR-122$^{loxp/loxp}$* Alb Cre+ mice with four i.p. injections of ASO-Ph1 over a two week time course (**Fig. 5a**). This treatment approach was associated with as selective repression of Phd1 transcript and protein levels, (**Fig. 5b,c; Supplementary Fig. 2**), while hepatic HIF1A stabilization during ischemia and reperfusion were restored (**Fig. 5d**). Subsequent studies of hepatic ischemia and reperfusion injury demonstrated normalization of hepatic injury in *miR-122$^{loxp/loxp}$* Alb Cre+ mice (**Fig. 5k-p**), while this treatment provided robust liver protection in wild-type mice (**Fig. 5e-i**), in the context of enhanced hepatic HIF1A levels (**Fig. 5j**). Taken together, these studies indicate a functional role for PHD1 repression in mediating miR-122-elicited liver protection during hepatic ischemia and reperfusion injury.

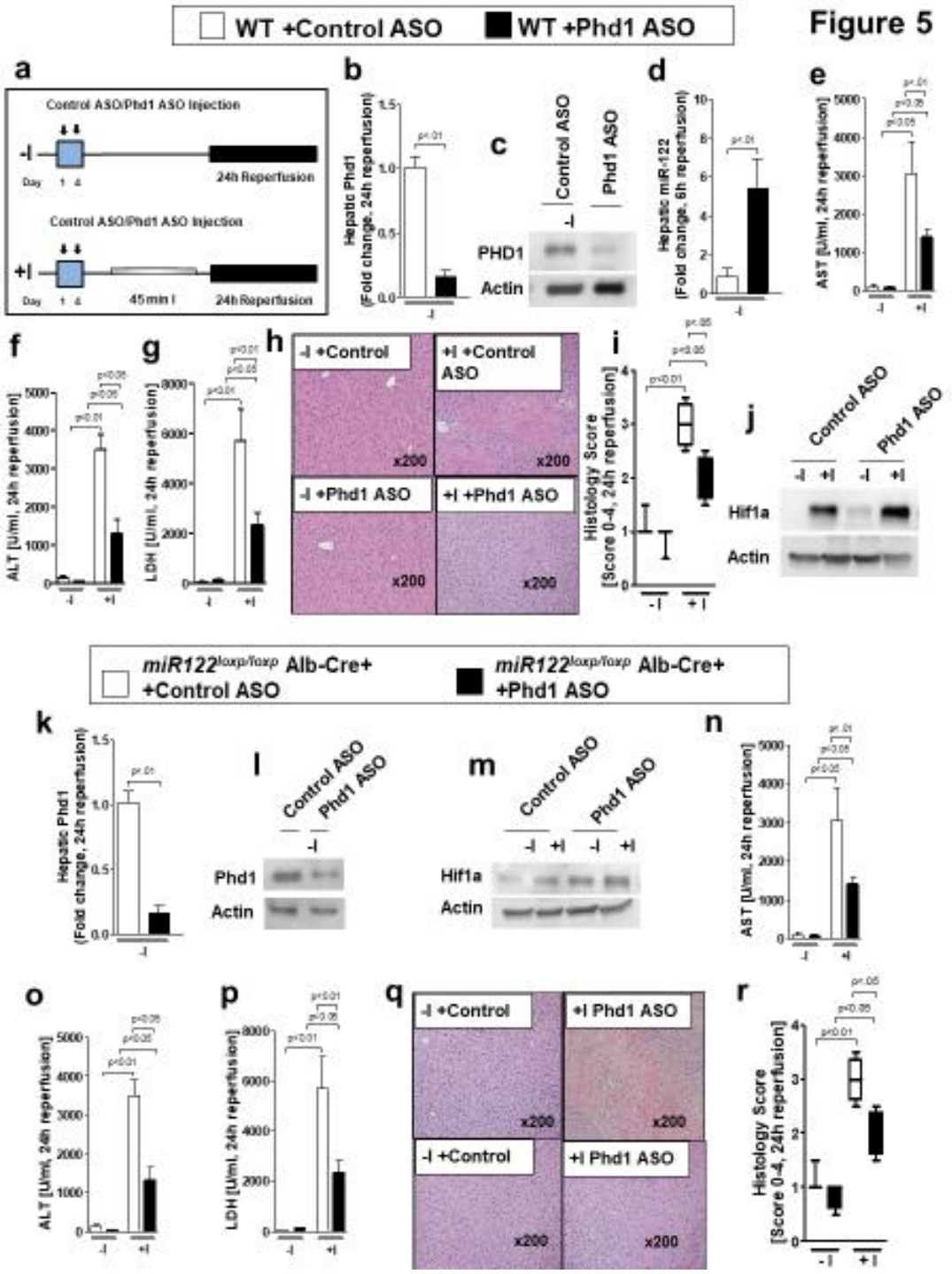

Figure 5*: Hepatic ischemia reperfusion injury in wild type mice and hepatocyte miR-122 deletion mice with PHD1 antisense oligo treatment.* (a) Schematic of antisense oligo treatment compared with control and PHD1 antisense oligo. (b) Phd1 mRNA level, (c) Phd1 protein expression, (d) hepatic miR-122 RT-PCR (e-g) serum levels in PHD1 ASO treated

wild type mice. (h) Histology and (i) histology scoring with Suzuki test. (j) HIF1 α expression level in Phd1 ASO wild type mice following sham and without ischemia. (k) Phd1 RT-PCR , (l) Phd1 protein level and (m) HIF1 α in hepatocyte miR-122 deletion with Phd1 antisense oligo treated mice. (n-p) Serum levels, (q) histology and (r) histology scoring. Data presented as mean ± SD of three to eight samples per group. $P<0.05$, one-way analysis of variance followed by Bonferroni's multiple comparison test.

Hepatic overexpression of miR122 provides liver protection during hepatic ischemia and reperfusion injury. To demonstrate that pharmacologic interventions to directly enhance hepatic miR-122 expression, we next performed treatment studies of wild-type mice with nano-particles containing miR-122 mimetic. Pretreatment with a single dose of NLE-formulated miR-122 mimetic 24h was associated with robust elevations of hepatic miR-122 levels after 24h (**Fig. 6a, b**). Studies of hepatic ischemia and reperfusion injury demonstrated robust liver protection in the context of repressed PHD1 transcript and protein levels, and elevated HIF1A protein stabilization (**Fig. 6c-j**). Together, these studies indicate that hepatic miR-122 overexpression represents a novel therapeutic approach for the treatment of ischemia and reperfusion injury of the liver.

Hepatic miR-122 levels are elevated during human liver transplantation. As final step we performed proof-of-principle studies in liver biopsies obtained during human liver transplantation (AMCIRB Protocol 16-0100). For this purpose, we obtained two subsequent liver biopsies during cadaveric liver transplantation – the first during cold ischemia and a second biopsy from the same liver following warm ischemia and reperfusion. Each liver biopsy served as its own control (n=10; average cold, warm ischemia and reperfusion time **Fig. 6k**; patient demographics **Supplementary Table 1**). Liver biopsies were immediately snap-frozen in the operating room. Consistent with the above studies in murine ischemia and reperfusion injury of the liver, we found that hepatic hsa-miR-122 levels were dramatically elevated during human liver transplantation (over 19-fold; **Fig. 6l**). Consistent with a

functional role for miR-122 induction in repressing its target gene PHD1, we found that transcript and protein levels of PHD1 were repressed during human liver transplantation (**Fig. 6m-p**). Taken together, these findings indicate that the observed transcriptional alterations such as induction of miR-122 and concomitant repression of PHD1 are occurring during human liver transplantation.

Table 1. Data represents the individual patient (5 patients) or mean ± SD. MELD score, model for end-stage liver disease score, cold ischemia time (CIT), cold ischemia until the first biopsy was taken (Biopsy CIT), total cold ischemia time (total CIT), warm ischemia ti me (WIT), reperfusion time (RT)

	Patient 1	**Patient 2**	**Patient 3**	**Patient 4**	**Patient 5**	**Mean ± SD**
Donor age	24	26	33	36	27	29.2 ± 5.06
Recipient age	31	61	69	50	30	48.2 ± 17.51
MELD	27	29	31	27	31	29 ± 2
Biopsy CIT (min)	407	450	185	311	396	349.8 ± 105.1
Total CIT (min)	515	581	324	360	558	467.6 ± 117.7
WIT (min)	45	54	44	45	48	47.2 ± 4.08
RT (min)	130	119	127	131	110	123.4 ± 8.84

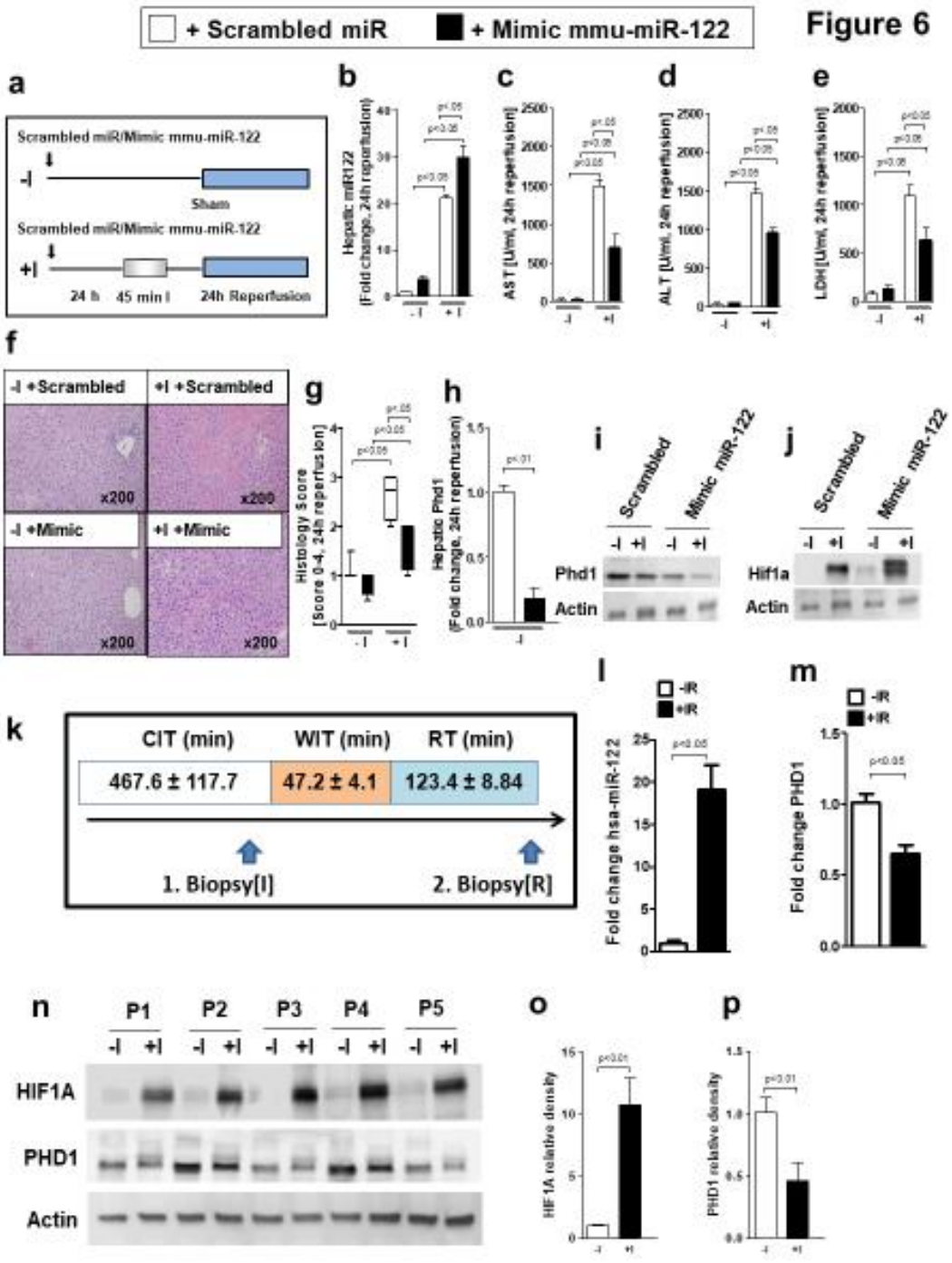

Figure 6: Hepatic overexpression of miR-122 during acute liver injury in WT mice and miR-122 induction in human liver following ischemia. (a) Schematic of murine mimic miR-122 treatment compared with control. (b) miR-122 transcripts level in 24h treated with mimic miR-122 mice. (b-e) Hepatic serum levels, (f) liver histology and (g) histology

scoring. (h) Phd1 RT-PCR, (i) western blot of Phd1, and (j) Hif1 α protein level in mimic murine miR-122 injected mice model. (k) First liver biopsy was taken during ischemia (I) at the conclusion of cold ischemia time (CIT) during back table preparation of the liver allograft. A second biopsy was taken during reperfusion (R) after warm ischemia time (WIT) and reperfusion time (RT) immediately before closure of the abdomen following drain placement. (i) Fold change of human miR-122 and (m) PHD1 level following ischemia reperfusion. (n) Western blot of HIF1A and PHD1 in patient liver specimen following ischemia compared to without ischemia.

(o) HIF1A densitometry and (p) PHD1 densitometry. Data presented as mean ± SD of three to eight samples per group. $P<0.05$, one-way analysis of variance followed by Bonferroni's multiple comparison test.

Discussion

The current studies are designed to identify miRNA targets for the treatment of hepatic ischemia and reperfusion injury. To make progress on this front we performed a targeted miRNA screen in a murine model of partial liver ischemia. This approach revealed the most prominent increase of hepatic miRNAs for miR-122 – a miRNA that has become famous for its role in propagating the replication of the hepatitis C virus[16]. Interestingly, we observed an opposing role for miR-122 during hepatic ischemia and reperfusion injury, where mice with hepatocyte-specific deletion of miR-122 experience increased disease susceptibility during hepatic ischemia and reperfusion injury. Subsequent studies demonstrated that mir-122 is a HIF1A target gene, and binding of HIF1A to the miR-122 promoter is critical for miR-122 induction. Moreover, a subsequent array in hepatocytes with miR-122 overexpression pointed us towards the PHD-1 – an oxygen sensing hydroxylase critical in targeting HIF1A for proteasomal degradation during normoxic conditions. As such, the present studies point towards a functional role of miR-122 induction and concomitant repression of PHD1, as a

feed-forward pathway to enhance hepatic HIF1A stabilization, and more robust HIF1A-elicited liver protection during hepatic ischemia and reperfusion injury.

Several previous studies had implicated the PHD-HIF pathway in promoting ischemia tolerance of different organs. For example, a recent study showed that loss of Phd1 lowers oxygen consumption in skeletal muscle by reprogramming glucose metabolism from oxidative to more anaerobic ATP production through activation of a Pparalpha pathway. This metabolic adaptation to oxygen conservation impairs oxidative muscle performance in healthy conditions, but it provides acute protection of myofibers against lethal ischemia. In this model, hypoxia tolerance is due to reduced generation of oxidative stress, which allows Phd1-deficient myofibers to preserve mitochondrial respiration[26]. Similarly, a recent study implicated an interaction of HIF1A with the circadian rhythm protein Period2 in promoting ischemia tolerance through the transcriptional induction of glycolytic enzymes, a pathway that can also be activated by light-treatment[28]. Similarly, inhibition of PHDs via succinate, and concomitant stabilization of HIF1A has been shown to optimize alveolar-epithelial carbohydrate metabolism, thereby attenuating lung inflammation during mechanical ventilation[29]. Again other studies of ischemia and reperfusion demonstrate that HIF can function to promote the generation and signaling of anti-inflammatory adenosine, and thereby promote ischemia tolerance or dampen post-ischemic vasoconstriction and inflammation[30,31]. Together, these studies indicate that PHD-dependent stabilization of HIF is a critical step in mediating ischemia tolerance[32,33].

The present studies have important translational implications. As a direct extension of the present findings, therapeutic approaches to enhance hepatic miR-122 via nano-particle treatment with miR-122 mimetics could be pursued in patients prior to liver transplantation. This could be achieved by treatment of the donor during cadaveric liver transplantation, the addition of miR-122 mimetic-containing nanoparticles to the solution used for extracorporal liver preservation, or by pre-operative treatment of the donor prior to living-donor related

liver transplantation[34]. Due to the fact that the liver functions as a filter for nanoparticles, overexpression of miRNAs in the liver is easier to be achieved compared to organs (e.g. the heart). Alternatively, miR-122-dependent liver protection can be mimicked by selectively targeting PHD1 utilizing PHD1-specific oligonucleotides. Indeed, previous studies have shown that specific ASOs can be successfully used for the treatment of human disease[35]. Alternatively, PHD inhibition could also be achieved pharmacologically by the use of PHD inhibitors. Indeed, a recent clinical study utilized a global PHD inhibitor successfully for the treatment of renal anemia in humans[36]. As no side-effects were reported, this study indicates safety for utilizing PHD inhibitors in human, and those findings could be extended towards human liver transplantation.

Finally, our findings also have important implications for patients with hepatitis C who are receiving treatment with ASOs targeting miR-122. Indeed, a recent study showed that Miravirsen - a locked nucleic acid-modified DNA phosphorothioate antisense oligonucleotide that sequesters mature miR-122 in a highly stable heteroduplex – showed prolonged dose-dependent reductions in HCV RNA levels without evidence of viral resistance. As such, patients receiving treatment with Miravirsen may soon be undergoing liver surgery, e.g. for the resection of hepatitis-C associated hepatocellular carcinoma. Based on our studies in mice with hepatocyte-specific deletion of miR-122, we would predict that Miravirsen treatment is associated with dramatic increases of hepatic ischemia and reperfusion injury. As such, it may be an important consideration to discontinue Miravirsen treatment prior to hepatic surgery.

Taken together, the present studies implicate HIF1A-elicited induction of miR-122 in hepato-protection during ischemia and reperfusion injury via repression of its target gene PHD1. While these studies have important implications for liver transplantation or major liver surgery, steps to take these findings from bench to bedside will be critical. Such efforts

could include testing pharmacologic approaches to achieve hepatic overexpression of miRNAs, or the design and safety testing of human ASOs to target PHD1.

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국문 요약

간이식에서 간허혈성 및 재관류에 의한 손상은 수술의 합병증 및 사망률에 중요한 영향을 미친다. microRNA는 mRNA를 억제하여, 전사후의 유전자 발현을 조절하는 비코딩 RNA의 일종으로서, 이번 연구에서는 간 허혈 관용을 향상시키는 특정 micrioRNA을 다루고자 한다. 이 실험에서 간 허혈의 쥐 모델에서 표적화된 microRNA는 miR-122이며, 이 microRNA는 C 형 간염 바이러스 전파에 관여하는 것으로 알려져 있다. 간세포 내에서 miR-122 (miR-122loxp / loxp Alb Cre + mice)의 결실을 보인 쥐는 심한 허혈성 간 손상을 보였다. 또한 miR-122의 유도에 따라 hypoxia-inducible factor HIF1A가 전사되어, prolyl-hydroxylase PHD1의 활성이 억제되는 것이 관찰되었다. 뿐만 아니라, 간이식을 시행한 5명의 인체 간세포에서도 비슷한 결과를 보였다. 여러 연구 결과에 따르면, miR-122의 HIF1A 의존 유도는 PHD1 억제를 일으키며, 간에서의 HIF 반응의 강화를 통하여, 간손상 억제를 위한 피드포워드 경로 관여한다는 것이 알려져 있다. 이러한 결과는 miR-122를 촉진하거나 PHD1 발현을 억제하는 약리학적 접근법이 간 허혈 관용을 향상시키기 위한 새로운 치료법으로 제시될 수 있음을 시사한다.