Chinese Medical Journal®

Direct reprogramming of fibroblasts into functional hepatocytes via CRISPRa activation of endogenous *Gata4* and *Foxa3*

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Abstract

Background: The ability to generate functional hepatocytes without relying on donor liver organs holds significant therapeutic promise in the fields of regenerative medicine and potential liver disease treatments. Clustered regularly interspaced short palindromic repeats (CRISPR) activator (CRISPRa) is a powerful tool that can conveniently and efficiently activate the expression of multiple endogenous genes simultaneously, providing a new strategy for cell fate determination. The main purpose of this study is to explore the feasibility of applying CRISPRa for hepatocyte reprogramming and its application in the treatment of mouse liver fibrosis.

Method: The differentiation of mouse embryonic fibroblasts (MEFs) into functional induced hepatocyte-like cells (iHeps) was achieved by utilizing the CRISPRa synergistic activation mediator (SAM) system, which drove the combined expression of three endogenous transcription factors—*Gata4*, *Foxa3*, and *Hnf1a*—or alternatively, the expression of two transcription factors, *Gata4* and *Foxa3*. *In vivo*, we injected adeno-associated virus serotype 6 (AAV6) carrying the CRISPRa SAM system into liver fibrotic *Col1a1-Cre*^{ER}; *Cas9*$^{fl/fl}$ mice, effectively activating the expression of endogenous *Gata4* and *Foxa3* in fibroblasts. The endogenous transcriptional activation of genes was confirmed using real-time quantitative polymerase chain reaction (RT-qPCR) and RNA-seq, and the morphology and characteristics of the induced hepatocytes were observed through microscopy. The level of hepatocyte reprogramming *in vivo* is detected by immunofluorescence staining, while the improvement of liver fibrosis is evaluated through Sirius red staining, alpha-smooth muscle actin (α-SMA) immunofluorescence staining, and blood alanine aminotransferase (ALT) examination.

Results: Activation of only two factors, *Gata4* and *Foxa3*, via CRISPRa was sufficient to successfully induce the transformation of MEFs into iHeps. These iHeps could be expanded *in vitro* and displayed functional characteristics similar to those of mature hepatocytes, such as drug metabolism and glycogen storage. Additionally, AAV6-based delivery of the CRISPRa SAM system effectively induced the hepatic reprogramming from fibroblasts in mice with live fibrosis. After 8 weeks of induction, the reprogrammed hepatocytes comprised 0.87% of the total hepatocyte population in the mice, significantly reducing liver fibrosis.

Conclusion: CRISPRa-induced hepatocyte reprogramming may be a promising strategy for generating functional hepatocytes and treating liver fibrosis caused by hepatic diseases.

Keywords: Liver fibrosis; CRISPRa; Cell reprogramming; Transcriptional activation; Hepatocytes

Introduction

Liver diseases, including liver metabolic diseases and fulminant liver failure, are important causes of death worldwide.[1–4] Replacing the diseased tissue with functional hepatocytes has translational value for the treatment of fibrotic liver diseases.[5–9] The induced hepatocyte-like cells (iHeps) derived from direct reprogramming can provide an adequate supply of sufficient functional hepatocytes, effectively addressing the scarcity of donor liver organs.[5,6,9] Moreover, the *in vivo* conversion of fibroblasts into iHeps provides one potential approach for the treatment of liver fibrosis.

Clustered regularly interspaced short palindromic repeats (CRISPR)-mediated gene activation, termed CRISPR activator (CRISPRa), involves the use of fusion

Access this article online

Quick Response Code:

Website: www.cmj.org

DOI: 10.1097/CM9.0000000000003088

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Chinese Medical Journal 2024;137(11)

Received: 06-07-2023; **Online:** 09-05-2024 **Edited by:** Jinjiao Li

proteins of catalytically dead Cas9 (dCas9) and transcriptional activation domains (e.g. VP64 and p65) to recruit transcription activators[10–14] in mammalian cells, activating the expression of endogenous genes and triggering chromatin remodeling of target genes.[15] The ability to program these transcription factors (TFs) to target any genomic locus of interest through simple exchange of the 20-nt targeting sequence of the single-guide RNA (sgRNA) enables a simple, robust, and highly scalable method for controlling of complex transcription networks. Several sets of CRISPR-based activators, such as dCas9-SunTag-p65-HSF1, VP64-dCas9-VP64, dCas9-SunTag-VP64, and synergistic activation mediator (SAM) system, have been developed.[16] The dCas9-SAM system contains three components: the dCas9-VP64 fusion protein, SAMs MS2-P65-HSF1 (MPH), and sgRNA. In the SAM system, the sgRNA scaffold is engineered with two MS2 hairpin aptamers, which can recruit the transactivator MPH to form a transcriptional activation complex. The SAM system can be packaged with adeno-associated virus (AAV) after VP64 is removed, indicating the SAM system is a promising tool for application *in vivo*.[17]

In this study, we report that CRISPRa-mediated activation of two key TFs, *Gata4* and *Foxa3,* could directly induce fibroblasts to differentiate into hepatocyte-like cells. These CRISPRa-activated *Gata4* and *Foxa3*-induced hepatocyte-like cells (CRGF-iHeps) could be expanded *in vitro* and displayed functional characteristics of mature hepatocytes. Notably, adeno-associated virus serotype 6 (AAV6)-mediated CRISPRa activated the expression of *Gata4* and *Foxa3 in vivo* and induced fibroblast reprogramming into functional hepatocytes, thus reducing liver fibrosis. Our study aims to provide a strategy for generating functional hepatocyte-like cells for liver engineering and regenerative medicine.

Methods

Cell procedures

Mouse embryonic fibroblasts (MEFs) were from C57BL/6 mouse embryos 13.5-day post-coitum, and the isolation process strictly followed the instruction in published articles. The embryos were carefully dissected to remove the head and visceral tissues. The remaining tissues were minced using forceps and then incubated in a solution containing 2.5 g/L trypsin with 1 mmol/L ethylenediaminetetraacetic acid (EDTA) for 20 min at 37°C. Following trypsinization, MEF medium (Dulbecco's Modified Eagle's Medium [DMEM; Gibco, #11965092, NY, USA] supplemented with 10% fetal bovine serum [FBS; Sigma, #12003C, Victoria, Australia], 2 mmol/L L-glutamine [Gibco, #A2916801], and penicillin/streptomycin [Gibco, #15140122]) supplemented with 25 μg/mL DNase I (Roche, #10104159001, Mannheim, Germany) was added to dissociate the tissue fragments. The triturated cells were collected by centrifugation, resuspended in MEF medium, and plated on 15-cm tissue culture dishes. The cells were cultured in MEF medium for 3–4 days at 37°C under 5% CO_2 before freezing.

Tail tip fibroblasts (TTFs) were prepared from 10-week-old adult C57BL/6 mice. The tail tips were obtained and minced into 5-mm pieces. The tissue fragments were then plated on gelatin-coated 12-well plates and grown in a 1:1 mixture of DMEM/F-12 medium (Gibco, #11320033) and MEF medium until they reached confluency.

Hepatocytes were isolated from 10-week-old adult C57BL/6 mouse livers using a two-step collagenase digestion method. Induced hepatocyte-like cells (iHeps) and adult mouse hepatocytes were cultured in hepato-medium, which is DMEM/F-12 (Gibco, #11320033) supplemented with 10% FBS (Gibco, #10099158), 1 μg/mL insulin (Sigma, #I9278, MO, USA), 100 nmol/L dexamethasone (Sigma, #D4902), 10 mmol/L nicotinamide (Gibco, A13449), 2 mmol/L L-glutamine (Gibco, #A2916801), 50 mmol/L β-mercaptoethanol (Gibco, #21985023), and penicillin/streptomycin (Gibco, #15140122). The medium was further supplemented with 20 ng/mL hepatocyte growth factor (R&D, #2207-HG, Minnesota, USA) and 20 ng/mL epidermal growth factor (R&D, #2028-EG).

sgRNA design and production

The design and assembly of sgRNA were performed following the methodology described by Silvana Konermann *et al*.[15] Specifically, sgRNAs were designed to target the promoter regions of the *Gata4*, *Foxa3*, and *Hnf1α* genes. The sgRNA sequences, along with their reverse complementary oligonucleotides bearing sticky ends, were synthesized by Tianyi Huiyuan Company (Beijing Tianyi Huiyuan Bioscience & Technology Inc., Beijing, China). The sgRNA oligonucleotide sequences were subsequently annealed to form double-stranded DNA. In parallel, the SAM vector was digested with *Bsm*BI enzyme, and the resulting digested products were extracted through gel extraction. Finally, the annealed sgRNA and SAM vector were ligated together using T4 ligase. A list of sgRNA oligonucleotides is provided in the Supplementary Table 1, http://links.lww.com/CM9/B964.

Lentiviral production

The human Embryonic Kidney 293 transformed (HEK293T; ATCC, #CRL-3216, MD, USA) cells were cultured in DMEM (Gibco, #11965092) supplemented with 10% FBS (Sigma, #12003C) and 1% penicillin/streptomycin (Invitrogen, #15140122, NY, USA) at 37°C with 5% CO_2. Approximately, 3.5 million cells were seeded per 10-cm tissue culture polystyrene (TCPS) dish. After 24 h, the cells were transfected via the lipid-mediated transfection method using Hieff Trans® Liposomal Transfection Reagent (Yeason, #40802ES, Shanghai, China) with pMD2.G (Addgene, #12259, Massachusetts, USA) and psPAX2 (Addgene, #12260) second-generation envelope and packaging plasmids (lenti-SAMv2 and lenti-MPHv2). The viral supernatant was collected at 24 h and 48 h after the addition of transfection liposomes. The collected viral supernatant was pooled and centrifuged at 600 × *g* for 10 min, followed by filtration through a 0.45-μm filter. Finally, the viral

supernatant was concentrated using Lentivirus Concentration Solution (Yeason, #41101ES) according to the manufacturer's protocol.

AAV vectors

The AAV6-MS2-dg*Gata4*-dg*Foxa3*-MPH virus was prepared in HEK293T cells using PEI transfection, as previously described.[19] AAV vectors were purified via cesium chloride gradient centrifugation and diluted in phosphate-buffered saline (PBS; Gibco, #10010023) for intravenous injection. Mice were injected with 4.3×10^{11} viral genomes (vg) of each vector via the tail vein, unless otherwise specified. The injection was performed at a slow rate, with a volume limit of 300 μL for tail vein injections.

Real-time quantitative polymerase chain reaction (RT-qPCR) and end point PCR

RT-qPCR analysis was performed on a QuantStudio™ 5 Real-Time PCR system (Applied Biosystems).

For end point PCR, cDNA was amplified with Ex Taq DNA Polymerase (TaKaRa, #RR001A, Beijing, China) by PCR on a thermocycler (Applied Biosystems). All primers used were listed in the Supplementary Table 2, http://links.lww.com/CM9/B964.

Periodic acid-Schiff (PAS) staining and DiI-labeled acetylated low-density lipoprotein (DiI-ac-LDL) assays, Albumin enzyme-linked immunosorbant assays (ELISAs) and cytochrome P450 (CYP) metabolism assays

The cells were subjected to staining using PAS (Abcam, ab150680, Cambridge, UK) and DiI-ac-LDL (Invitrogen, L3484) following the manufacturer's guidelines. For analysis of albumin secretion, MEFs transduced with the three factors were cultured in phenol red-free medium. After 24 h of culture, the culture supernatant was collected. The concentration of albumin (ALB) in the supernatant was measured using the mouse albumin ELISA kit (Abcam, ab207620) according to the manufacturer's instructions. To measure CYP enzyme activity, MEFs and iHep cells were cultured in medium containing 50 mmol/L 3-methylcholanthrene for 48 h. The cells were dissociated and incubated with substrates in 200 mL of incubation medium at various concentrations for 3 h at 37°C. The reaction was stopped by the addition of 800 mL of cold methanol followed by centrifugation. The resulting supernatants were collected for analysis of the indicated products using liquid chromatography-tandem mass spectrometry (LC–MS/MS; BD Biosciences 1200 HPLC and API 4000 mass spectrometer, California, USA). Freshly isolated hepatocytes were utilized as a positive control. The total protein content of the cells was used to normalize the data. Substrates and standard metabolic products such as phenacetin (Sigma, 77440), diclofenac (Sigma, SML3086), bufuralol (Sigma, 615668), acetaminophen (Sigma, A-064), 4′-OH diclofenac (Sigma, H3661), testosterone (Sigma, T5411), 6′-OH-testosterone (Sigma, H-059), and 1′-OH-bufuralol (BD Biosciences, 451040, CA, USA) were obtained from commercial suppliers.

Immunofluorescence

For immunofluorescence (IF) staining, the cells were fixed with 4% paraformaldehyde for 15 min at room temperature. Subsequently, the sections were incubated with 0.2% Triton X-100 (Sigma, #X100) in PBS for 15 min and washed three times with PBS. After blocking with 3% bovine serum albumin (BSA) in PBS for 60 min at room temperature, the cells were incubated overnight at 4°C with primary antibodies. After three PBS washes, the cells were incubated with the appropriate fluorescence-conjugated secondary antibodies in the dark for 60 min at room temperature. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma, D9542). The primary and secondary antibodies were diluted in PBS containing 3% BSA. The following antibodies were used for IF: mouse anti-albumin (Abcam, ab79827, 1:200), rabbit anti-E-cadherin (Cell Signaling Technology, #3195S, 1:500, MA, USA), and mouse anti-MUP-1 (R&D, MAB6560, 1:200). The secondary antibodies used were Cy5-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Inc., #115-175-205, 1:1000, PA, USA), Cy3-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Inc., #111-165-144, 1:1000), and Cy3-conjugated donkey anti-goat IgG (Jackson ImmunoResearch Inc., #705-166-147, 1:1000).

Mice

All animal experiments were conducted following the principles outlined in the Guide for the Care and Use of Laboratory Animals. The animal experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Fuwai Hospital, Chinese Academy of Medical Sciences.

Cas9-EGFP mice [B6;129-Gt(ROSA)26Sor$^{tm1(CAG-cas9*,-EGFP)Fezh/J}$] and *Col1a1-Cre/ER* mice [B6.Cg-Tg(*Col1a1*-cre/ER,-DsRed) 1Smkm/J] (Strain #029241) were obtained from the Jackson Laboratory (Bar Harbor, Maine, USA). Recombination was induced by intraperitoneal injection of tamoxifen (75 mg tamoxifen/kg body weight) once every 24 h, for four consecutive days, to induce gene recombination in the hybrid mouse strains. Cas9 mice carry a knock-in allele (RCL-Cas9-EGFP) that enables Cre recombinase-dependent expression of the CRISPR-associated protein 9 (Cas9) endonuclease and enhanced green fluorescent protein (EGFP). When combined with dead guid RNA (dg RNA)-MPH and Cre recombinase, these mice can activate single or multiple genes *in vivo*. The mice used in this study were C57BL/6 mice and ranged in age from 5 weeks to 16 weeks. The experimental and control groups, which included both male and female mice, had an even distribution of the littermates.

Liver fibrosis models

Mice were administered with CCl_4 diluted at 1:3 in corn oil at a dosage of 0.5 μL/g of body weight via intraperitoneal injection every other day for 16 doses, and the livers

were harvested and analyzed on the second or the third day after the last dose of CCl_4.

RNA-seq and analysis pipeline

The total RNA of MEFs and iHeps was extracted from 5×10^6 cells using TRIzol reagent (Invitrogen, #15596026). Sequencing libraries were prepared according to the instructions of Illumina RNA-Seq library kit with minor modifications. Samples were sequenced using an Illumina HiSeq platform with 150 bp paired-end reads. The raw reads were processed with Trim-Galore (Version 0.6.6) to remove adaptors and low-quality sequences, and the clean reads were aligned to the mouse mm9 reference genome using STAR (Version 2.7.1a, https://github.com/alexdobin/STAR). The expression of genes fragments per kilobase of exon per million reads mapped (FPKM) and raw read counts were calculated using the RSEM (Version 1.3.1, https://deweylab.github.io/RSEM) program "rsem-calculate-expression". Only genes with expression values FPKM >1 in at least one cell type were considered for subsequent analysis. The DESeq2 (Version 1.34.0) R package was used to normalize raw read counts and detect differentially expressed genes. GSEA was performed using the R package clusterProfiler (Version 4.2.2). According to the log2 fold change as determined by DESeq2, the genes were ranked as the input. *P*-values were adjusted using the Benjamini-Hochberg FDR (BH-FDR) method. Pathway enrichment analysis was conducted using function gseGO from the clusterProfiler R package, with parameters ont = "biological process (BP)" and pAdjustMethod = "BH."

Calculation of hepatic reprogramming efficiency and liver repopulation

The hepatic reprogramming efficiency was determined by calculating the percentage of induced hepatocyte-like cells derived from fibroblasts (F-iHeps; single cells or nodules) among all hepatocytes present in the liver. The total number of hepatocytes per 10^3 fields in a liver section was quantified using fumarylacetoacetate hydrolase (FAH) IF and DAPI staining. To account for hepatocyte-free areas such as blood vessels and mesenchyme, F-iHeps were quantified in more than 2000 hepatocytes per mouse and randomly selected from 10^3 images of R2 liver sections from R2 liver lobes to account for hepatocyte-free areas such as blood vessels and mesenchyme. F-iHeps were identified based on the coexpression of EGFP and FAH. The extent of liver repopulation was determined by calculating the percentage of all F-iHeps among all hepatocytes in the liver.

Statistical analyses

The data are presented as means ± standard errors of the means (SEMs). Statistical comparisons between the experimental and control groups were performed using an unpaired, two-tailed Student's *t* test, unless stated otherwise. The homogeneity of variances was tested by Levene's test. A *P*-value of less than 0.05 was considered to be statistically significant. Statistical analyses were conducted using Prism 6.0 (GraphPad, MA, USA) and R (Version 4.10, https://www.R-project.org/).

Results

Induction of hepatocyte-like cells from MEFs by SAM-mediated endogenous Gata4, Foxa3, and Hnf1a activation

The overexpression of transgenes encoding the TFs *Gata4*, *Foxa3,* and *Hnf1a* (GFH) has been shown to directly convert cultured MEFs to functional iHeps. We hypothesized that CRISPRa-targeted activation of endogenous genes encoding these same factors in their native chromatin context could provide an alternative method to achieve the reprogramming of MEFs into iHeps [Figure 1A]. For targeted gene activation, we used the SAM system to activate endogenous GFH expression by targeting the transcription start site (TSS) of these genes in MEFs [Figure 1A, B and Supplementary Table 1, http://links.lww.com/CM9/B964]. Stable MEF-MPH were infected with SAM-sgRNA lentivirus [Figure 1A]. After 5 days of infection, sg*Gata4*-1 and sg*Gata4*-2 led to approximately 1500-fold and 1000-fold increases in *Gata4* transcription, respectively (compared with those in the SAM-sgMock or no-infection controls) [Figure 1C]; *Foxa3* expression was elevated 100-fold, 90-fold, and 5-fold by sg*Foxa3*-1, sg*Foxa3*-2, and sg*Foxa3*-3, respectively [Figure 1D]. The gRNA (gRNA1-3) targeting regions proximal to the TSS of the *Hnf1a* locus did not induce detectable levels of activation [Figure 1E]. We combined sg*Hnf1a*-7, -8, and -9 to infect MEFs, which resulted in a 25-fold increase in the expression of *Hnf1a* [Figure 1B, E]. In addition, immunostaining and western blotting revealed that the protein expression levels of GFH could be substantially increased after infection with SAM-sg*Gata4*-sg*Foxa3*-sg*Hnf1a* (SAM-sg*GFH*) [Figure 1F, G].

Next, we introduced the most efficient gRNAs into MEF-MPH [Figure 2A]. After three days of infection with SAM-sgRNAs of three genes, we replanted the cells on collagen-coated dishes and culture them further. Within 7–10 days after replanting, morphologically identifiable epithelial-like cells appeared in the fibroblast cultures and proliferated in clusters [Figure 2B]; these cells were designated as CRISPRa-activated GFH induced hepatocyte-like cells (CRGFH-iHeps). IF staining revealed that the epithelial cell marker E-cadherin (ECAD) was coexpressed with albumin (ALB) in CRGFH-iHeps [Figure 2C], indicating a successful mesenchymal-epithelial transition in CRGFH-iHeps. The CRGFH-iHeps also expressed hepatic genes, such as *Alb*, *Ttr*, *Aat*, and *CK18* [Figure 2D]. Functionally, CRGFH-iHeps exhibited glycogen storage, as demonstrated by PAS staining [Figure 2E] and uptake of DiI-ac-LDL [Figure 2E]. Furthermore, CRGFH-iHeps exhibited CYP activity [Figure 2F], secreted large amounts of ALB into the medium [Figure 2G] and metabolized drugs [Figure 2H]. These results showed that MEFs were converted into iHeps by the CRISPRa SAM system, which resulted in major changes in hepatic gene expression and hepatic function.

Induced hepatic reprogramming by CRISPRa-activated endogenous Gata4 and Foxa3

To investigate the feasibility of CRISPRa-mediated activation of fewer TFs for hepatic reprogramming, we infected

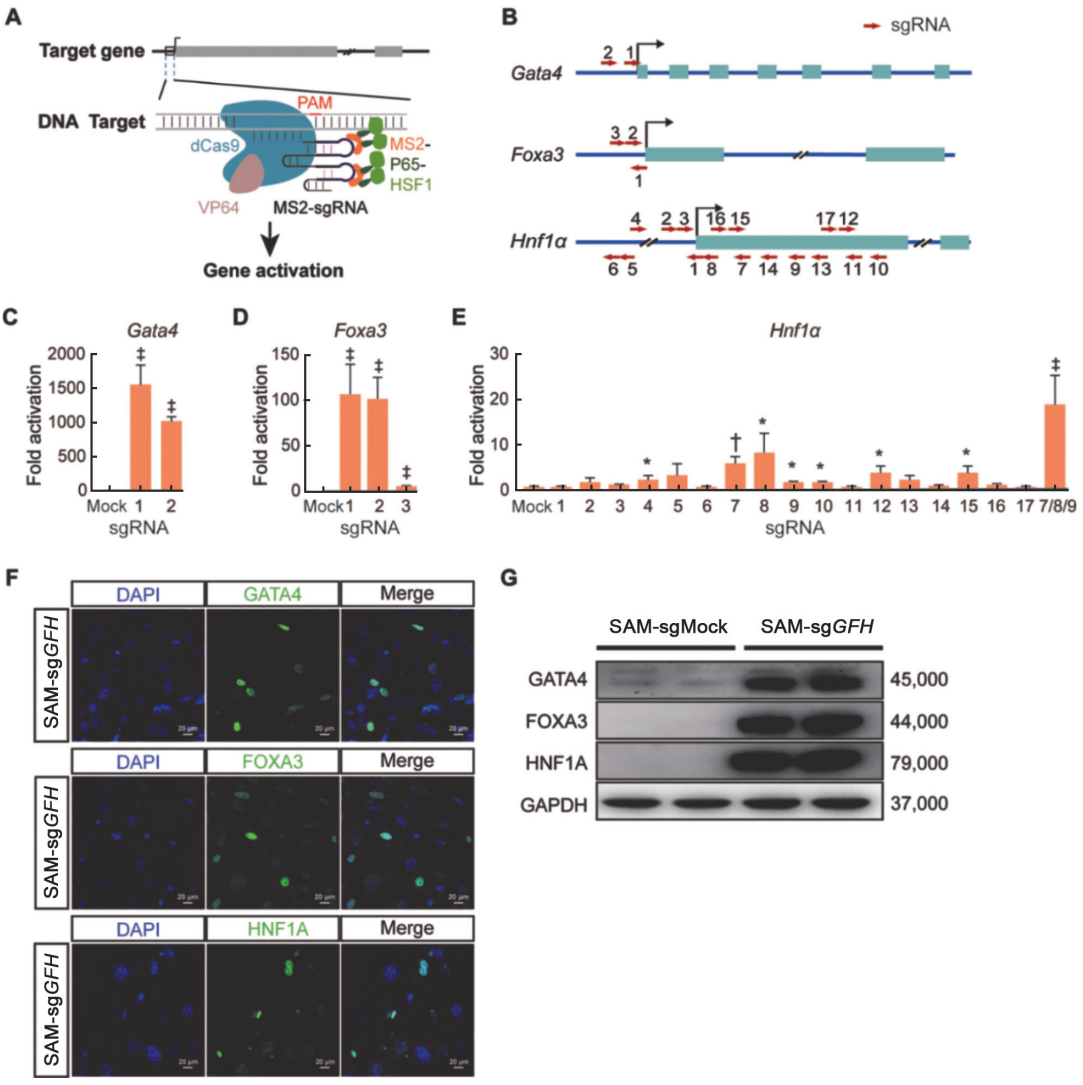

Figure 1: CRISPRa activates endogenous *GFH*. (A) Schematic diagram of SAM system activated gene expression. (B) The mouse *GFH* genes. gRNA targets are indicated (red arrows). (C–E) MEFs were transfected with indicated SAM-gRNAs. The levels of *GFH* activation were analyzed via RT-qPCR 3 days after infection ($n = 3$). (F) IF staining of GFH in MEFs demonstrated protein expression through targeted activation of the endogenous loci. Scale bar, 20 μm. (G) GFH proteins were detected in CRISPRa-activated cells by Western blot analysis. All the gRNAs used are listed in Supplementary Table 1, http://links.lww.com/CM9/B964. All the assays were performed on day 3 post-transfection ($n = 3$). Student's *t* test, $^{*}P < 0.05$, $^{\dagger}P < 0.01$ and $^{\ddagger}P < 0.001$. CRISPRa: Clustered regularly interspaced short palindromic repeats (CRISPR) activator; DAPI: 4′,6-diamidino-2-phenylindole; dCas9: dead Cas9; GFH: *GATA4, FOXA3 and HNF1A*; HSF1: Heat shock factor 1; IF: Immunofluorescence; MEFs: Mouse embryonic fibroblasts; MPH: MS2-P65-HSF1, Transcriptional activation complexes; PAM: Protospacer adjacent motif; RT-qPCR: Real-time quantitative polymerase chain reaction; SAM: Synergistic activation mediator; SAM-sg*GFH*: SAM-sg*Gata4*-sg*Foxa3*-sg*Hnf1a*, sgRNA: Single-guide RNA; VP64: Transcriptional activator.

SAM-sg*Gata4*-sg*Foxa3* (SAM-sg*GF*), SAM-sg*Gata4*-sg*Hnf1a* (SAM-sg*GH*), and SAM-sg*Foxa3*-sg*Hnf1a* (SAM-sg*FH*), and the single factors SAM-sg*Gata4*, SAM-sg*Foxa3*, and SAM-sg*Hnf1a* in MEF-MPH, respectively. After 12 days of induction, we found that the combination of the two factors *Gata4* and *Foxa3* activated by CRISPRa can also induce MEF reprogramming into induced epithelial-like cells, namely CRGF-iHeps, while iHeps were not observed in the sg*GH*, sg*FH* or single factor activation groups [Figure 3A and Supplementary Figure 1A, B, http://links.lww.com/CM9/B964]. The growth curve of epithelial-like cells from CRISPR-activated *Gata4* and *Foxa3* was similar to that of CRGFH-iHeps [Supplementary Figure 1C, http://links.lww.com/CM9/B964]. The IF results showed that the epithelial marker E-cadherin was coexpressed with ALB in the CRGF-iHeps [Figure 3B]. RNA-Seq analysis revealed that the transcriptome profile of CRGF-iHeps were similar to that of CRGFH-iHeps, with a significant decrease in the expression of marker genes in MEFs and a significant upregulation of marker genes in hepatocytes [Figure 3C]. Through gene set enrichment analysis (GSEA), we found that the genes involved in drug metabolism and xenobiotic metabolism, which are important markers in mature hepatocytes, were expressed at higher levels in CRGF-iHeps [Figure 3D]. In addition, similar to CRGFH-iHeps, CRGF-iHeps showed glycogen storage [Figure 3E], DiI-ac-LDL uptake [Figure 3E] and drug metabolism [Figure 3F]. These results indicated that endogenous activation of *Gata4* and *Foxa3* is sufficient to induce the conversion of MEFs to iHeps.

Additionally, we used TTFs from adult mice to induce iHeps under the same conditions. We immortalized TTFs by stably expressing SV40LT. Ten days after GF was

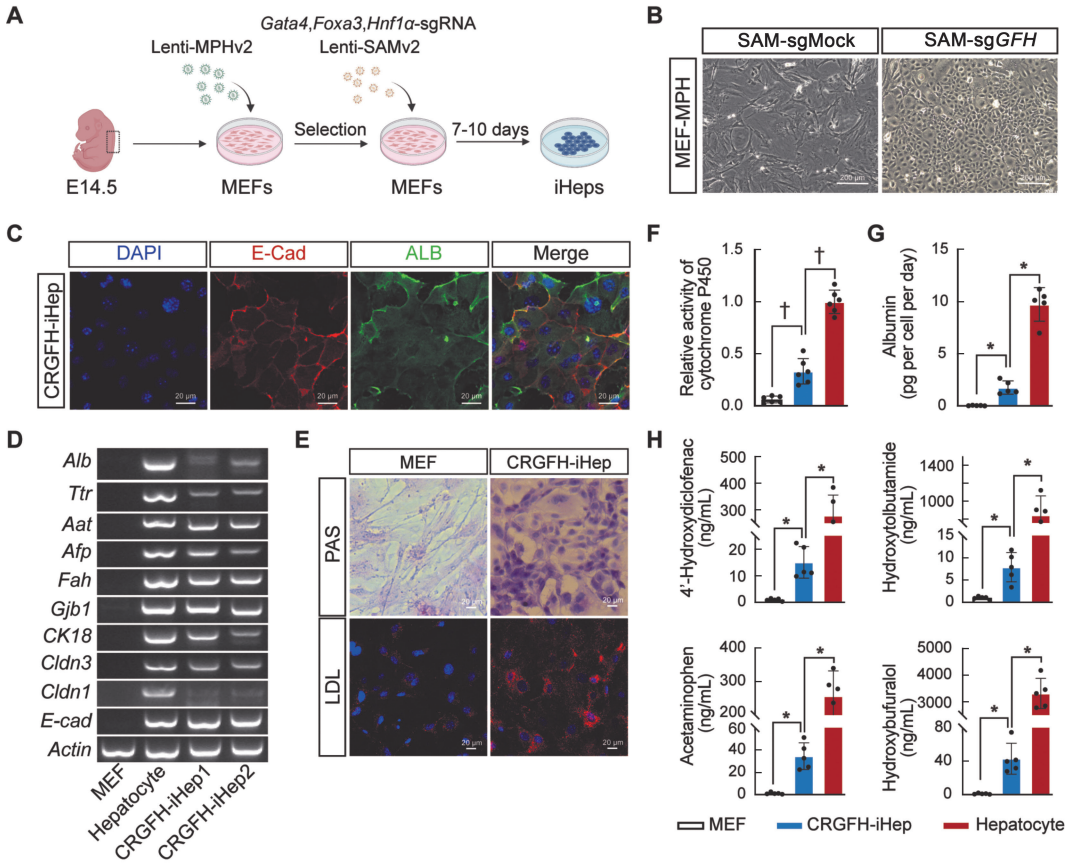

Figure 2: Generation of iHep cells from MEF cultures and assessment of hepatocyte-specific properties in iHep cells. (A) Experimental design for iHep cell induction. MEFs were transduced with a lentivirus encoding the dCas9-VP64 transactivator and subsequently transfected with gRNAs targeting *Gata4*, *Foxa3*, and *Hnf1a*. After 7–10 days of hepatogenic TF activation, the MEFs were transformed into iHeps. (B) CRGFH activation–induced iHep cells showed a typical epithelial morphology. Scale bars, 200 μm. (C) The coexpression of E-Cadherin (E-Cad, red) and albumin (ALB, green) in the CRGFH-iHeps was observed by immunostaining. (D) Expression of the indicated genes was measured by end point PCR in induced hepatocyte-like cells (CRGFH-iHeps), primary hepatocytes and MEFs. (E) Cytoplasmic accumulation of glycogen was determined by PAS staining (purple cytoplasmic staining). Intake of DiI-ac-LDL in CRGFH-iHep cells (red staining). Scale bars, 20 μm. (F,G) CYP activity (F) and the amount of albumin in the culture media (G) and were measured after culture of cells, $n = 6$ in (F) and $n = 5$ in (G) biological replicates]. Pairwise Wilcoxon rank-sum tests. (H) The metabolites of diclofenac, tolbutamide, phenacetin and bufuralol in the culture media, including 4′-hydroxydiclofenac, hydroxytolbutamide, acetaminophen, hydroxybufuralol, were quantified after cell culture. Pairwise Wilcoxon rank-sum tests, $^{*}P < 0.05$, $^{\dagger}P < 0.01$ and $^{\ddagger}P < 0.001$. CRGFH: *Gata4*, *Foxa3* and *Hnf1a* activated by CRISPRa; CRGFH-iHeps: CRISPRa-activated *Gata4*, *Foxa3* and *Hnf1a* induced hepatocyte-like cells; CRISPRa: Clustered regularly interspaced short palindromic repeats activator; CYP: Cytochrome P450; DAPI: 4′,6-diamidino-2-phenylindole; DiI-ac-LDL: DiI-labeled acetylated low-density lipoprotein; E14.5: Mouse embryonic day 14.5; iHeps: Induced hepatocyte-like cells; LDL: Low-density lipoprotein; Lenti-MPHv2: Lenti vector encoding the MS2-P65-HSF1 activator helper complex with a 2A Hygro resistance marker (EF1a-MS2-p65-HSF1-2A-Hygro-WPRE); Lenti-SAMv2: A lenti sgRNA cloning backbone with MS2 loops at tetraloop/stemloop 2, dCas9-VP64, and puro resistance marker; MEFs: Mouse embryonic fibroblasts; MPH: MS2-P65-HSF1, transcriptional activation complexes; PAS: Periodic acid-Schiff; PCR: Polymerase chain reaction; SAM: Synergistic activation mediator; SAM-sg*GFH*: SAM-sg*Gata4*, SAM-sg*Foxa3* and SAM-sg*Hnf1a*; sgRNA: Single-guide RNA; TF: Transcription factor.

activated by CRISPRa, epithelial clones, morphologically similar to MEF-derived iHeps were observed in the TTF culture [Supplementary Figure 2, http://links.lww.com/CM9/B964]. In summary, we reprogrammed MEFs and TTFs into induced hepatocytes by activating *Gata4* and *Foxa3* through CRISPRa.

In vivo hepatic reprogramming of fibroblasts with AAV-mediated CRISPRa

To investigate whether CRISPRa could reprogram fibroblasts into functional hepatocytes *in vivo* to reduce liver fibrosis, we used AAV6-mediated CRISPRa to activate *Gata4* and *Foxa3* in liver fibroblasts. Cas9 mice[20,21] can express Cas9-EGFP in specific cells after activation in the presence of Cre recombinase, and MS2-dgRNA-MPH can be specifically transfected with the AAV virus to activate target genes *in vivo* [Figure 4A]. We used the AAV6 capsid to generate an AAV vector coexpressing MS2-dg*Gata4*-dg*Foxa3* from the U6 promoter and the MPH element from the CMV promoter. We crossed Cas9 mice with *Col1a1-CreER* mice to trace the fibroblast fate and express Cas9 in fibroblasts by Cre recombinase expressed from the collagen alpha-1 (*Col1a1*) promoter after induction by tamoxifen. We treated *Col1a1-CreER*; Cas9-EGFP mice with 16 doses of CCl_4, injected the mice with 4×10^{11} viral genomes of AAV6-MS2-dg*Gata4*-dg*Foxa3*-MPH through the tail vein, and analyzed their livers 4 weeks later [Figure 4A].

After 16 doses of CCl_4, through Sirius red staining and alpha-smooth muscle actin (α-SMA) IF staining, the mice were found to have severe fibrosis, and α-SMA was coexpressed with EGFP [Figure 4B]. Four weeks after AAV6-MS2-dg*Gata4*-dg*Foxa3*-MPH injection, we found that the GFP-positive cells no longer expressed α-SMA [Figure 4C]. These GFP-positive cells were oval in shape, similar to hepatocytes *in vivo* [Figure 4C]. We found that

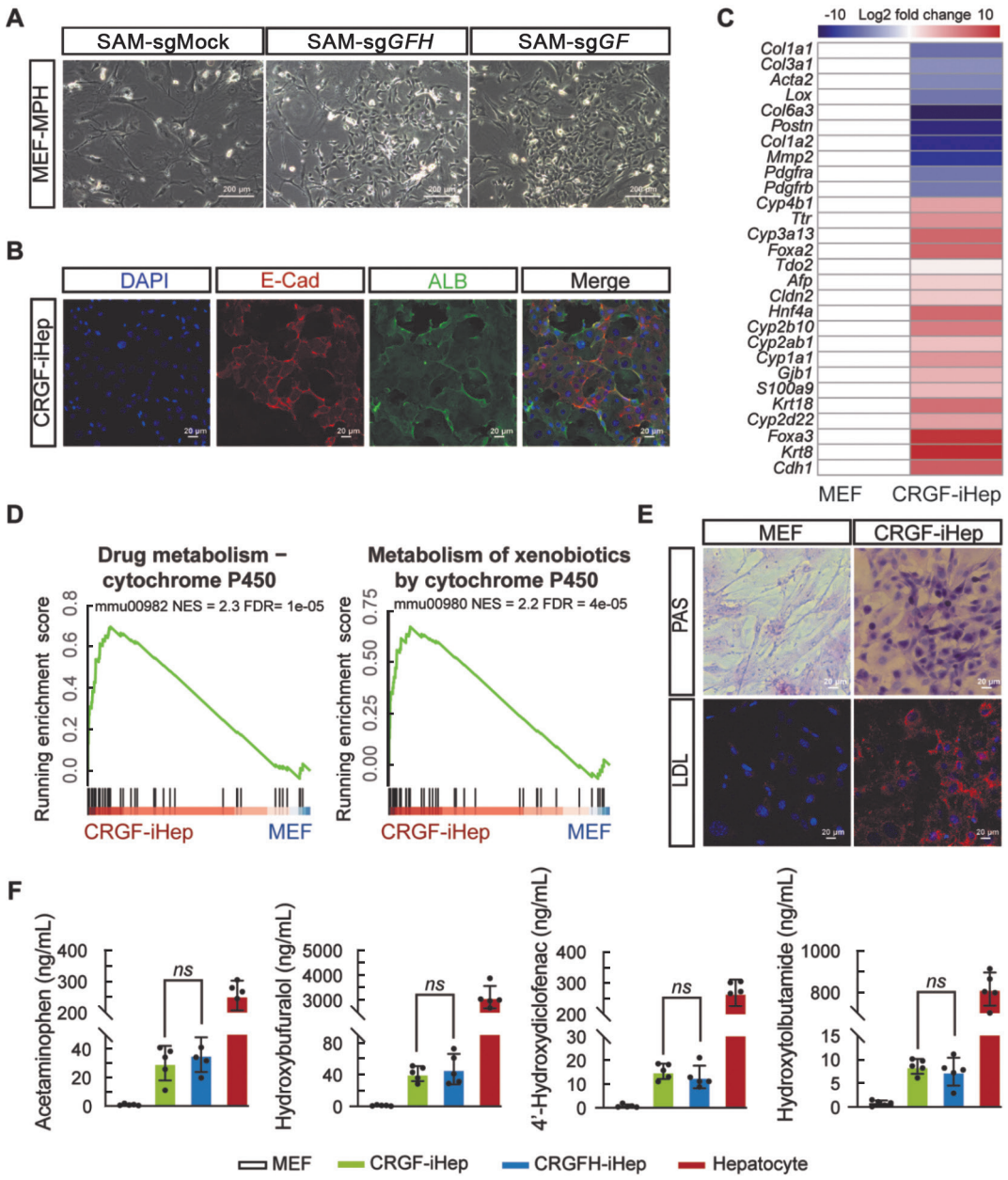

Figure 3: Endogenous CRGF induce hepatic conversion of MEFs. (A) Activation of endogenous GF or GH by CRISPRa induced fibroblast reprogramming into epithelial-like cells. Scale bars, 200 μm. (B) The coexpression of E-Cadherin and ALB in the CRGF-iHeps were detected by immunostaining. (C) Heatmap of the log2 fold changes in the expression of MEF and hepatocyte marker genes. RNA-Seq analysis of MEFs, CRGF-iHeps, CRGFH-iHeps, and primary hepatocytes ($n = 3$). Compared to that in MEFs, the expression of MEF marker genes was significantly downregulated in CRGF-iHeps and CRGFH-iHeps, while the expression of hepatocyte marker genes was significantly upregulated. (D) GSEA comparing CRGF-iHep samples *vs.* MEF samples. The pathways involved in "drug metabolism—CYP" and "metabolism of xenobiotics by CYP" were significantly enriched in the CRGF-iHeps. (E) Cytoplasmic accumulation of glycogen was determined by PAS staining (purple cytoplasmic staining). Intake of DiI-ac-LDL in CRGF-iHep cells (red staining). Scale bars, 20 μm. (F) The metabolites of phenacetin, bufuralol, diclofenac and tolbutamide in the culture media were quantified after cell culture ($n = 5$). Pairwise Wilcoxon rank-sum tests. ALB: Albumin; CRGF: *Gata4* and *Foxa3* activated by CRISPRa; CRGFH-iHeps: CRISPRa-activated *Gata4*, *Foxa3* and *Hnf1a* induced hepatocyte-like cells; CRGF-iHep: CRISPRa-activated *Gata4* and *Foxa3*-induced hepatocyte-like cells; CRISPRa: Clustered regularly interspaced short palindromic repeats activator; CYP: Cytochrome P450; DAPI: 4′,6-diamidino-2-phenylindole; DiI-ac-LDL: DiI-labeled acetylated low-density lipoprotein; FDR: False discovery rate; *GF*: *Gata4* and *Foxa3*; *GH*: *Gata4* and *Hnf1a*; GSEA: Gene Set Enrichment Analysis; HSF1: Heat shock factor 1; iHeps: Induced hepatocyte-like cells; LDL: Low-density lipoprotein; MEF: Mouse embryonic fibroblast; MPH: MS2-P65-HSF1, transcriptional activation complexes; NES: Normalized enrichment score; *ns*: Not significant; PAS: Periodic acid-Schiff; SAM: Synergistic activation mediator; SAM-sg*GF*: SAM-sg*Gata4*-sg*Foxa3*; SAM-sg*GFH*: SAM-sg*Gata4*-sg*Foxa3*-sg*Hnf1a*; sg*Foxa3*: Single guide RNA of *Foxa3*; sg*Gata4*: Single guide RNA of *Gata4*; sg*Hnf1a*: Single guide RNA of *Hnf1a*.

the cells expressed the fibroblast fate-tracing marker GFP, the hepatocyte marker FAH, and major urinary protein (MUP), suggesting that the iHeps were derived from fibroblasts (F-iHeps) [Figure 4D, E]. Next, we assessed the efficiency of hepatic reprogramming of fibroblasts via AAV6-mediated CRISPRa activation. We observed that F-iHep accounted for 0.42% of all hepatocytes in the liver, and most F-iHeps in these mice were single cells [Figure 4F]. We observed reduced liver fibrosis in AAV6-MS2-dg*Gata4*-dg*Foxa3*-MPH recipients, as shown by Sirius red staining, α-SMA IF, and hydroxyproline measurement [Figure 4G,H]. In addition, the levels of the hepatocyte injury marker serum alanine aminotransferase (ALT) were reduced in these mice [Figure 4H].

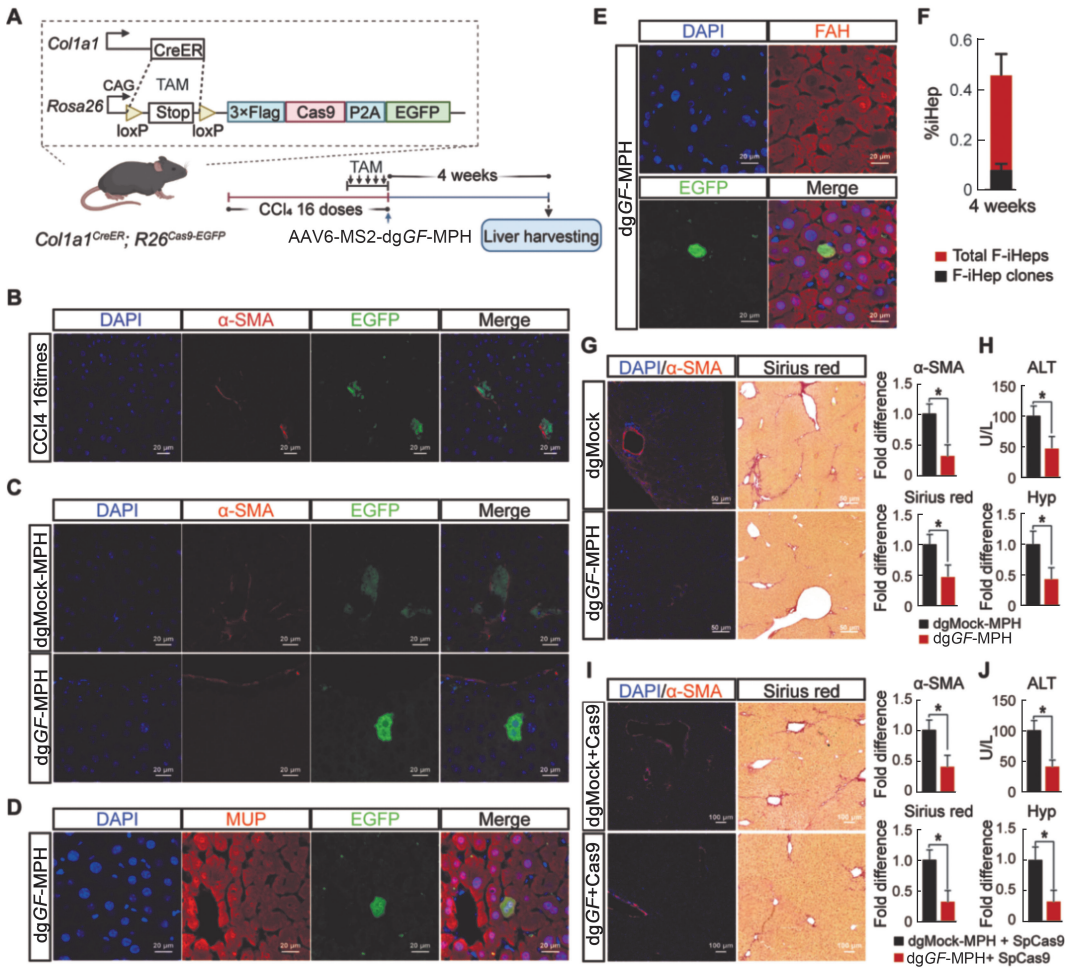

Figure 4: *In vivo* hepatic reprogramming of fibroblasts with AAV mediated CRISPRa-mediated activation of *Gata4* and *Foxa3*. (A) Schematic diagram of CRISPRa activation-mediated induction of hepatocyte reprogramming *in vivo*. (B) IF staining demonstrated that α-SMA and EGFP were co-expressed in fibroblasts after 16 CCl_4 injections. Scale bars, 20 μm. (C) After four weeks of injection of AAV6-MS2-dg*GF*-MPH into mice with liver fibrosis, cells that did not coexpress α-SMA and EGFP are formed, and the cell morphology was similar to that of liver cells. Scale bars, 20 μm. (D,E) EGFP positive cells were positive for both MUP (D) and FAH (E). Scale bars, 20 μm. (F) Hepatic reprogramming efficiency was assessed by quantifying F-iHep clones and total F-iHeps, respectively ($n = 5$). (G) Quantification of Sirius red staining and α-SMA IF staining ($n = 3$). Scale bars, 50 μm. (H) The serum levels of ALT and analysis of whole liver collagen content by Hyp assays ($n = 3$). (I) Quantification of Sirius red staining and α-SMA IF staining of coinjecting AAV6-MS2-dg*GF*-MPH with AAV6-CMVc-SpCas9 mice. Scale bars, 50 μm. (J) The Hyp and ALT levels in mice coinjected AAV6-MS2-dgGF-MPH with AAV6-CMVc-SpCas9 ($n = 3$). Student's *t* test, $^*P < 0.05$. α-SMA: Alpha-smooth muscle actin; AAV6: Adeno-associated virus serotype 6; ALT: Alanine aminotransferase; CRISPRa: Clustered regularly interspaced short palindromic repeats (CRISPR) activator; DAPI: 4′,6-diamidino-2-phenylindole; dg*GF*: Dead guide RNA of *Gata4* and *Foxa3*; EGFP: Enhanced green fluorescent protein; FAH: Fumarylacetoacetate hydrolase, hepatocyte markers; F-iHeps: Induced hepatocytes derived from fibroblasts; HSF1: Heat shock factor 1; Hyp: Hydroxyproline; IF: Immunofluorescence; iHep: Induced hepatocyte-like cells; LoxP: Locus of X-over P1; MPH: MS2-P65-HSF1, transcriptional activation complexes; MUP: Major urinary protein; P2A: Porcine teschovirus-1 2A; TAM: Tamoxifen.

To demonstrate the potential therapeutic utility of CRISPRa in liver fibrosis, we co-coinjected AAV6-MS2-dg*Gata4*-dg*Foxa3*-MPH with AAV6-CMVc-SpCas9 into mouse liver fibrosis models. Four weeks after the injection, we observed a significant reduction in liver fibrosis compared to that in the control littermates that were injected with AAV6-MS2-dgMock-MPH, as well as a reduction in the serum levels of the hepatocyte injury marker ALT [Figure 4I, J]. These results demonstrated that the CRISPRa system can activate hepatocyte TFs *in vivo* to induce hepatocyte reprogramming and reduce liver fibrosis.

Discussion

CRISPRa approaches show potential for controlling cellular reprogramming. In this study, we directly induced fibroblasts to differentiate into functional hepatocytes by CRISPRa-mediated activation of two key TFs, *Gata4* and *Foxa3*. CRGF-iHeps exhibited mature hepatic functions. Notably, we reduced the liver fibrosis through CRISPRa reprogramming *in vivo*. This study provides a strategy for generating functional hepatocyte-like cells for the purpose of liver engineering and regenerative medicine.

Direct cell lineage transdifferentiation *in vivo* is promising for disease treatment, especially for cells with poor transplantation efficacy, such as cardiac muscle cells and neurons.[19,22–26] Due to the limited carrying capacity of AAVs, traditional *in vivo* reprogramming requires the synthesis of multiple AAV viruses. Rezvani *et al*[19] developed an *in vivo* hepatocyte reprogramming system in which *Gata4*, *Hnf1a*, *Foxa1*, *Foxa2*, *Foxa3*, and *Hnf4a* were introduced into mice via multiple AAV6 vectors. After 8 weeks of induction, 0.87% of the total hepatocytes were induced from fibroblasts, which effectively reduced hepatic fibrosis. CRISPRa can activate the expression of

multiple endogenous genes expression simultaneously with a fixed cargo size, regardless of the complexity of the gene. This process preserves the native transcription pattern of the gene itself and gene products via various mechanism, such as alterative splicing. In this way, we activated the two factors *Gata4* and *Foxa3 in vivo* with CRISPRa to transform fibroblasts into functional hepatocytes, effectively reducing liver fibrosis in mice. In addition, transdifferentiation from fibroblasts toward hepatocytes can be well achieved both *in vivo* and *in vitro*, although the efficiency of reprogramming was partially compromised compared with that of the strategy of Rezvani *et al*.[19] Moreover, the injection of AAV6-SpCas9 and AAV6-MS2-dg*Gata4*-dg*Foxa3*-MPH into mice with liver fibrosis through the tail vein *in vivo* also effectively reduced liver fibrosis. These findings demonstrated the therapeutic potential of CRISPRa in liver fibrosis.

In summary, we used CRISPRa to activate endogenous *Gata4* and *Foxa3* to induce the transformation of fibroblasts into functional hepatocytes. We injected the CRISPRa system into liver fibrosis model mice, which effectively reduced liver fibrosis. Comprehensive analysis revealed that hepatocytes generated by the CRISPRa strategy have broad application potential for drug discovery and liver fibrosis treatment.

Funding

This work was supported by the grants from the National Key Research and Development Program of China (No. 2019YFA0801500), National High Level Hospital Clinical Research Funding (No. 2023-GSP-ZD-2-01), and the Chinese Academy of Medical Sciences Innovation Fund for Medical Sciences (No. 2021-I2M-1-008).

Conflicts of interest

None.

Data availability

All the data included in this study are available upon request by contacting with the corresponding author.

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How to cite this article: Li JC, Li RP, Bai X, Zhang WL, Nie Y, Hu SS. Direct reprogramming of fibroblasts into functional hepatocytes via CRISPRa activation of endogenous *Gata4* and *Foxa3*. Chin Med J 2024;137:1351–1359. doi: 10.1097/CM9.0000000000003088