Epigenetic Targeting of Granulin in Hepatoma Cells by Synthetic CRISPR dCas9 Epi-suppressors

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The CRISPR-associated Cas9 system can modulate disease-causing alleles both in vivo and ex vivo, raising the possibility of therapeutic genome editing. In addition to gene targeting, epigenetic modulation by the catalytically inactive dCas9 may also be a potential form of cancer therapy. Granulin (GRN), a potent pluripotent mitogen and growth factor that promotes cancer progression by maintaining self-renewal of hepatic stem cancer cells, is upregulated in hepatoma tissues and is associated with decreased tumor survival in patients with hepatoma. We synthesized a group of dCas9 epi-suppressors to target GRN by tethering the C terminus of dCas9 with three epigenetic suppressor genes: DNMT3a (DNA methyltransferase), EZH2 (histone 3 lysine 27 methyltransferase), and KRAB (the Krüppel-associated box transcriptional repression domain). In conjunction with guide RNAs (gRNAs), the dCas9 epi-suppressors caused significant decreases in GRN mRNA abundance in Hep3B hepatoma cells. These dCas9 epi-suppressors initiated de novo CpG DNA methylation in the GRN promoter, and they produced histone codes that favor gene suppression, including decreased H3K4 methylation, increased H3K9 methylation, and enhanced HP1a binding. Epigenetic knockdown of GRN led to the inhibition of cell proliferation, decreased tumor sphere formation, and reduced cell invasion. These changes were achieved at least partially through the MMP/TIMP pathway. This study thus demonstrates the potential utility of using dCas9 epi-suppressors in the development of epigenetic targeting against tumors.

RESULTS
Development of CRISPR dCas9 as an Epigenetic Targeting Therapy
To target GRN epigenetically in hepatoma, we modified the CRISPR/Cas9 system by tethering it with three epi-suppressors: DNMT3a...
(de novo DNA methyltransferase), EZH2 (histone 3 K27 methyltransferase), and KRAB (heterochromatin binding suppressor) (Figure S1; Tables S1–S3, construct and gRNA sequences). To avoid genomic DNA breaks, a catalytically deactivated Cas9 variant (dCas9) was used to guide epigenetic targeting. This dCas9 variant is defective in DNA cleavage but maintains the ability to bind to the gRNA-guided gene target.18,19 The binding specificity is determined by both gRNA-DNA base pairing and by a short DNA motif (protospacer adjacent motif [PAM] sequence: NGG) juxtaposed to the DNA complementary region.20–24 In our epigenetic targeting system, the dCas9 protein bound to the target gene promoter, while the epi-suppressors silenced the activity of the target gene (Figure 1A).

We first conducted a proof-of-concept study for this approach in a cytomegalovirus (CMV) promoter-luciferase reporter system, where the CMV promoter was used to drive the luciferase reporter gene (Figure 1B). Presumably, the dCas9-epigenetic suppressors would introduce epigenetic inhibition in the CMV promoter. When the CMV promoter was epigenetically silenced, luciferase would be inhibited. We designed five gRNAs from various locations in the CMV promoter sequence (Figure S2; Table S1). The reporter vector, dCas9-suppressor vectors, and gRNA vectors were co-transfected into 293T cells. By measuring luciferase activity, we found that the potency of the dCas9 epi-suppressors was closely related to the location of the gRNA-binding sites in the promoter (Figure 1C). For example, gRNAs 1 and 2, which were located relatively far away from the transcription initiation site, did not produce significant suppression of the luciferase activity. In contrast, gRNAs 4 and 5, which were proximal to the initiation site, exhibited the maximum inhibition of the reporter gene. This pattern was observed for all three epi-suppressors (dCas9-DNMT3a, dCas9-EZH2, and dCas9-KRAB). Among the three epigenetic suppressors tested, the heterochromatin-binding suppressor dCas9-KRAB showed the best inhibition, particularly when a mixture of gRNAs (1–5) was used.

CRISPR interference has been reported to prevent transcription by steric blockage of the RNA polymerase complex.18,25 We also observed significant inhibition of the CMV promoter activity by dCas9-gRNAs without epi-suppressors. However, greater inhibitory activity was observed in the dCas9-epi-suppressor construct groups (dCas9-DNMT3a, dCas9-EZH2, and dCas9-KRAB) than in the dCas9 group that lacked the epi-suppressors (Figure 1D). The controls, including mock-transfected cells without dCas9 (vector) or untargeted dCas9 (dCas9-gCT), had little effect on the activity of the reporter gene.

We further validated the role of the dCas9 epi-suppressors in a second reporter system, where the CMV promoter drove the copGFP gene (Figure 1E). When the mixture of gRNAs (1–5) was used for targeting, we found that the potency of epi-suppressors was KRAB > EZH2 > DNMT3a (Figure 1F). These data suggest that the heterochromatin-binding factor dCas9-KRAB yielded the best inhibition of the target gene.

Epigenetic Targeting of Oncogenic GRN in Hepatoma

GRN is a putative biomarker of hepatic cancer stem cells. To define its role in hepatoma, we first examined its expression in normal control and hepatoma patients using the dataset from the The Cancer Genome Atlas (TCGA) database of the National Cancer Institute. GRN was overexpressed in hepatoma as compared with normal liver (Figure 2A). When the hepatoma samples were classified based on the gene copy number variations, GRN was significantly upregulated in the group with GRN gain and amplification (Figure 2B). The hepatoma patients were then divided into high and low groups based on GRN expression (Figure 2C). The patients with high GRN expression had significantly lower survival than those with low GRN expression (Figure 2D).

We then used the same dCas9-epi-suppressor methodology to target the endogenous GRN gene in hepatoma cells. The human GRN promoter has been well characterized (Figure S3A),26 and its activity is associated with epigenetic modifications.27–29 We designed four gRNAs complementary to the GRN promoter (Figure 2E; Figures S3B and S3C). The dCas9 epi-suppressors and gRNAs were co-transfected into 293T cells. Using qPCR and western blotting, we found that endogenous GRN was significantly decreased by this epigenetic system, with a potency order of KRAB > EZH2 > DNMT3a (Figures 2F and 2G), similar to that seen in the pCMV–luciferase/copGFP reporter systems. Therefore, we focused on the dCas9-KRAB epigenetic system in this study.

We then tested this system in the Hep3B hepatoma cell line, with the focus on the dCas9-KRAB construct. Using qPCR, we demonstrated that this dCas9-KRAB epigenetic approach significantly inhibited the expression of GRN in Hep3B tumor cells as compared with the random gRNA (gCT) and dCas9 controls (Figure 2H). Western blotting also confirmed the epigenetic knockdown of GRN oncoprotein in Hep3B cells (Figure 2I). Taken together, these data demonstrate that it is feasible to harness this epigenetic approach to knock down GRN in hepatoma cells.

Epigenetic Silencing of GRN Is Associated with De Novo DNA Methylation

To delineate the mechanisms that silence GRN epigenetically, we first examined DNA methylation in the promoter of GRN (Figure 3A). In both dCas9 control (no epi-suppressor) and random gRNA (gCT) control groups, the GRN promoter was minimally methylated, in parallel with the abundant expression of GRN in Hep3B cells. However, epigenetic targeting by dCas9-KRAB induced de novo DNA methylation in the GRN promoter (Figures 3B and 3C), resulting in the silencing of GRN in tumor cells. Similarly, this induced DNA methylation was also observed with dCas9-DNMT3a and dCas9-KRAB treatments in 293T cells (Figure 3D). The TCGA data also showed a negative correlation between the status of DNA methylation in the GRN promoter (cg24420717) and GRN mRNA abundance in patients with hepatoma (Figure S4; Spearman correlation coefficient −0.29, p = 1.30E–08).
We found that the treatment with dCas9-KRAB-gRNAs significantly reduced H3K4 methylation compared with three control groups (Figure 4A).

Methylation of H3K9 and H3K27 are suppressive markers on gene promoters. Transfection of cells with the dCas9-KRAB construct enhanced the H3K9 suppression signals in the GRN promoter (Figure 4B). However, no significant difference was noticed in H3K27 methylation as compared with the controls (Figure 4C).

Heterochromatin protein 1 (HP1α) functions as an epigenetic gatekeeper to inhibit gene activity by binding to H3K9 methylation marks. We found that dCas9-KRAB also induced a significant increment in HP1α binding to the GRN promoter, in parallel with increased H3K9 methylation and gene silencing (Figure 4D).

We also compared histone modifications induced by each dCas9 epi-suppressor treatment (Figure 4E). As compared with the dCas9-gRNA control, dCas9-EZH2 altered H3K4, H3K9, and H3K27. However, dCas9-DNMT3a did not significantly affect
Epigenetic Targeting of GRN Reduces the Growth of Hep3B Hepatoma Cells

GRN is a cancer stem cell marker and plays an important role in regulating self-renewal of cancer stem cells. We examined if epigenetic silencing of GRN would affect the growth of tumor cells. We first examined cell proliferation using Hep3B cells. After epigenetic knockdown of GRN, there was a decrease in cell growth in the GRN-knocked down tumor cells as compared with the random gRNA control (gCT) and dCas9 control groups (Figure 5A).

We also examined if epigenetic knockdown of GRN altered the formation of tumor spheres (Figure 5B). After transfection with the dCas9-KRAB, Hep3B tumor cells were cultured in stem cell culture medium for 1 week. As expected, both the dCas9 control and random gRNA control cells formed clone spheres with compact structure. However, after knockdown of the cancer stem cell marker GRN with dCas9-KRAB, Hep3B cells exhibited a significant reduction in tumor spheres under the same culturing conditions.

We then tested the activity of the dCas9-KRAB treatment in altering cell invasion. Transwell was coated with Matrigel, a solubilized basement membrane preparation derived from the Engelbreth-Holm-Swarm mouse sarcoma. After epigenetic treatment, Hep3B cells were seeded on a Matrigel-coated transwell chamber,
and the number of cells that passed through the Matrigel to the bottom of the transwell was quantified as a measure of cell invasion. We found that, after treatment with dCas9 control and gCT control, Hep3B cells still exhibited the malignant phenotype of invading across the Matrigel membrane. However, epigenetic knockdown of GRN by dCas9-KRAB resulted in significantly lower invasion activity (Figure 5C; p < 0.01).

**GRN Epigenetic Targeting Inhibits Hepatoma Cells through the TIMP/MMP Pathway**

The TIMP/MMP pathway plays a critical role in cell migration and tumor metastasis. We investigated whether epigenetic knockdown of GRN affected the invasion of hepatoma cells through this pathway. As compared with the gCT non-targeting control, knockdown of GRN with dCas9-gRNAs significantly downregulated all the MMP family genes tested, including MMP-7, MMP-9, MMP-11, and MMP14 (Figure 6A; p < 0.01). Among the TIMP family genes examined, both TIMP1 and TIMP2 were significantly upregulated after epigenetic knockdown of GRN. TIMP3, however, was slightly downregulated. TIMP4 was not affected by this epigenetic targeting (Figure 6B). The TCGA data also showed the co-expression pattern of GRN with the TIMP/MMP family genes (Figure S5).

**DISCUSSION**

The CRISPR-associated nuclease Cas9 provides an effective means of introducing targeted loss of function at specific sites in the genome, raising the hope for therapeutic genome editing. By combining with co-expressed gRNAs, the nuclear-tagged Cas9 leads to the assembly of a specific endonuclease complex that can target any chromosomal DNA sequence for cleavage at a site immediately 5’ to an NGG PAM site. In this study, we show that, when linked with epigenetic suppressors DNMT3a, EZH2, and KRAB, the catalytically inactive dCas9 is able to introduce suppressive epigenetic marks into the promoter of a target gene.

The hepatic cancer stem cell marker GRN is located at 17q21.32, a region frequently involved in chromosome gain in liver cancers. GRN contributes to human cancers by potentiating neoplastic transformation, tumor growth, metastases, tissue invasion, and therapeutic resistance. The GRN promoter has been characterized in several cell lines, including A549 (lung carcinoma), CaSkii (cervical carcinoma), NIH 3T3 (mouse fibroblast), and COS-7 cells (monkey kidney fibroblast). It lacks a conventional TATA box, but it contains several potential CCAAT boxes and GC box elements. Mutations in the GRN gene result in autosomal dominant frontotemporal lobar degeneration (FTLD) associated with personality changes and progressive dementia. In addition, the promoter activity is associated with epigenetic mutations, including increased DNA methylation in patients with sporadic FTLD, and altered histone acetylation.

In this study, we utilized synthetic dCas9 epi-suppressors to epigenetically suppress GRN as a potential intervention for hepatoma. To precisely target the GRN promoter, we synthesized three dCas9 epigenetic constructs by tethering the C terminus of dCas9 to three suppressor domains derived from DNMT3a, EZH2, and KRAB. The synthetic suppressor factors contain a dCas9 DNA-binding domain that specifically binds to its GRN gene target under the guidance of gRNAs. The regulatory domain suppresses the promoter activity of GRN using an epigenetic mechanism. We demonstrate that all three
The suppressor domain of the synthetic dCas9 constructs can inhibit the target gene by multiple mechanisms, including decreased H3K4 methylation and enhanced H3K9 methylation, DNA methylation, and alteration of local chromatin structure. DNA methylation-dependent repression is well established, especially for a CpG island-rich promoter. DNMT3a is a de novo DNA methyltransferase that methylates the C5 position of all cytosine nucleotides having the dinucleotide sequence 5’-CpG-3’. Once tethered to a target site, the enzyme methylates CpG islands in DNA sequences near the region where it binds. In this study, we demonstrate that the dCas9-DNMT3a is able to silence the CMV promoter in both luciferase and copGFP reporter systems.

Histone H3K9 or H3K27 methylation is normally associated with chromatin compaction and transcriptional silencing. The H3K27 methyltransferase EZH2 relies on polycomb repression complex 2 (PRC2) partners to achieve optimal activity. In this study, we showed that, when tethered to the C terminus of dCas9, the EZH2 catalytic domain effectively inhibits the target promoters in 293T cells.

It is interesting to note that dCas9 epi-suppressors, like dCas9-KRAB, are able to use overlapping epigenetic pathways to suppress their target promoters. It is known that KRAB, the Krüppel-associated box, is present in the transcriptional repression domains of hundreds of human zinc-finger transcription factors. KRAB functions as a transcriptional repressor to scaffold a silencing complex consisting of histone methyltransferase, the nucleosome remodeling and deacetylation (NuRD) complex, heterochromatin protein 1 (HP1), and DNA methyltransferases. It can be directed to assemble multiprotein repression complexes on its target promoter. In this study, we demonstrate that, when tethering to the GRN promoter, dCas9-KRAB was the most potent inhibitor in our system. It inhibits the expression of GRN in Hep3B tumor cells not only by introducing de novo DNA methylation but also by altering histone marks in the promoter, including decreased H3K4 methylation and enhanced H3K9 methylation. In addition, it recruits the heterochromatin protein HP1a. HP1 proteins are gatekeepers of epigenetic gene silencing. Working together, the synthetic dCas9-KRAB induces a substantial knockdown of the oncogenic GRN in hepatoma cells.

In summary, our data demonstrate that three epigenetic suppressors (DNMT3a, EZH2, and KRAB), when tethered to the gene promoter through the gRNA-guided dCas9, significantly inhibit the expression of target genes using distinct epigenetic mechanisms. dCas9-KRAB inhibits the target gene by multiple mechanisms, including de novo DNA methylation, H3K9 hypermethylation, and the recruitment of HP1a. Epigenetic silencing of GRN reduces invasion and tumor sphere formation in Hep3B tumor cells via the MMP/TIMP pathway. Our data demonstrate that this dCas9 epi-suppressor system may serve as a powerful epigenetic approach to inhibit therapeutic target genes in cancer cells.

**MATERIALS AND METHODS**

**Cell Lines and Cell Culture**

Hep3B (ATCC HB-8064) was purchased from ATCC and cultured in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS).
Figure 5. GRN Knockdown Suppresses Cell Proliferation, Invasion, and Tumor Sphere Formation

(A) Cell proliferation in Hep3B tumor cells after epigenetic silencing of GRN. (B) Tumor sphere formation in Hep3B cells following GRN suppression. (C) Reduced invasion of Hep3B cells by epigenetic inhibition of GRN. **p < 0.001 as compared with dCas9 control vector. All data shown are mean ± SD.

and 100 U/mL penicillin-streptomycin. 293T cells were purchased from ATCC and cultured in DMEM (Invitrogen, CA) supplemented with 10% FBS (Invitrogen, CA), 1× Non-Essential Amino Acid (NEAA, Invitrogen, CA), and 100 U/mL penicillin-streptomycin (Invitrogen, CA). Cells were incubated at 37°C in 5% CO2 air atmosphere.

Construction of the dCas9 Epi-suppressors

The CRISPR dCas9 plasmid was purchased from Addgene as a gift from Prashant Mali. The humanized KRAB domain was synthesized from human cDNAs by overlapping PCR and verified by DNA sequence. The full-length cDNA of DNMT3a (MHS6278-20275692) and EZH2 (MHS6278-202756846) were purchased from GE (Dharmacon, CO), and they were used for PCR cloning. The catalytically inactive dCas9 was created by D10A and H840A point mutation with Q5 Site-Directed Mutagenesis Kit (New England Biolabs, MA) and cloned into pCDH1 plasmid (SBI, CA). Three epigenetic suppressors, KRAB, EZH2, and DNMT3 (DNMT3a catalytic domain C-terminal), were amplified by PCR, and they were ligated into the C terminus of the dCas9 sequence in the pCDH1 vector, respectively, to obtain dCas9-KRAB, dCas9-EZH2, and dCas9-DNMT3a vector constructs. gRNAs that target the GRN promoter were cloned into pGreenPuro (SBI, CA).

Plasmid Transfection

Plasmids were transfected into target cell lines (293T and Hep3B) using Lipofectamine 2000 (Invitrogen, CA), following the protocol provided by the manufacturer. Briefly, cells were seeded in a 6-well plate at 4 × 10^5/well. After overnight culture, 4 μg constructed plasmids and 10 μL Lipofectamine 2000 were mixed in 250 μL Opti-Media (Opti-MEM I Reduced Serum Medium) (Gibco, CA). After incubation for 5 min at room temperature, the DNA-Lipofectamine 2000 mixture was added to target cells, and the culture medium was replaced with fresh medium 6 hr following transfection.

For the lentivirus package, the dCas9 epi-suppressor plasmids were co-transfected with pSPAX2 and pMD2.G packaging vectors in 293T cells using Lipofectamine 2000. The supernatants containing the lentiviruses were collected at 48 and 72 hr after transfection for cell study. The lentiviruses were collected at 48 and 72 hr after transfection for cell study.

Gene Activity by Luciferase Assay

Fresh 293T cells were seeded into 48-well plates at a density of 1 × 10^5 cells per well. The dCas9 epi-suppressor vectors, luciferase reporter vector, and pRL-TK control vector were co-transfected into cells using Lipofectamine 2000 (Invitrogen, CA). Cell lysates were harvested 48 hr after transfection, and dual-luciferase reporter assays were performed using a Turner Biosystems Single Tube Luminometer (Promega, WI).

Quantitation of GFP Fluorescence by Luminometer

293T cells were seeded in 12-well plates at a density of 3 × 10^5 cells per well. The dCas9 epi-suppressor vectors and GFP reporter vector were co-transfected into 293T cells. At 48 hr after transfection, the GFP fluorescence was measured using a Turner Biosystems Single Tube Luminometer (Promega, WI).

Promoter Histone Suppression Code by ChIP

ChIP assays for histone methylation and HP1a recruitment were performed using an EZ-Magna ChIP G Kit (Millipore, CA). Briefly, monoclonal Hep3B cells with stable expression of copGFP in 10-cm dishes were transiently transfected with 15 μg suppressor vectors. At 48 hr after transfection, cells were cross-linked with...
DNA Methylation by Bisulfate Sequencing

Transiently transfected 293T and stably transfected monoclonal Hep3B cells with dCas9-KRAB were selected by puromycin. Stable cells (1 × 10⁶) were collected and genomic DNA was extracted. Genomic DNAs were converted by bisulfite sodium using an EZ DNA MethylationGold kit (Zymo Research, CA). DNA samples were amplified using PCR primers JH1369F: 5'-GGATGTATGGATTGATGTGG-3' and JH1370R: 5'-AAGACCTTACAAACAACTAATGACACGTTGTACGACAT-3'. Individual ChIP assays were repeated three times to confirm the reproducibility of the qPCR.

Cell Invasion by Transwell Assay

In the invasion assay, cells were seeded on the upper chamber (4 × 10⁵ cells per well) of six-well Transwell plates equipped with polycarbonate filters coated with 1.0 mL 1:8 diluted Matrigel (300 μg/mL/well) (BD Biosciences, CA, 356231) in serum-free medium. DMEM with 5% FBS was added to the lower chamber as the chemoattractant. After 24 hr of incubation at 37°C, filters were cleaned on the upper side with a cotton swab, fixed with 1% formaldehyde (Sigma, MO) and harvested for immunoprecipitation. Antibodies used in ChIP assays included anti-H4K4Me3, anti-H3K9Me3, anti-H3K27Me3, and anti-HIP1a (Millipore, CA).32 An aliquot of cell lysates was saved to serve as the input DNA control. After the reversal of cross-linking at 62°C for 2 hr and 95°C for 10 min, ChIP samples were purified and subjected to real-time qPCR. Individual ChIP assays were repeated three times to confirm the reproducibility of the qPCR. Real-time qPCR was performed using 2xKapa SYBR (Applied Biosystems, CA) on an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems, CA) with GRN primers (forward, 5'-GCGGTTTTGGCAGTACATC-3'; reverse, 5'-GGGCGGAGTTGTTACGACAT-3'). Individual ChIP assays were repeated three times to confirm the reproducibility of the qPCR.

Cell Proliferation by MTT Assay

Cell survival was measured using the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium (MTT) assay.48,49 Briefly, cells (1 × 10⁵/well) were plated onto 96-well plates, and they were incubated with 20 μL 5 mg/mL MTT (Sigma, MO) per well at 37°C for 4 hr. The absorbance was measured at 490 nm using a microplate reader (BioTek Instruments). Cell viability (%) was calculated based on the following equation: cell viability (%) = (A_sample/A_control) × 100%, where A_sample and A_control represent the absorbance of the sample and control wells, respectively.

Western Blot Analysis

Western blot was used to detect the secreted GRN proteins in cell supernatants. Briefly, 293T cells were transfected with dCas9-KRAB + scramble gRNA plasmids for control group and dCas9-KRAB + GRN gRNA plasmids for treatment group. At 48 hr after transfection, cell supernatants (20 μL) were separated on MiniPROTEIN TGX gradient gel (Bio-Rad, CA) and transferred to a nitrocellulose membrane. After blocking with Odyssey Blocking buffer for 1 hr, membranes were incubated with specific primary antibodies against GRN (Abcam, ab55167) and Anti-beta Actin antibody (Abcam, ab8227) overnight at 4°C. After washing with PBS 4 times, the IRDye 680 secondary antibody (LI-COR Biosciences, Lincoln, NE) was added and the infrared fluorescence was visualized with the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE).50
100% methanol, and stained with 0.1% crystal violet. Crystal violet was washed from the cells using 4 mL 33% acetic acid. The absorbance of the washed liquid was determined at 590 nm. All samples were performed in triplicate.  

**Real-Time qPCR**

Genomic, bisulfate-converted DNA and mRNA were extracted using DNeasy Blood & Tissue Kit (QIAGEN, CA), EZ DNA MethylationGold kit (Zymo Research, CA), and RNeasy Mini Kit (QIAGEN, CA), and mRNAs were reverse-transcribed using M-MLV Reverse Transcriptase (Thermo Fisher Scientific, CA). Real-time qPCR was performed using 2xKapa mixed with SYBR (Applied Biosystems, CA) on an ABI PRISM 7900 HT Sequence Detector System (Applied Biosystems, CA) with primers. All experiments were performed in triplicate. PCR primers used for qPCR are listed in Table S1.  

**Sphere Formation Assay**

Hep3B cells were seeded in serum-free medium (SFM), containing DMEM/F12 (Gibco, CA) supplemented with 20 ng/mL epidermal growth factor (EGF) (Invitrogen, CA), 10 ng/mL basic fibroblast growth factor (bFGF) (Invitrogen, CA), 5 μg/mL insulin (Invitrogen, CA), and 10 μL/mL B27 (Invitrogen, CA), at 1 × 10^4 cells/well in Sigma. Cells were then incubated at 37°C in a humidified atmosphere containing 5% CO₂. The spheres were counted on day 10; we counted 10 fields of spheres at 40× phase view for each group, and all samples were performed in triplicate.  

**Acquisition of TCGA Data**

Clinical data of GRN expression were generated from the TCGA Research Network (https://cancergenome.nih.gov). The liver hepatocellular carcinoma (LIHC) TCGA provisional RNA sequencing (RNA-seq) cohorts with 442 samples were used for calculation. Clinical annotation of samples was obtained from http://firebrowse.org/?cohort=LIHC. The differential expression of GRN between normal liver and liver cancer was generated by the Gene expression viewer of FIREBROWSE (http://firebrowse.org/viewGene.html?gene=GRN). The GRN putative copy number alterations plot, mRNA and methylation correlation plot, survival plot, GRN co-occurrence plot, and TIMP/MMP pathway gene co-expression plot were generated by chipоторал.  

**Statistical Analysis**

All experiments were performed in triplicate and the data were expressed as mean ± SD. The comparative CT method was applied in the real-time qRT-PCR assay according to the delta-delta CT method. Data were analyzed using SPSS software (version 20.0; SPSS, IL). One-way ANOVA (Bonferroni test) was used to compare statistical differences for variables among treatment groups. The data were considered statistically significant at p < 0.05.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes five figures and three tables and can be found with this article online at https://doi.org/10.1016/j.omtn.2018.01.002.

**AUTHOR CONTRIBUTIONS**

J.-F.H., J.C., W.L., and A.R.H. conceived and designed the experiment, H.W., R.G., Z.D., L.B., and L.L. performed the experiments; H.W. and R.G. analyzed data; J.-F.H. and H.W. wrote the paper.

**CONFLICTS OF INTEREST**

No conflicts of interest were disclosed.

**ACKNOWLEDGMENTS**

This work was supported by the National Natural Science Foundation of China grants (31430021 and 81272294), the National Basic Research Program of China (973 Program) (2015CB943303), and California Institute of Regenerative Medicine (CIRM) grant (RT2-01942) to J.-F.H.; the National Natural Science Foundation of China grant (81500116) to H.W.; the National Natural Science Foundation of China grant (81601449) to R.G.; by Key Project of Chinese Ministry of Education grant (311015), National Natural Science Foundation of China grant (81672275), Nation Key Research and Development Program of China grant (2016YFC13038000), National Science Foundation of Jilin Province grant (20150101176JC), Research on Chronic Noncommunicable Diseases Prevention and Control of National Ministry of Science and Technology (2016YFC1303804), and National Health Development Planning Commission Major Disease Prevention and Control of Science and Technology Plan of Action, Cancer Prevention and Control (ZX-07-C2016004) to J.C.; the National Natural Science Foundation of China grants (81372835 and 81670143) and Jilin Science and Technique International Collaboration grant (20130413010GH) to W.L.; and the Department of Veterans Affairs Research Service.

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