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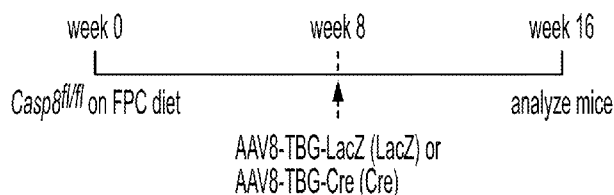


FIG. 1A

(57) Abstract: The subject matter described herein relates to a method for treating or preventing metabolic dysfunction-associated steatohepatitis (MASH) in a subject in need thereof, the method comprising administering to the subject a composition targeting the caspase 8 (CASP8) pathway in hepatocytes. The compositions and methods disclosed herein can be used as disease modifying therapies to enable treatment of MASH fibrosis and related disorders earlier in disease progression and improve clinical outcomes.



METHODS AND COMPOSITIONS TO TREAT METABOLIC DYSFUNCTION-ASSOCIATED STEATOHEPATITIS (MASH) BY SILENCING A CASPASE 8-METEORIN PATHWAY

[0001] This International Patent Application claims the benefit of and priority to U.S. Application No. 63/489,622, filed March 10, 2023, entitled “SILENCING A CASPASE 8-METEORIN PATHWAY IN HEPATOCYTES TO TREAT NONALCOHOLIC STEATOHEPATITIS (NASH)”, the contents of which is hereby incorporated by reference in its entirety.

STATEMENT REGARDING GOVERNMENT SUPPORT

[0002] This invention was made with government support under DK133694 awarded by the National Institutes of Health. The government has certain rights in the invention.

[0003] This patent disclosure contains material that is subject to copyright protection. The copyright owner has no objection to the facsimile reproduction by anyone of the patent document or the patent disclosure as it appears in the U.S. Patent and Trademark Office patent file or records, but otherwise reserves any and all copyright rights.

INCORPORATION BY REFERENCE

[0004] All patents, patent applications and publications, and other literature references cited herein are hereby incorporated by reference in their entirety. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art as known to those skilled therein as of the date of the invention described and claimed herein.

TECHNICAL FIELD

[0005] The present invention relates generally to the treatment or prevention of metabolic dysfunction-associated steatohepatitis (MASH). More particularly, the present invention relates to silencing of the caspase 8 (CASP8)-meteorin (METRN) pathway. MASH was previously referred to as nonalcoholic liver disease, specifically nonalcoholic steatohepatitis (NASH). References to MASH herein are intended to refer to MASH as well as the prior nomenclature of NASH.

BACKGROUND

[0006] MASH is the leading cause of chronic liver disease worldwide; however, there are limited treatments for NASH due to a poor understanding of MASH pathology. More specifically, there is limited understanding of the conversion of the relatively benign steatosis to MASH. Recent genetic evidence has shown that the gene CASP8 promotes MASH progression.

SUMMARY

[0007] In certain aspects, described herein is a method for treating or preventing metabolic dysfunction-associated steatohepatitis (MASH) in a subject in need thereof, comprising administering to the subject a composition targeting a caspase 8 (CASP8) pathway in hepatocytes.

[0008] In some embodiments, MASH is diagnosed in the subject by determining *METRN* mRNA levels, meteorin protein levels, or both *METRN* mRNA levels and meteorin protein levels in a sample from the subject. In some embodiments, the subject has increased levels of *METRN* mRNA, meteorin protein, or both *METRN* mRNA and meteorin protein as compared to a sample from a healthy subject. In some embodiments, the subject is determined to have fibrotic MASH if the sample levels of *METRN* mRNA levels, meteorin protein levels, or both *METRN* mRNA levels and meteorin protein levels of the subject are increased by a 3 to 4-fold compared to the expression of *METRN* mRNA and/or meteorin protein in a subject (or cohort of subjects) not suffering from MASH. In some embodiments, the subject is determined to have fibrotic MASH if the sample levels of *METRN* mRNA of the subject are increased by a 3 to 4-fold compared to the expression of *METRN* mRNA in a subject (or cohort of subjects) not suffering from MASH.

[0009] In some embodiments, the sample is blood or blood plasma or liver cells.

[0010] In some embodiments, the composition reduces CASP8 expression in the subject when compared to untreated subjects or to expression level of CASP8 in the subject pre-treatment. In some embodiments, the expression of CASP8 is reduced in liver cells of the subject.

[0011] In some embodiments, the composition comprises a CASP8 small interfering ribonucleic acid (siCASP8). In some embodiments, the siCASP8 comprises at least one of the sequences encoding a small interfering ribonucleic acid (siCASP8) of Table 1.

[0012] In some embodiments, siCASP8 is a hepatocyte-targeted siRNA. In some embodiments, the siCASP8 comprises a ligand-based targeting molecule. In some embodiments, the ligand-based targeting molecule comprises an aptamer. In some embodiments, the siCASP8 comprises N-acetyl galactosamine (GalNac). In other embodiments, the siCASP8 comprises a naked siRNA. In other embodiments, the siCASP8 comprises an antibody-protamine. In some embodiments the antibody-protamine is conjugated to siCASP8. In some embodiments, the composition comprises an anti-sense oligonucleotide.

[0013] In some embodiments, the composition comprises a CASP8 short-hairpin ribonucleic acid (shCASP8). In some embodiments, the shCASP8 comprises at least one of the nucleic acid sequences of Table 1.

[0014] In some embodiments, the composition comprises a viral vector comprising a nucleic acid sequence encoding a shCASP8. In some embodiments, the viral vector is an adeno-associated vector (AAV). In some embodiments, the viral vector is AAV8. In some embodiments, the viral vector is a hepatocyte-targeted AAV.

[0015] In some embodiments, the subject is a mammal. In some embodiments, the mammal is a human.

[0016] In some embodiments, the composition reduces or inhibits *METRN* mRNA expression, meteorin protein expression, or both *METRN* mRNA expression and meteorin protein expression when compared to untreated subjects or when compared to an expression level of *METRN* mRNA, level of meteorin protein, or both levels *METRN* mRNA and meteorin protein in the subject pre-treatment. In some embodiments, the *METRN* mRNA expression is reduced or inhibited in liver cells of the subject. In some embodiments, meteorin protein expression is reduced or inhibited in liver cells of the subject.

[0017] In some embodiments, the composition comprises a METRN small interfering ribonucleic acid (siMETRN), a Signal transducer and activator of transcription 3 (STAT3)

siRNA (siSTAT3), or a STAT3-activating receptor (Kit) siRNA (siKit). In some embodiments, the composition comprises at least one of the sequences encoding the small interfering ribonucleic acid (siMETRN, siSTAT3, or siKit) of Table 1.

[0018] In some embodiments, the siMETRN, siSTAT3, or siKit is a hepatocyte-targeted siRNA. In some embodiments, the siMETRN, siSTAT3, or siKit comprises a ligand-based targeting molecule. In some embodiments, the ligand-based targeting molecule comprises an aptamer. In some embodiments, the siMETRN, siSTAT3, or siKit comprises N-acetyl galactosamine (GalNac). In other embodiments, the siMETRN, siSTAT3, or siKit comprises a naked siRNA. In other embodiments, the siMETRN, siSTAT3, or siKit comprises an antibody-protamine. In some embodiments the antibody-protamine is conjugated to siMETRN, siSTAT3, or siKit. In some embodiments, the composition comprises an anti-sense oligonucleotide.

[0019] In some embodiments, the composition comprises a METRN short-hairpin ribonucleic acid (shMETRN), a STAT3 shRNA (shSTAT3), or Kit shRNA (shKit). In some embodiments, the shMETRN, shSTAT3, or shKit comprises at least one of the nucleic acid sequences of Table 1.

[0020] In some embodiments, the composition comprises a viral vector comprising a nucleic acid sequence encoding a shRNA. In some embodiments, the viral vector is an adeno-associated vector (AAV). In some embodiments, the viral vector is AAV8. In some embodiments, the viral vector is a hepatocyte-targeted AAV.

[0021] In some embodiments, the composition reduces or inhibits *METRN*, *STAT3*, or *Kit* expression when compared to untreated subjects or to expression level of *METRN*, *STAT3*, or *Kit* in the subject pre-treatment. In some embodiments, *METRN*, *STAT3*, or *Kit* expression is reduced or inhibited in liver cells of the subject.

[0022] In some embodiments, the composition comprises a guide RNA (gRNA) or a single-molecule guide RNA (sgRNA) comprising a spacer sequence that is complementary to a portion of a nucleic acid sequence encoding CASP8, METRN, STAT3, or KIT.

[0023] In some embodiments, the gRNA or the sgRNA is pre-complexed with a DNA endonuclease. In some embodiments, the DNA endonuclease is a Cas9 endonuclease.

[0024] In some embodiments, the composition reduces or inhibits expression of one or more of *CASP8*, *METRN*, *STAT3*, *Kit* when compared to untreated subjects or to expression level of *CASP8*, *METRN*, *STAT3*, or *Kit* in the subject pre-treatment. In some embodiments, *METRN*, *STAT3*, or *Kit* expression is reduced or inhibited in liver cells of the subject.

[0025] In some embodiments, the subject is a mammal. In some embodiments, the mammal is a human. In some embodiments, the composition is delivered systemically.

[0026] In certain aspects, described herein is a composition for treating or preventing MASH comprising a hepatocyte-targeted nucleic acid capable of targeting the caspase 8 (*CASP8*) pathway. In some embodiments, the composition comprises a viral vector comprising the nucleic acid.

[0027] In some embodiments, the composition reduces or inhibits expression of one or more of *CASP8*, *METRN*, *STAT3*, or *Kit*.

[0028] In some embodiments, the viral vector is an AAV vector. In some embodiments, the viral vector is AAV8.

[0029] In some embodiments, the hepatocyte-targeted nucleic acid comprises N- acetyl galactosamine (GalNAc). In some embodiments, the viral vector is a hepatocyte-targeted AAV.

[0030] In some embodiments, composition comprises a guide ribonucleic acid (gRNA) or a single-molecule guide RNA (sgRNA) comprising a spacer sequence that is complementary to a portion of a nucleic acid sequence encoding *CASP8*, *METRN*, *STAT3*, or *Kit*.

[0031] In some embodiments, the gRNA or the sgRNA is pre-complexed with a DNA endonuclease. In some embodiments, the DNA endonuclease is a Cas9 endonuclease.

[0032] In some embodiments, the composition comprises at least one of the small interfering ribonucleic acid (siRNA) sequences from Table 1.

[0033] In some embodiments, the hepatocyte-targeted nucleic acid comprises any one of the small interfering ribonucleic acid (siRNA) sequences from Table 1.

[0034] In some embodiments, the composition comprises an expression vector targeting the caspase 8 (CASP8) pathway. In some embodiments, the expression vector targeting the caspase 8 (CASP8) pathway encodes a CASP8, METRN, STAT3, or Kit short-hairpin RNA (shRNA).

[0035] In certain aspects, described herein is a nucleic acid comprising a sequence of any one of the small interfering ribonucleic acid (siRNA) designs from Table 1.

[0036] In certain aspects, described herein is a nucleic acid comprising a sequence encoding a short-hairpin RNA (shRNA), wherein the shRNA comprises a nucleic acid sequence provided in Table 1.

[0037] In some embodiments, the nucleic acid comprises a hepatocyte-targeting motif. In some embodiments, the hepatocyte-targeting motif comprises N-acetyl galactosamine (GalNac).

[0038] In certain aspects, described herein is a vector comprising the nucleic acid sequence disclosed above.

[0039] In certain aspects, described herein is a viral vector comprising the nucleic acid disclosed above. In some embodiments, the viral vector is an AAV vector. In some embodiments, the AAV vector is AAV8. In some embodiments, the viral vector is a hepatocyte-targeted AAV.

[0040] In certain aspects, described herein is a method of diagnosing metabolic dysfunction-associated steatohepatitis (MASH) in a subject comprising: determining the level of meteorin (METRN) in a sample from the subject; and diagnosing the subject with MASH if the level of METRN expression in the sample is increased compared to a sample from a healthy subject.

[0041] In some embodiments, the subject is diagnosed with fibrotic MASH.

[0042] In some embodiments, the sample is blood or blood plasma or liver cells.

[0043] In some embodiments, the level of METRN CASP8, METERN, STAT3, or Kit is determined using an antibody. In some embodiments, the level of METRN CASP8,

METERN, STAT3, or Kit is determined by the enzyme-linked immunosorbent assay (ELISA). In some embodiments, the level of METRN CASP8, METERN, STAT3, or Kit mRNA is determined using RT-qPCR or RNA-seq.

BRIEF DESCRIPTION OF THE DRAWINGS

[0044] The patent or application file contains at least one drawing executed in color. To conform to the requirements for PCT patent applications, many of the figures presented herein are black and white representations of images originally created in color.

[0045] **FIGS. 1A-H** show deletion of hepatocyte-CASP8 lowers fibrosis and inflammation in MASH without affecting HC death. **FIG. 1A** shows That Casp8^{fl/fl} mice fed the MASH-inducing fructose-palmitate-cholesterol (FPC) for 8 wks to induce hepatosteatosis were treated with AAV8-TBG-LacZ (LacZ) or AAV8-TBG-Cre (Cre). After an additional 8 wks on the FPC diet, livers were assayed for **FIG. 1B** shows body weight and liver/body ratio; **FIG. 1C** shows Casp8 protein; **FIG. 1D** shows inflammation (H&E) and fibrosis (Sirius red); **FIG. 1E** shows aSMA⁺ area (HSCs); **FIG. 1F** shows activated HSC mRNAs (Tgfb1, Spp1); (G) F4/80⁺ area; and (H) cleaved Casp3⁺ and TUNEL⁺ cells. Mean $\pm$ SEM; n=5 mice; *p < 0.05; **p < 0.01; n.s., not significant.

[0046] **FIGS. 2A-B** show deletion of Casp8 in hepatocytes lowers hepatic stellate cells (HSC) activation by conditioned medium (CM) from the hepatocytes. **FIG. 2A** shows that AML12 mouse hepatocytes were transfected with scrambled RNA (Scr) or siCasp8. After 24 h, the medium was changed to DMEM/0.1% FBS. After another 24 h, CM was transferred to quiescent HSCs. After 72 h, the HSCs were assayed for Timp1, Spp1, and Colla1 mRNA. Mean $\pm$ SEM; n=4; *p < 0.05. Inset, Casp8 immunoblot. **FIG. 2B** shows THLE2 human hepatocytes were transfected with scrambled RNA (Scr) or siCasp8. After 24 h, the medium was changed to DMEM/0.1% FBS. After another 24 h, CASP8 mRNA was measured in THLE2 cells (left), and CM from THLE2 cells was transferred to quiescent HSCs. After 72 h, the HSCs were assayed for Timp1, Spp1, and Colla1 mRNA (right). Mean $\pm$ SEM; n=4; *p < 0.05.

[0047] **FIG. 3** shows heat-inactivation of hepatocyte (HC) conditioned medium (CM) blocks hepatic stellate cell (HSC) activation and negates the difference between Scr-HC and Casp8-silenced-HC CM. Control or heat-inactivated (HI; 100°C x 5 min) CM from Scr- or

siCasp8-treated HCs was added to HSCs as in **FIG. 2** shows that after 72 h, the HSCs were assayed for the indicated mRNAs. Mean $\pm$ SEM; n=4; *p < 0.05; ns, non-significant.

[0048] **FIGS. 4A-D** show RNAseq and LC-MS/MS data indicating that METRN is downregulated in Casp8-silenced AML12 hepatocytes and culture medium. **FIG. 4A** shows screening scheme. **FIG. 4B** shows that 11 candidates were selected from the overlap of the RNAseq and LC-MS/MS data. **FIG. 4C** shows qPCR verification of the candidate genes from **FIG. 4B** in Casp8 hepatocyte-KO livers. Mean $\pm$ SEM; n=5 mice; *p < 0.05; **p < 0.01. **FIG. 4D** shows Metrn protein was measured in the samples from **FIG. 1C** and the quantification result. Mean $\pm$ SEM; n=4; ***p < 0.001.

[0049] **FIGS. 5A-E** show METRN expression is increased in human and mouse MASH livers. **FIG. 5A** shows METRN mRNA and protein were assayed in normal and MASH human livers. **FIG. 5B** shows METRN mRNA from RNAseq GEO dataset 126848, comparing livers from healthy and non-alcoholic fatty liver disease (NAFLD) subjects. **FIG. 5C** shows METRN mRNA expression in liver single cells (normal liver) from the Human Protein Atlas database. **FIGS. 5D-E** shows that Meteorin protein was measured in the livers of mice fed chow or the MASH-inducing FPC diet for 16 wks **FIG. 5D** shows HF-CDA diet for 8 wks **FIG. 5E**. For **FIG. 5A** and **FIG. 5B**, mean $\pm$ SEM; *p < 0.05.

[0050] **FIGS. 6A-C** show siCasp8 decreases Metrn expression in mouse and human hepatocytes. **FIG. 6A** shows Scr- and siCasp8-treated AML12 cells were assayed for Caspase 8 (Casp8) and Meteorin (Metrn) by immunoblot. **FIG. 6B** shows that Scr- and siCasp8-treated THLE2 cells were assayed for Casp8 and Metrn mRNA and protein. **FIG. 6C** shows that Scr- and siCasp8-treated primary human hepatocytes were assayed for Casp8 and Metrn mRNA and protein. Mean $\pm$ SEM; n=3; *p < 0.05; ***, P < 0.001.

[0051] **FIG. 7** shows METRN induces pro-fibrotic gene expression in hepatic stellate cells (HSCs). Primary mouse HSCs were treated with 400 mM recombinant (r) Meteorin or vehicle for 48 h. The HSCs were then assayed for Timp1, Colla1 and Acta2 mRNA by qPCR. Mean $\pm$ SEM; n=4; *p < 0.05, **p<0.01.

[0052] **FIGS. 8A-B** show HSC activation by the conditioned medium (CM) of Casp8-silenced AML 12 hepatocytes is enhanced by genetically restoring METRN. **FIG. 8A** shows

that AML12 cells were transfected with scrambled (Scr) or siCasp8 siRNA and GFP or Metrn plasmid, as indicated. After 24 h, the medium was changed to DMEM/0.1% FBS. After another 24 h, Meteorin protein was measured in the AML12 cells. **FIG. 8B** shows that CM from the AML12 cells in **FIG. 8A** was transferred to quiescent HSCs. After 72 h, the HSCs were assayed for Timp1 and Spp1 mRNA (B). Mean $\pm$ SEM; n=4; ***p < 0.001.

[0053] **FIGS. 9A-B** show deletion of METRN in THLE-2 human hepatocytes lowers hepatic stellate cell (HSC) activation by THLE-2 conditioned medium (CM). **FIG. 9A** shows THLE2 cells were transfected with scrambled RNA (Scr) or siMetrn. After 24 h, the medium was changed to DMEM/0.1% FBS. After another 24 h, METRN mRNA and protein were measured in THLE2 cells. **FIG. 9B** shows CM from THLE2 cells from **FIG. 9A** was transferred to quiescent HSCs. After 72 h, the HSCs were assayed for Timp1 and Spp1, mRNA **FIG. 9B** shows Mean $\pm$ SEM; n=4; *p < 0.05, ***p<0.001.

[0054] **FIGS. 10A-B** show METRN activates Stat3 in hepatic stellate cells (HSCs). **FIG. 10A** shows primary mouse HSCs treated with 400 ng/ml recombinant (r) Meteorin or vehicle for 48 h. The HSCs were then assayed for phospho (p-Y705) and total Stat3 by immunoblot. **FIG. 10B** shows primary mouse HSCs treated with 100 ng/ml recombinant (r) Meteorin, vehicle, or both Meteorin and Stat3 for 2 h. The HSCs were then assayed for phospho (p-Y705) Stat3.

[0055] **FIGS. 11A-B** show hepatic stellate cell activation is reduced by silencing Kit/Stat3 in primary mouse hepatic stellate cells. **FIG. 11A** shows primary mouse HSCs transfected with scrambled (Scr) or siKit/siStat3 siRNA as indicated. After 48 h, Kit/Stat3 mRNA was measured in the HSC cells. N=3-4. *P<0.05. **FIG. 11B** shows primary mouse HSCs transfected with scrambled (Scr) or siKit/Stat3 siRNA, as indicated. After 24 h, the cells were treated with vehicle or rMetrn. After another 48 h, Timp1 and Spp1 mRNA was measured in the HSC cells. N=4. **P<0.01, ***P<0.001, ****P<0.0001.

[0056] **FIGS. 12A-C** show METRN expression in AML12 hepatocytes does not require the caspase activity of caspase 8. **FIG. 12A** shows AML12 cells treated for 48 h with 20 mM of the pan-caspase inhibitor ZVAD or the caspase 8 inhibitor ITED, or DMSO vehicle control, and then Metrn mRNA was measured by qPCR. **FIG. 12B** shows AML12 mouse hepatocytes treated with IETD or Veh in DMEM/0.1% FBS. After 24 h, the conditioned

medium (CM) was transferred to quiescent HSCs. After 72 h, the HSCs were assayed for Timp1 and Colla1 mRNA. **FIG. 12C** shows a positive control for the inhibitors, AML12 cells treated for 24 h with or without 5 mg/ml Jo2 to induce apoptosis together with DMSO or 20 mM ZVAD or ITED. The active form of Casp3 (cl-Casp3), as a measure of pan-caspase or upstream caspase 8 activity, was then assayed by immunoblot. N.S., not significant.

[0057] **FIG. 13** shows the protective effect of silencing hepatocyte Casp8 on hepatic stellate cell activation was lost when hepatocytes were transfected with the CASP8 C360A mutant. AML12 cells were transfected with scrambled (Scr) or siCasp8 siRNA and GFP, Casp8 WT or C360A mutation plasmid, as indicated. After 24 h, the medium was changed to DMEM/0.1% FBS. After another 24 h, the conditioned medium (CM) from hepatocytes was transferred to quiescent HSCs. After 72 h, the HSCs were assayed for Colla1 and Timp1 mRNA. These data suggest that CASP8-induced HSC activation is independent of its protease activity.

[0058] **FIGS. 14A-D** show elevation of CASP8 full length protein in MASH livers. **FIG. 14A** shows *CASP8* mRNA in livers of healthy and MASLD subjects, based on RNAseq GEO dataset 126848. **FIG. 14B** shows immunoblot of full-length caspase-8 in normal and MASH human livers, with β -actin as loading control. **FIG. 14C** shows immunoblot of full-length caspase-8 in chow- and FPC-fed mouse livers, with β -actin as loading control. **FIG. 14D** shows immunoblot of full-length caspase-8 in chow- and HF-CDAA-fed mouse livers, with β -actin as loading control.

[0059] **FIGS. 15A-L** show suppression of the progression to early MASH fibrosis with deletion of hepatocyte caspase-8 in mice with established diet-induced hepatosteatosis without blocking hepatocyte apoptosis. Male *Casp8^{fl/fl}* mice were fed the FPC diet for 8 weeks to induce steatosis. The mice were then injected with AAV8-TBG-Cre or AAV8-TBG-LacZ control and continued on the FPC diet for an additional 8 weeks. **FIG. 15A** shows the experimental scheme. **FIG. 15B** shows immunoblot of caspase-8 in liver, with β -actin as the loading control. **FIGS. 15C-E** shows body weight, body weight:liver weight ratio, and fasting blood glucose (FBG). **FIG. 15F** shows staining of liver sections for Sirius red (upper panels; bar, 500 μ m) and H&E (lower panels; bar, 100 μ m), with quantification of Sirius red-positive area and hepatic mononuclear cells. **FIG. 15G** shows α -SMA immunofluorescence (upper panels) and quantification; DAPI counterstain (lower panels) for

nuclei is shown in bottom panels; bar, 200 mm. **FIG. 15H** shows F4/80 immunofluorescence (upper panels) and quantification; DAPI counterstain (lower panels) for nuclei is shown in bottom panels; bar, 200 mm. **FIG. 15I** shows mRNA expression of *Tgfb1*, *Spp1*, *Acta2* and *Mcp1*. **FIG. 15J** shows Plasma ALT. **FIG. 15K** Cleaved caspase-3 (cl-Casp3) immunofluorescence (upper panels) and quantification; DAPI counterstain (lower panels) for nuclei is shown in bottom panels; bar, 500 mm. **FIG. 15L** TUNEL staining (upper panels) and quantification; DAPI counterstain (lower panels) for nuclei is shown in bottom panels; bar, 500 mm.

[0060] **FIGS. 16A-I** show suppression of the progression of MASH fibrosis with deletion of hepatocyte caspase-8 in mice with a high cell death rate without blocking hepatocyte apoptosis. Male *Casp8^{fl/fl}* mice were fed the HF-CDAA diet for 4 weeks to induce steatosis. The mice were then injected with AAV8-TBG-Cre or control AAV8-TBG-GFP and continued on the HF-CDAA diet for an additional 4 weeks. **FIG. 16A** shows the experimental scheme. **FIG. 16B** shows immunoblot of caspase-8 in liver, with β -actin as the loading control. **FIGS. 16C-E** show body weight, body weight:liver weight ratio, and fasting blood glucose (FBG). **FIG. 16F** shows staining of liver sections for Sirius red, with quantification. **FIG. 16G** show H&E staining. **FIG. 16H** show α SMA immunofluorescence (upper panels) of liver sections of mice fed the HF-CDAA diet for 8 weeks with or without hepatocyte caspase-8 knockout at 4 weeks. In M, DAPI counterstain for nuclei (lower panels) is shown in the bottom panels. Bars, 200 mm. **FIG. 16I** shows cleaved caspase-3 immunofluorescence (upper panels) and quantification; DAPI counterstain (lower panels) for nuclei is shown in bottom panels; bar, 200 mm.

[0061] **FIGS. 17A-E** show blunting hepatic stellate cell activation with deletion of Casp8 in hepatocytes *in vitro*. **FIG. 17A** shows immunoblot of caspase-8 in control (Scr) and Casp8-silenced AML12 cells. **FIG. 17B** shows AML12 mouse hepatocytes (HCs) transfected with scrambled RNA (Scr) or siCasp8. After 24 hours, the media were changed to DMEM/0.1% FBS, and after an additional 24 hours, the conditioned media (CM) were transferred to quiescent HSCs. After 72 hours, the HSCs were assayed for *Timp1*, *Spp1*, and *Colla1* mRNA. Mean $\pm$ SEM; n=4; *p < 0.05. **FIG. 17C** shows *CASP8* mRNA in control (Scr) and *CASP8*-silenced THLE2 cells. **FIG. 17D** shows THLE2 human HCs were transfected with scrambled RNA (Scr) or siCasp8. After 24 hours, the media were changed to

DMEM/0.1% FBS, and after an additional 24 hours, the conditioned media from the cells was transferred to quiescent HSCs. After 72 hours, the HSCs were assayed for *Timp1*, *Spp1*, and *Col1a1* mRNA (right). Mean $\pm$ SEM; n=4; *p < 0.05. **FIG. 17E** shows control or heat-inactivated (HI; 100°C x 5 min) CM from Scr- or siCasp8-treated AML12 HCs was added to HSCs as in panel A. After 72 h, the HSCs were assayed for the indicated mRNAs. Mean $\pm$ SEM; n=4; *p < 0.05; ns, non-significant.

[0062] FIGS. 18A-P shows identification of CASP8 downstream gene – Metrn. **FIG. 18A** shows the scheme for screening caspase-8-dependent secretory genes/proteins in hepatocytes that can activate HSCs. **FIG. 18B** shows Volcano plot from the RNAseq data. **FIG. 18C** shows heat plot from the RNAseq data. **FIG. 18D** shows venn diagram plot from the RNAseq and LC-MS/MS data, integrated with a database of secretory proteins. **FIG. 18E** shows mRNA levels of candidate genes in the livers from the mice in **FIGS. 15A-I**. **FIG. 18F** shows Scr- and siCasp8-treated AML12 cells were assayed for caspase-8 (Casp8) and meteorin (Metrn) by immunoblot. **FIG. 18G** shows Scr- and siCasp8-treated THLE2 cells were assayed for *CASP8* and *METRN* mRNA and caspase-8 and meteorin protein. **FIG. 18H** shows Scr- and siCasp8-treated primary human hepatocytes were assayed for *CASP8* and *METRN* mRNA and caspase-8 and meteorin protein. Mean $\pm$ SEM; n=3; *p < 0.05; ***, P < 0.001. **FIG. 18I** shows Immunoblot of meteorin, with quantification, in the livers of control or hepatocyte caspase-8 knockout mice fed the FPC diet for 16 weeks. **FIG. 18J** shows immunoblot of meteorin, with quantification, in the livers of control or hepatocyte caspase-8 knockout mice fed the HF-CDAA diet for 8 weeks. **FIG. 18K** shows Hepatocyte nuclear extracts from AML12 cells treated with Casp8 or Scr siRNA that were subjected to Pol II ChIP analysis using anti-Pol II or IgG control. The promoter region sequence was amplified by qPCR and normalized to the values obtained from input DNA. ***p < 0.001; mean $\pm$ SEM; n = 4. **FIG. 18L** Hepatocyte nuclear extracts from AML12 cells treated with Casp8 or Scr siRNA were subjected to YY1 ChIP analysis using anti-YY1 or IgG control. The promoter region sequence was amplified by qPCR and normalized to the values obtained from input DNA. **p < 0.01; mean $\pm$ SEM; n = 4. **FIG. 18M** shows Scr- and siYy1-treated AML12 cells assayed for Yy1 and meteorin (Metrn) by immunoblot. **FIG. 18N** shows Scr- and siYY1-treated primary human hepatocytes assayed for *YY1* and *METRN* mRNA and caspase-8 and meteorin protein. **FIG. 18O** shows AML12 cells treated for 48 hours with 20 mM of the pan-caspase inhibitor ZVAD or the caspase 8 inhibitor ITED, or DMSO vehicle

control, followed by quantification of *Metrn* mRNA. **FIG. 18P** shows AML12 cells treated for 24 hours with or without 5 mg/ml Jo2 to induce apoptosis together with DMSO or 20 mM ZVAD or IETD. The active, cleaved form of caspase-3 (cl-Casp3), which is a measure of caspase-8 activity, was assayed by immunoblot. n.s., not significant.

[0063] FIGS. 19A-L shows that hepatocyte caspase-8 induces meteorin, which promotes the expression of pro-fibrotic genes in hepatic stellate cells. **FIG. 19A** shows Primary mouse HSCs treated with 200 ng/ml recombinant (r) meteorin or vehicle for 48 hours. The HSCs were then assayed for *Timp1*, *Colla1*, and *Acta2* mRNA by qPCR. Mean $\pm$ SEM; n=4; *p < 0.05, **p<0.01. **FIG. 19B** shows primary human HSCs from healthy liver treated with 200 ng/ml r-meteorin or vehicle for 48 hours. The HSCs were then assayed for *TIMP1*, *SPP1*, *COL1A1*, and *ACTA2* mRNA by qPCR. Mean $\pm$ SEM; n=4; **p < 0.01, ***p<0.001. **FIG. 19C** shows primary HSCs from the livers of individuals with MASH were treated with 200 ng/ml r-meteorin or vehicle for 48 hours. The HSCs were then assayed for *TIMP1*, *COL1A1*, and *ACTA2* mRNA by qPCR. Mean $\pm$ SEM; n=4; *p < 0.05, **p<0.01. **FIG. 19D** AML12 cells transfected with scrambled (Scr) or siCasp8 siRNA and with GFP or *Metrn* plasmid, as indicated. After 24 hours, the conditioned media were changed to DMEM/0.1% FBS, and after an additional 24 hours, the AML12 cells were assayed for meteorin protein. **FIG. 19E** shows conditioned media from the AML12 cells in panel A transferred to quiescent HSCs. After 72 h, the HSCs were assayed for *Timp1* and *Spp1* mRNA. Mean $\pm$ SEM; n=4; ***p < 0.001. **FIG. 19F** shows THLE2 cells transfected with scrambled RNA (Scr) or siMETRN. After 24 hours, the media were changed to DMEM/0.1% FBS, and after an additional 24 hours, the THLE2 cells were assayed for *METRN* mRNA and meteorin protein. **FIG. 19 G** shows conditioned media from THLE2 cells from panel C transferred to quiescent HSCs. After 72 h, the HSCs were assayed for *Timp1* and *Spp1* mRNA. Mean $\pm$ SEM; n=4; *p < 0.05, ***p<0.001. **FIG. 19H** shows AML12 mouse hepatocytes treated with IETD or Veh in DMEM/0.1% FBS. After 24 hours, the conditioned media were transferred to quiescent primary mouse HSCs. After 72 hours, the HSCs were assayed for *Timp1* and *Colla1* mRNA. **FIG. 19I** shows AML12 cells transfected with scrambled (Scr) or siCasp8 siRNA and with GFP, wild-type *Casp8*, or C360A-mutant *Casp8*. After 24 hours, the media were changed to DMEM/0.1% FBS, and after another 24 hours, the conditioned media from the hepatocytes were transferred to quiescent primary mouse HSCs. After 72 hours, the HSCs were assayed for *Colla1* and *Timp1* mRNA. **FIG. 19J** shows primary mouse HSCs treated with 400 ng/ml

r-meteorin or vehicle for 48 hours. The HSCs were then assayed for phospho (p-Y705) and total Stat3 by immunoblot. **FIG. 19K** shows primary mouse HSCs transfected with scrambled (Scr), siKit, or siStat3 siRNA. After 48 hours, *Kit* and *Stat3* mRNA were measured in the HSC cells. n=3-4. *p < 0.05. **FIG. 19L** shows primary mouse HSCs transfected with scrambled (Scr), siKit, or Stat3 siRNA. After 24 hours, the cells were treated with vehicle or r-meteorin. After another 48 hours, *Timp1* and *Spp1* mRNA was measured in the HSCs. n=4. **P<0.01, ***P<0.001, ****P<0.0001.

[0064] **FIGS. 20A-I** show increased Meteorin and YY1 in human and mouse MASH liver. **FIG. 20A** shows *METRN* mRNA and meteorin protein assayed in the livers of subjects with or without MASH. **FIG. 20B** shows *METRN* mRNA levels in livers of healthy and metabolic-associated steatotic liver disease (MASLD) subjects, based on the RNAseq GEO dataset 126848. **FIG. 20C** shows increased Meteorin protein in the livers of mice fed chow or the FPC diet for 16 weeks. **FIG. 20D** shows increased Meteorin protein in the livers of mice fed chow or the HF-CDA diet for 8 weeks. **FIG. 20E** shows Meteorin immunoblot of liver extracts from C57BL/6J mice fed chow or FPC diet for 8 or 16 weeks. **FIG. 20F** shows Meteorin immunoblot of liver extracts of normal and steatotic human liver. **FIG. 20G** shows YY1 protein assayed in the livers of subjects with or without MASH. **FIG. 20H** shows increased YY1 protein in the livers of mice fed chow or the FPC diet for 16 weeks. **FIG. 20I** shows increased YY1 protein in the livers of mice fed chow or the HF-CDA diet for 8 weeks.

[0065] **FIGS. 21A-I** show that genetic restoration of hepatocyte meteorin blocks the protective effect of hepatocyte caspase-8 knockout on MASH fibrosis. Male *Casp8^{fl/fl}* mice were fed the FPC diet for 8 weeks and then injected with AAV8-TBG-Cre (KO), AAV8-TBG-Cre/Metn (KO+Metn) or AAV8-TBG-GFP (control) and continued on the FPC diet for an additional 8 weeks. **FIG. 21A** shows the experimental scheme. **FIG. 21B** shows immunoblot of caspase-8 and meteorin in liver, with β -actin as loading control. **FIGS. 21C-E** show body weight, body weight:liver weight ratio, and fasting blood glucose (FBG). **FIG. 21F** shows staining of liver sections for Sirius red (upper panels; bar, 200 μ m) and H&E (lower panels; bar, 200 μ m), with quantification of Sirius red-positive area and hepatic mononuclear cells. **FIG. 21G** shows liver mRNA levels of *Timp1*, *Spp1*, *Col1a1*, *Tnfa*, *Mcp1*,

and *Emr1*. **FIG. 21H** shows COL1A1 and F4/80 immunohistochemistry, with quantification; bar, 200 μ m. **FIG. 21I** shows plasma ALT.

[0066] **FIGS. 22A-H** show that silencing of hepatocyte meteorin after the development of early MASH blocks the progression of liver fibrosis. Male *Casp8^{fl/fl}* mice were fed the FPC diet for 16 weeks and then injected with AAV8-H1-shMetrn (shMetrn) or AAV8-H1-Scr (control) and continued on the FPC diet for an additional 12 weeks. **FIG. 22A** shows the experimental scheme. **FIG. 22B** shows liver *Metrn* mRNA and immunoblot of meteorin protein, with β -actin as loading control (right panel). **FIGS. 22C-E** show body weight, body weight:liver weight ratio, and fasting blood glucose (FBG). **FIG. 22F** shows staining of liver sections for Sirius red (upper panels; bar, 200 μ m) and H&E (lower panels; bar, 200 μ m), with quantification of Sirius red-positive area and hepatic mononuclear cells. **FIG. 22G** shows liver mRNA levels of *Timp1*, *Spp1*, *Col1a1*, *Col1a2*, *Tgfb1*, *Tnfa*, *Mcp1*, and *Emr1*. **FIG. 22H** shows plasma ALT.

DETAILED DESCRIPTION

[0067] All patent applications, published patent applications, issued and granted patents, texts, and literature references cited in this specification are hereby incorporated herein by reference in their entirety to more fully describe the state of the art to which the present disclosed subject matter pertains.

[0068] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Methods and materials are described herein for use in the present invention; other, suitable methods and materials known in the art can also be used. The materials, methods, and examples are illustrative only and not intended to be limiting. All publications, patent applications, patents, sequences, database entries, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control.

[0069] The present disclosure provides compositions and methods for treating and/or preventing nonalcoholic liver disease. Nonalcoholic liver disease, specifically metabolic dysfunction-associated steatohepatitis (MASH), is the leading cause of chronic liver disease worldwide; however, there are limited treatments for MASH due to a poor understanding of

MASH pathology. More specifically, there is limited understanding of the conversion of the relatively benign steatosis to MASH. Recent genetic evidence has shown that the gene CASP8 promotes MASH progression. Moreover, it has been found that MASH induces the gene METRN which has never been implicated in any liver function or disease.

[0070] As used herein, the term “subject” refers to a vertebrate animal. In one embodiment, the subject is a mammal or a mammalian species. In one embodiment, the subject is a human. In one embodiment, the subject is a healthy human adult. In other embodiments, the subject is a non-human vertebrate animal, including, without limitation, non-human primates, laboratory animals, livestock, racehorses, domesticated animals, and non-domesticated animals. In one embodiment, the term “human subjects” means a population of healthy human adults.

[0071] The singular forms “a”, “an” and “the” include plural reference unless the context clearly dictates otherwise.

[0072] As used herein the term “about” is used herein to mean approximately, roughly, around, or in the region of. When the term “about” is used in conjunction with a numerical range, it modifies that range by extending the boundaries above and below the numerical values set forth.

[0073] As used herein the term “variant” covers nucleotide or amino acid sequence variants which have about 99%, about 98%, about 97%, about 96%, about 95%, about 94%, about 93%, about 92%, about 91%, about 90%, about 85%, about 80%, about 75%, about 70%, or about 65% nucleotide identity, or about 99%, about 98%, about 97%, about 96%, about 95%, about 94%, about 93%, about 92%, about 91%, about 90%, about 85%, about 80%, about 75%, about 70%, or about 65% amino acid identity, including but not limited to variants comprising conservative, or non-conservative substitutions, deletions, insertions, duplications, or any other modification.

[0074] As used herein MASH refers to metabolic dysfunction-associated steatohepatitis. MASH was previously referred to as NASH.

[0075] In certain aspects, described herein is the use of small interfering RNA (siRNA) platforms to silence the caspase 8 (CASP8) or meteorin (METRN) genes in hepatocytes (liver

cells). *See* Table 1. Mouse models described herein show that the knockout of the CASP8 gene blocked the progression of steatosis to MASH. Moreover, scRNA-seq data has shown that hepatocytes express the METRN gene at higher levels in fibrotic tissue when compared to normal human liver. As such, blocking the expression of CASP8 or METRN in hepatocytes can lead to better outcomes for patients with MASH.

[0076] Nonalcoholic fatty liver disease is a major cause of liver disease worldwide (Younossi ZM, Koenig AB, Abdelatif D, Fazel Y, Henry L, Wymer M. Global epidemiology of nonalcoholic fatty liver disease-Meta-analytic assessment of prevalence, incidence, and outcomes. *Hepatology*. 2016 Jul; 64(1): pp. 73-84). Nonalcoholic steatohepatitis (MASH) is a subtype of nonalcoholic fatty liver disease and is associated with development of cirrhosis and liver transplants (Sheka AC, Adeyi O, Thompson J, Hameed B, Crawford PA, Ikramuddin S. Nonalcoholic Steatohepatitis: A Review. *JAMA*. 2020 Mar 24;323(12): pp. 1175-1183). The CASP8 gene is critical for the pathogenesis of MASH (Hatting M, Zhao G, Schumacher F, Sellge G, Masaoudi MA, et al. Hepatocyte caspase-8 is an essential modulator of steatohepatitis in rodents. *Hepatology*. 2013 Jun;57(6): pp. 2189-2201). CASP8 knockout mice experienced slower progression to MASH than their wildtype counterparts (Hatting M, Zhao G, Schumacher F, Sellge G, Masaoudi MA, et al. Hepatocyte caspase-8 is an essential modulator of steatohepatitis in rodents. *Hepatology*. 2013 Jun;57(6): pp. 2189-2201).

[0077] Described herein is the discovery that CASP8 induces the expression of the gene METRN and fibrotic liver tissue has been shown to have higher levels of METRN expression than normal liver tissue. Silencing RNA (siRNA) can downregulate gene expression of specific genes (Agrawal N, Dasaradhi PVN, Mohammed A, Malhotra P, Bhatnagar RK, Mukherjee SK. RNA Interference: Biology, Mechanism, and Applications. *Microbiol Mol Biol Rev*. 2003 Dec; 67(4): pp. 657-685).

[0078] In certain aspects, described herein is the use of siRNA to silence the expression of CASP8 or METRN in hepatocytes. Also described herein is the use of METRN as a plasma biomarker for diagnosing fibrotic MASH.

[0079] The disclosure further provides methods for treating and/or preventing MASH fibrosis in patients possessing increased expression of METRN. The compositions and methods disclosed herein can be used as disease modifying therapies to enable prevention or

treatment of MASH fibrosis and related disorders earlier in disease progression and improve clinical outcomes. The disclosure is based, at least in part, on the discovery, that CASP8 and METRN gene expressions in humans can lead to the development of MASH fibrosis, and that this result is due the gene's critical role activation of the Kit/STAT3 signaling pathway.

Using a personalized medicine approach, methods of treatment or preventing MASH fibrosis comprising either silencing or reducing expression of hepatocyte CASP8 or METRN among subjects who have high METRN expression compared to a subject who is not suffering from MASH fibrosis are described.

[0080] Methods of treatment or prevention

[0081] In certain aspects, described herein is a method for treating or preventing MASH in a subject in need thereof, comprising administering to the subject a composition targeting the caspase 8 (CASP8) pathway in hepatocytes.

[0082] The practice of aspects of the present invention can employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Molecular Cloning A Laboratory Manual, 3rd Ed., ed. by Sambrook (2001), Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1989); DNA Cloning, Volumes I and II (D. N. Glover ed., 1985); Oligonucleotide Synthesis (M. J. Gait ed., 1984); Mullis et al. U.S. Pat. No: 4,683,195; Nucleic Acid Hybridization (B. D. Hames & S. J. Higgins eds. 1984); Transcription and Translation (B. D. Hames & S. J. Higgins eds. 1984); Culture Of Animal Cells (R. I. Freshney, Alan R. Liss, Inc., 1987); Immobilized Cells and Enzymes (IRL Press, 1986); B. Perbal, A Practical Guide To Molecular Cloning (1984); the series, Methods In Enzymology (Academic Press, Inc., N.Y.), specifically, Methods In Enzymology, Vols. 154 and 155 (Wu et al. eds.); Gene Transfer Vectors For Mammalian Cells (J. H. Miller and M. P. Calos eds., 1987, Cold Spring Harbor Laboratory); Immunochemical Methods In Cell And Molecular Biology (Caner and Walker, eds., Academic Press, London, 1987); Handbook Of Experimental Immunology, Volumes I-IV (D. M. Weir and C. C. Blackwell, eds., 1986); Manipulating the Mouse Embryo, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986). All patents, patent applications and references cited herein are incorporated by reference in their entireties.

[0083] In some embodiments, MASH is diagnosed in the subject by determining *METRN* mRNA levels, meteorin protein levels, or both *METRN* mRNA levels and meteorin protein levels in a sample from the subject. In some embodiments, the subject has increased levels of *METRN* mRNA, meteorin protein, or both *METRN* mRNA and meteorin protein as compared to a sample from a healthy subject. In some embodiments, the subject is determined to have fibrotic MASH if the sample levels of *METRN* mRNA levels, meteorin protein levels, or both *METRN* mRNA levels and meteorin protein levels of the subject are increased by a 3 to 4 fold compared to the expression of *METRN* mRNA and/or meteorin protein of a subject (or cohort of subjects) not suffering from MASH. In various embodiments, expression of *METRN* mRNA levels, meteorin protein levels, or both *METRN* mRNA levels and meteorin protein levels of a subject may be increased by at least 1.5 times greater than expression of *METRN* mRNA levels, meteorin protein levels, or both *METRN* mRNA levels and meteorin protein levels of a subject (or cohort of subjects) not suffering from MASH. Expression of *METRN* mRNA levels, meteorin protein levels, or both *METRN* mRNA levels and meteorin protein levels of a subject may be increased by at least 3 times greater than expression of *METRN* mRNA levels, meteorin protein levels, or both *METRN* mRNA levels and meteorin protein levels of a subject (or cohort of subjects) not suffering from MASH. Expression of *METRN* mRNA levels, meteorin protein levels, or both *METRN* mRNA levels and meteorin protein levels of a subject may be increased by at least 5 times greater than expression of *METRN* mRNA levels, meteorin protein levels, or both *METRN* mRNA levels and meteorin protein levels of a subject (or cohort of subjects) not suffering from MASH. Expression of *METRN* mRNA levels, meteorin protein levels, or both *METRN* mRNA levels and meteorin protein levels of a subject may be increased by at least 10 times greater than expression of *METRN* mRNA levels, meteorin protein levels, or both *METRN* mRNA levels and meteorin protein levels of a subject (or cohort of subjects) not suffering from MASH.

[0084] In some embodiments, the subject is determined to have fibrotic MASH if the sample levels of *METRN* mRNA levels are increased by a 3 to 4 fold compared to the expression of *METRN* mRNA of a subject (or cohort of subjects) not suffering from MASH. In various embodiments, expression of *METRN* mRNA levels of a subject may be increased by at least 1.5 times greater than expression of *METRN* mRNA levels of a subject (or cohort of subjects) not suffering from MASH. Expression of *METRN* mRNA levels of a subject may be increased by at least 3 times greater than expression of *METRN*

mRNA levels of a subject (or cohort of subjects) not suffering from MASH. Expression of *METRN* mRNA levels of a subject may be increased by at least 5 times greater than expression of *METRN* mRNA levels of a subject (or cohort of subjects) not suffering from MASH. Expression of *METRN* mRNA levels of a subject may be increased by at least 10 times greater than expression of *METRN* mRNA levels of a subject (or cohort of subjects) not suffering from MASH.

[0085] In some embodiments, the sample is blood or blood plasma, or liver cells. In some embodiments, MASH is diagnosed in the subject by determining *METRN* mRNA plasma levels, meteorin protein plasma levels, or both *METRN* mRNA plasma levels and meteorin protein plasma levels in a sample from the subject. In some embodiments, the subject has increased plasma levels of *METRN* mRNA, meteorin protein, or both *METRN* mRNA and meteorin protein as compared to a sample from a healthy subject.

[0086] In some embodiments, the composition reduces CASP8 expression in the subject when compared to untreated subjects or to expression level of CASP8 in the subject pre-treatment. The expression of CASP8 may be reduced by at least about 5% to about 95%, e.g., about 5%, 10%, 15%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, relative to corresponding expression of CASP8 in a subject suffering from MASH. The expression of CASP8 may be silenced relative to corresponding expression of CASP8 in a subject suffering from MASH. In some embodiments, the expression of CASP8 is reduced in liver cells of the subject.

[0087] In some embodiments, the composition comprises a CASP8 small interfering ribonucleic acid (siCASP8). In some embodiments, the siCASP8 comprises at least one of the sequences encoding the small interfering ribonucleic acid (siCASP8) of Table 1. For example, in various embodiments the composition comprises at least one of the siRNA designs of Table 1. In various embodiments, the siRNA comprises a nucleic acid sequence at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identical to the siRNA designs listed in Table 1. In some embodiments, the siRNA consists of a siRNA nucleic acid sequence of Table 1.

[0088] In some embodiments, siCASP8 is a hepatocyte-targeted siRNA. In some embodiments, the siCASP8 comprises a ligand-based targeting molecule. In some

embodiments, the ligand-based targeting molecule comprises an aptamer. For example, galactose and galactose derivatives can be used to target molecules to hepatocytes in vivo through their binding to the asialoglycoprotein receptor (ASGPr) expressed on the surface of hepatocytes. Binding of such galactose targeting moieties to the ASGPr(s) induces cell-specific targeting of a polymer to hepatocytes and endocytosis of the delivery polymer into hepatocytes. ASGPr targeting molecules may be selected from the group comprising: lactose, galactose, N-acetylgalactosamine (GalNAc), galactosamine, N-formylgalactosamine, N-acetyl-galactosamine, N-propionylgalactosamine, N-n-butanoylgalactosamine, and N-iso-butanoyl-galactosamine (Iobst, S.T. and Drickamer, K., Selective Sugar Binding to the Carbohydrate Recognition Domains of the Rat Hepatic and Macrophage Asialoglycoprotein Receptors, J.B.C. 1996, 271, 6686). ASGPr targeting moieties can be monomeric (e.g., having a single galactosamine) or multimeric (e.g., having multiple galactosamines). In some embodiments, the siCASP8 comprises N-acetyl galactosamine (GalNAc). In other embodiments, the siCASP8 comprises a naked siRNA. In other embodiments, the siCASP8 comprises an antibody-protamine. In some embodiments the antibody-protamine is conjugated to siCASP8. In some embodiments, the composition comprises an anti-sense oligonucleotide. CASP8 expression may be reduced using any known method in the art.

[0089] In various embodiments the composition comprises an RNA nanoparticle. In various embodiments, the RNA nanoparticle comprises CASP8 siRNA. In various embodiments, the RNA nanoparticle comprises METRN siRNA. In various embodiments, the RNA nanoparticle comprises Kit siRNA. In various embodiments, the RNA nanoparticle comprises Stat3 siRNA.

[0090] In some embodiments, the composition comprises a CASP8 short-hairpin ribonucleic acid (shCASP8). In some embodiments, the shCASP8 comprises at least one of the nucleic acid sequences of Table 1. In some embodiments, the composition comprises a viral vector comprising a nucleic acid sequence encoding a shCASP8. In some embodiments, the viral vector is an adeno-associated vector (AAV). In various embodiments, the viral vector is a vector that preferentially targets the liver or liver cells. In various embodiments, the AAV is AAV 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 or variant thereof. In some embodiments, the viral vector is AAV8. In some embodiments, the viral vector is a hepatocyte-targeted AAV. In various embodiments, the shRNA comprises a nucleic acid sequence at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identical to the

nucleic acid sequence as provided in Table 1. In some embodiments, the shRNA consists of a nucleic acid sequence of Table 1. In various embodiments, the vector is a viral vector comprising a nucleic acid encoding a CASP8 or METRN short-hairpin RNA (shRNA).

[0091] In some embodiments, the subject is a mammal. In some embodiments, the mammal is a human.

[0092] In some embodiments, the composition reduces or inhibits *METRN* mRNA expression, meteorin protein expression, or both *METRN* mRNA expression and meteorin protein expression when compared to untreated subjects or to expression level of *METRN* mRNA, level of meteorin protein, or both levels *METRN* mRNA and meteorin protein in the subject pre-treatment. In some embodiments, the *METRN* mRNA expression is reduced or inhibited in liver cells of the subject. In some embodiments, meteorin protein expression is reduced or inhibited in liver cells of the subject. The expression of *METRN* mRNA, meteorin protein, or both *METRN* mRNA and meteorin protein may be reduced by at least about 5% to about 95%, e.g., about 5%, 10%, 15%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, relative to corresponding expression of CASP8, METRN, Kit and/or Stat3 in a subject suffering from MASH. The expression of CASP8, METRN, Kit and/or Stat3 may be silenced relative to corresponding expression level of *METRN* mRNA, level of meteorin protein, or both levels *METRN* mRNA and meteorin protein in a subject suffering from MASH.

[0093] In some embodiments, the composition comprises a METRN small interfering ribonucleic acid (siMETRN), a Signal transducer and activator of transcription 3 (STAT3) siRNA (siSTAT3), or a STAT3-activating receptor (Kit) siRNA (siKit). In some embodiments, the composition comprises at least one of the sequences encoding the small interfering ribonucleic acid (siMETRN, siSTAT3, or siKit) of Table 1. METRN, Kit or Stat3 expression may be reduced using any known method in the art. For example, in various embodiments the composition comprises at least one of the siRNA designs of Table 1. In various embodiments, the siRNA comprises a nucleic acid sequence at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identical to the siRNA designs listed in Table 1. In some embodiments, the siRNA consists of a siRNA nucleic acid sequence of Table 1.

[0094] In some embodiments, the siMETRN, siSTAT3, or siKit is a hepatocyte-targeted siRNA. In some embodiments, the siMETRN, siSTAT3, or siKit comprises a ligand-based targeting molecule. For example galactose and galactose derivatives can be used to target molecules to hepatocytes in vivo through their binding to the asialoglycoprotein receptor (ASGPr) expressed on the surface of hepatocytes. Binding of such galactose targeting moieties to the ASGPr(s) induces cell-specific targeting of a polymer to hepatocytes and endocytosis of the delivery polymer into hepatocytes. ASGPr targeting molecules may be selected from the group comprising: lactose, galactose, N-acetylgalactosamine (GalNAc), galactosamine, N-formylgalactosamine, N-acetyl-galactosamine, N-propionylgalactosamine, N-n-butanoylgalactosamine, and N-iso-butanoyl-galactosamine (Iobst, S.T. and Drickamer, K., Selective Sugar Binding to the Carbohydrate Recognition Domains of the Rat Hepatic and Macrophage Asialoglycoprotein Receptors, J.B.C. 1996, 271, 6686). ASGPr targeting moieties can be monomeric (e.g., having a single galactosamine) or multimeric (e.g., having multiple galactosamines). In some embodiments, the ligand-based targeting molecule comprises an aptamer. In some embodiments, the siMETRN, siSTAT3, or siKit siRNA comprises N-acetyl galactosamine (GalNAc). In other embodiments, the siMETRN, siSTAT3, or siKit siRNA comprises a naked siRNA. In other embodiments, the siMETRN, siSTAT3, or siKit siRNA comprises an antibody-protamine. In some embodiments the antibody-protamine is conjugated to siMETRN, siSTAT3, or siKit siRNA. In some embodiments, the composition comprises an anti-sense oligonucleotide.

[0095] In some embodiments, the composition comprises a METRN short-hairpin ribonucleic acid (shMETRN), a STAT3 shRNA (shSTAT3), or Kit shRNA (shKit). In some embodiments, the shMETRN, shSTAT3, or shKit comprises at least one of the nucleic acid sequences of Table 1. In various embodiments, the shRNA comprises a nucleic acid sequence at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identical to the siRNA nucleic acid sequences listed in Table 1.

[0096] In some embodiments, the composition comprises a viral vector comprising a nucleic acid sequence encoding a shRNA. In some embodiments, the viral vector is an adeno-associated vector (AAV). In some embodiments, the viral vector is AAV8. In some embodiments, the composition comprises a viral vector encapsulating a nucleic acid sequence encoding a shRNA. In some embodiments, the viral vector is a hepatocyte-targeted AAV. In some embodiments, the composition reduces or inhibits *METRN*, *STAT3*, or *Kit* expression

when compared to untreated subjects or to expression level of *METRN*, *STAT3*, or *Kit* in the subject pre-treatment. In some embodiments, *METRN*, *STAT3*, or *Kit* expression is reduced or inhibited in liver cells of the subject. In various embodiments, the shRNA comprises a nucleic acid sequence at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identical to the nucleic acid sequence as provided in Table 1. In some embodiments, the shRNA consists of a nucleic acid sequence of Table 1. The expression of *METRN*, *Kit* and/or *Stat3* may be reduced by at least about 5% to about 95%, e.g., about 5%, 10%, 15%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, relative to corresponding expression of *METRN*, *Kit* and/or *Stat3* in a subject suffering from MASH. The expression of *CASP8*, *METRN*, *Kit* and/or *Stat3* may be silenced relative to corresponding expression of *CASP8*, *METRN*, *Kit* and/or *Stat3* in a subject suffering from MASH.

[0097] In some embodiments, the composition comprises a guide RNA (gRNA) or a single-molecule guide RNA (sgRNA) comprising a spacer sequence that is complementary to a portion of a nucleic acid sequence encoding *CASP8*, *METRN*, *STAT3*, or *KIT*. In some embodiments, the composition comprises a viral vector comprising a nucleic acid sequence encoding a gRNA or sgRNA. In some embodiments, the viral vector is an adeno-associated vector (AAV). In some embodiments, the viral vector is AAV8. In some embodiments, the composition comprises a viral vector encapsulating a nucleic acid sequence encoding a gRNA or sgRNA. In some embodiments, the viral vector is a hepatocyte-targeted AAV.

[0098] In some embodiments, the gRNA or the sgRNA is pre-complexed with a DNA endonuclease. In some embodiments, the DNA endonuclease is a Cas9 endonuclease.

[0099] In some embodiments, the composition reduces or inhibits expression of one or more of *CASP8*, *METRN*, *STAT3*, *Kit* when compared to untreated subjects or to expression level of *CASP8*, *METRN*, *STAT3*, or *Kit* in the subject pre-treatment. In some embodiments, *METRN*, *STAT3*, or *Kit* expression is reduced or inhibited in liver cells of the subject. The expression of *CASP8*, *METRN*, *Kit* and/or *Stat3* may be reduced by at least about 5% to about 95%, e.g., about 5%, 10%, 15%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, relative to corresponding expression of *CASP8*, *METRN*, *Kit* and/or *Stat3* in a subject suffering from MASH. The expression of *CASP8*, *METRN*, *Kit* and/or *Stat3* may

be silenced relative to corresponding expression of CASP8, METRN, Kit and/or Stat3 in a subject suffering from MASH.

[0100] In some embodiments, the subject is a mammal. In some embodiments, the mammal is a human. In some embodiments, the composition is delivered systemically.

[0101] In some embodiments, a population of cells can be contacted with a compound or agent which, for example, includes subjecting the cells to an appropriate culture media which comprises the indicated compound or agent. Where the cell population is in vivo, contacting the cell population includes administering the compound or agent in a pharmaceutical composition to a subject via an appropriate administration route such that the compound or agent contacts the cell population in vivo.

[0102] For in vivo methods, a therapeutically effective amount of a compound described herein can be administered to a subject. Methods of administering compounds to a subject are known in the art and easily available to one of skill in the art.

[0103] As described herein, the methods of treatment described herein refer generally to obtaining a desired pharmacological and/or physiological effect. The effect may be prophylactic in terms of completely or partially preventing a disease or symptom thereof and/or may be therapeutic in terms of a partial or complete stabilization or cure for a disease and/or adverse effect attributable to the disease. Methods described herein covers any treatment of a disease in a subject, and includes: (a) preventing the disease or symptom from occurring in a subject which may be predisposed to the disease or symptom, may or may not be diagnosed as having it; (b) inhibiting the disease symptom, i.e., arresting its development; or (c) relieving the disease symptom, i.e., causing regression of the disease or symptom.

[0104] A therapeutically effective amount of an agent or composition disclosed herein, for example, is one that is effective for preventing, ameliorating, treating or delaying the onset of a disease or condition.

[0105] In various embodiments, the present application discloses methods for treating or preventing MASH in a subject in need thereof, comprising administering to said subject a composition that reduces either CASP8 or METRN expression in hepatocytes. In some embodiments, the CASP8 or METRN expression level is reduced in the subject as compared

to an untreated subject suffering from MASH. In various embodiments, the present application discloses methods for treating or preventing MASH in a subject in need thereof, comprising administering to said subject a composition that reduces either CASP8 or METRN expression compared to the CASP8 or METRN expression before administration of the composition. In various embodiments, the present application discloses methods for treating or preventing MASH in a subject in need thereof, comprising administering to said subject a composition that reduces either Kit or signal transducer and activator of transcription 3 (Stat3) expression. In some embodiments, the Kit or Stat3 expression level is reduced in the subject as compared to an untreated subject suffering from MASH. In various embodiments, the present application discloses methods for treating or preventing MASH in a subject in need thereof, comprising administering to said subject a composition that reduces either Kit or Stat3 expression compared to the Kit or Stat3 expression before administration of the composition.

[0106] In some embodiments, the present application discloses methods for treating or preventing MASH in a subject in need thereof, comprising administering a composition that reduces hepatic stellate cell activation by reducing CASP8 or METRN expression in hepatocytes. In some embodiments, the present application discloses methods for treating or preventing MASH in a subject in need thereof, comprising administering a composition that reduces hepatic stellate cell activation by reducing Kit or Stat3. CASP8, METRN, Kit or Stat3 expression may be reduced using any known method in the art. For example, in various embodiments the composition comprises at least one of the siRNA designs of Table 1. In various embodiments, the siRNA comprises a nucleic acid sequence at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identical to the siRNA designs listed in Table 1. In some embodiments, the siRNA consists of a siRNA nucleic acid sequence of Table 1. In various embodiments, the present application discloses a composition comprising CASP8 shRNA. In various embodiments, the present application discloses a composition comprising METRN shRNA. In various embodiments, the present application discloses a composition comprising Kit shRNA. In various embodiments, the present application discloses a composition comprising Stat3 shRNA. For example, in various embodiments the composition is a vector encoding a shRNA wherein the shRNA comprises a nucleic acid sequence encoding the nucleic acid sequences provided in Table 1. In various embodiments, the shRNA comprises a nucleic acid sequence at least 75%, 80%, 85%, 90%, 95%, 96%, 97%,

98% or 99% identical to the nucleic acid sequence as provided in Table 1. In some embodiments, the shRNA consists of a nucleic acid sequence of Table 1. In various embodiments, the vector is a viral vector containing a nucleic acid encoding a CASP8 or METRN short-hairpin RNA (shRNA). In various embodiments, the viral vector is an AAV vector. In various embodiments, the viral vector is a vector that preferentially targets the liver or liver cells. In various embodiments, the AAV is AAV 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 or variant thereof. In various embodiments, the AAV is AAV8 or variant thereof. In some embodiments, the AAV, including the AAV8, is a hepatocyte-targeted AAV. In some embodiments, the composition comprises hepatocyte-targeted AAV8 containing a nucleic acid encoding CASP8 or METRN short-hairpin RNA (shRNA). In various embodiments the subject is a mammal. In various embodiments, the mammal is a human. In various embodiments, the human has increased expression of METRN compared to a human not suffering from MASH. In various embodiments the composition comprises an RNA nanoparticle. In various embodiments, the RNA nanoparticle comprises CASP8 siRNA. In various embodiments, the RNA nanoparticle comprises METRN siRNA. In various embodiments, the RNA nanoparticle comprises Kit siRNA. In various embodiments, the RNA nanoparticle comprises Stat3 siRNA.

[0107] In some embodiments, a subject has increased expression of METRN compared to a subject suffering from MASH. For example, expression of METRN of a subject may be increased by at least 1.5 times greater than expression of METRN of a subject not suffering from MASH. Expression of METRN of a subject may be increased by at least 3 times greater than expression of METRN of a subject not suffering from MASH. Expression of METRN of a subject may be increased by at least 5 times greater than expression of METRN of a subject not suffering from MASH. Expression of METRN of a subject may be increased by at least 10 times greater than expression of METRN of a subject not suffering from MASH.

[0108] In various embodiments, the present application discloses methods for treating or preventing MASH in a subject in need thereof, comprising administering a composition that reduces hepatic stellate cell activation by reducing CASP8, METRN, Kit3 and/or Stat3 expression. The expression of CASP8, METRN, Kit and/or Stat3 may be reduced by at least about 5% to about 95%, e.g., about 5%, 10%, 15%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, relative to corresponding expression of CASP8, METRN, Kit and/or Stat3 in a subject suffering from MASH. The expression of CASP8, METRN, Kit and/or Stat3 may be

silenced relative to corresponding expression of CASP8, METRN, Kit and/or Stat3 in a subject suffering from MASH. In some embodiments, the expression of CASP8, METRN, Kit and/or Stat3 is reduced in liver cells of the subject.

[0109] In various embodiments, the present application discloses methods for treating or preventing MASH in a subject in need thereof, comprising administering to said patient a composition that targets the CASP8 through a non-apoptotic pathway in MASH. In various embodiments, the composition targets CASP8 or METRN in hepatocytes. In various embodiments, the method involves administering a composition that reduces expression of CASP8 or METRN. CASP8 or METRN expressions may be reduced using any known method in the art. For example, in various embodiments the composition comprises at least one of siRNA designs of Table 1. In various embodiments, the present application discloses a composition comprising CASP8 shRNA. In various embodiments, the present application discloses a composition comprising METRN shRNA. In various embodiments, the present application discloses a composition comprising Kit shRNA. In various embodiments, the present application discloses a composition comprising Stat3 shRNA. For example, in various embodiments the composition is a vector encoding a shRNA wherein the shRNA comprises a nucleic acid sequence encoding the nucleic acid sequences provided in Table 1. In various embodiments, the shRNA comprises a nucleic acid sequence at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identical to the nucleic acid sequence as provided in Table 1. In some embodiments, the shRNA consists of a nucleic acid sequence of Table 1. In various embodiments, the vector is a viral vector containing a nucleic acid encoding a CASP8 or METERN short-hairpin RNA (shRNA). In various embodiments, the viral vector is an AAV vector. In various embodiments, the viral vector is a vector that preferentially targets the liver or liver cells. In various embodiments the AAV is AAV 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 or variants thereof. In various embodiments, the AAV is AAV8 or variant thereof. In some embodiments, the AAV, including the AAV8, is a hepatocyte-targeted AAV. In some embodiments, the composition comprises hepatocyte-targeted AAV8 containing a nucleic acid encoding CASP8 or METERN short-hairpin RNA (shRNA). In various embodiments, the subject is a mammal. In various embodiments, the mammal is a human. In various embodiments, the human has increased expression of METRN compared to a human not suffering from MASH.

Table 1

Targeting Gene	siRNA sequence
CASP8	rGrGrArArCrCrUrGrGrUrArUrArUrUrCrArGrUrCrArCrUTT
	rGrUrCrArUrGrCrUrCrUrArUrCrArGrArUrUrUrCrArGrAAG
METRN	rGrArGrCrUrGrCrArArGrArArUrCrArGrUrCrArUrCrArCTG
	rGrGrUrGrArUrGrGrGrArCrUrArUrCrArArUrArArGrArACT
Kit	rGrUrCrCrArArCrUrGrUrUrGrArCrArGrUrUrCrUrGrArAGA
Stat3	rGrCrArCrUrUrUrCrArArCrCrUrUrGrCrUrArArUrArUrCCA

[0110] In some embodiment, targeted gene expression can be reduced by several genome editing techniques such as RNAi (RNA interference), zinc finger nucleases (ZFNs), a TALE-effector domain nuclease (TALLEN), prime editing and base editing, CRISPR/Cas9 systems which are known in the art. In some embodiment, the CRISPR/Cas9 systems comprise a guide RNA (gRNA) or a single-molecule guide RNA (sgRNA). In some embodiment, the gRNA or sgRNA comprises a spacer sequence that is complementary to a portion of a nucleic acid sequence encoding CASP8. In some embodiment, the gRNA or sgRNA comprises a spacer sequence that is complementary to a portion of a nucleic acid sequence encoding METRN. In some embodiment, the gRNA or sgRNA comprises a spacer sequence that is complementary to a portion of a nucleic acid sequence encoding Kit. In some embodiment, the gRNA or sgRNA comprises a spacer sequence that is complementary to a portion of a nucleic acid sequence encoding Stat3.

[0111] Inhibition of RNA encoding CASP8, METRN, Kit and/or Stat3 can effectively modulate the expression of these proteins. Inhibitors can include shRNAs encoding siRNAs, siRNA; interfering RNA or RNAi; dsRNA; RNA Polymerase III transcribed DNAs; ribozymes; GalNac-siRNA; GalNac-Antisense Oligonucleotide (ASO) and antisense nucleic acids, which can be RNA, DNA, or an artificial nucleic acid.

[0112] Antisense oligonucleotides, including antisense DNA, RNA, and DNA/RNA molecules, act to directly block the translation of mRNA by binding to targeted mRNA and

preventing protein translation. For example, antisense oligonucleotides of at least about 15 bases and complementary to unique regions of the DNA sequence encoding an EGFR fusion molecule can be synthesized, e.g., by conventional phosphodiester techniques. Antisense nucleotide sequences include, but are not limited to: morpholinos, 2'-O-methyl polynucleotides, DNA, RNA and the like.

[0113] siRNA comprises a double stranded structure containing from about 15 to about 50 base pairs, for example from about 21 to about 25 base pairs, and having a nucleotide sequence identical or nearly identical to an expressed target gene or RNA within the cell. The siRNA comprise a sense RNA strand and a complementary antisense RNA strand annealed together by standard Watson-Crick base-pairing interactions. The sense strand comprises a nucleic acid sequence which is substantially identical to a nucleic acid sequence contained within the target miRNA molecule. "Substantially identical" to a target sequence contained within the target mRNA refers to a nucleic acid sequence that differs from the target sequence by about 3% or less. The sense and antisense strands of the siRNA can comprise two complementary, single-stranded RNA molecules, or can comprise a single molecule in which two complementary portions are base-paired and are covalently linked by a single-stranded "hairpin" area.

[0114] The siRNA can be altered RNA that differs from naturally-occurring RNA by the addition, deletion, substitution and/or alteration of one or more nucleotides. Such alterations can include addition of non-nucleotide material, such as to the end(s) of the siRNA or to one or more internal nucleotides of the siRNA, or modifications that make the siRNA resistant to nuclease digestion, or the substitution of one or more nucleotides in the siRNA with deoxyribo-nucleotides. One or both strands of the siRNA can also comprise a 3' overhang. As used herein, a 3' overhang refers to at least one unpaired nucleotide extending from the 3'-end of a duplexed RNA strand. For example, the siRNA can comprise at least one 3' overhang of from 1 to about 6 nucleotides (which includes ribonucleotides or deoxyribonucleotides) in length, or from 1 to about 5 nucleotides in length, or from 1 to about 4 nucleotides in length, or from about 2 to about 4 nucleotides in length. For example, each strand of the siRNA can comprise 3' overhangs of dithymidylic acid ("TT") or diuridylic acid ("uu").

[0115] siRNA can be produced chemically or biologically, or can be expressed from a recombinant plasmid or viral vector. Methods for producing and testing dsRNA or siRNA molecules are known in the art. A short hairpin RNA (shRNA) encodes an RNA molecule with a tight hairpin turn that can be used to silence target gene expression via RNA interference (RNAi). Expression of shRNA in cells is typically accomplished by delivery of plasmids or through viral or bacterial vectors.

[0116] RNA polymerase III transcribed DNAs contain promoters, such as the U6 promoter. These DNAs can be transcribed to produce small hairpin RNAs in the cell that can function as siRNA or linear RNAs, which can function as antisense RNA. The CASP8, METRN, Kit and/or Stat3 inhibitor can comprise ribonucleotides, deoxyribonucleotides, synthetic nucleotides, or any suitable combination such that the target RNA and/or gene is inhibited. In addition, these forms of nucleic acid can be single, double, triple, or quadruple stranded.

Pharmaceutical compositions

[0117] In certain aspects, described herein is a composition for treating or preventing MASH comprising a hepatocyte-targeted nucleic acid targeting the caspase 8 (CASP8) pathway. In some embodiments, the composition comprises a viral vector comprising the nucleic acid. In some embodiments, the composition comprises a viral vector encapsulating the nucleic acid. In various embodiments, the viral vector is a vector that preferentially targets the liver or liver cells. In some embodiments, the viral vector is an AAV vector. In various embodiments, the AAV is AAV 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 or variant thereof. In some embodiments, the viral vector is AAV8. In some embodiments, the hepatocyte-targeted nucleic acid comprises N- acetyl galactosamine (GalNac). In some embodiments, the viral vector is a hepatocyte-targeted AAV.

[0118] In some embodiments, the composition reduces or inhibits expression of one or more of CASP8, METRN, STAT3, or Kit. In some embodiments, the expression of one or more of CASP8, METRN, STAT3, or Kit is reduced or inhibited in liver cells of the subject. The expression of CASP8, METRN, Kit and/or Stat3 may be reduced by at least about 5% to about 95%, e.g., about 5%, 10%, 15%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, relative to corresponding expression of CASP8, METRN, Kit and/or Stat3 in

a subject suffering from MASH. The expression of CASP8, METRN, Kit and/or Stat3 may be silenced relative to corresponding expression of CASP8, METRN, Kit and/or Stat3 in a subject suffering from MASH.

[0119] In some embodiments, composition comprises a guide ribonucleic acid (gRNA) or a single-molecule guide RNA (sgRNA) comprising a spacer sequence that is complementary to a portion of a nucleic acid sequence encoding CASP8, METRN, STAT3, or Kit.

[0120] In some embodiments, the gRNA or the sgRNA is pre-complexed with a DNA endonuclease. In some embodiments, the DNA endonuclease is a Cas9 endonuclease.

[0121] In some embodiments, the composition comprises at least one of the small interfering ribonucleic acid (siRNA) sequences from Table 1. In various embodiments, the siRNA is at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identical to the siRNA designs listed in Table 1. In some embodiments, the siRNA consists of a siRNA nucleic acid sequence of Table 1.

[0122] In some embodiments, the hepatocyte-targeted nucleic acid comprises any one of the small interfering ribonucleic acid (siRNA) sequences from Table 1. In various embodiments, the siRNA is at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identical to the siRNA designs listed in Table 1. In some embodiments, the siRNA consists of a siRNA nucleic acid sequence of Table 1.

[0123] In some embodiments, the composition comprises an expression vector capable of targeting the caspase 8 (CASP8) pathway. In some embodiments, the expression vector capable of targeting the caspase 8 (CASP8) pathway encodes a CASP8, METERN, STAT3, or Kit short-hairpin RNA (shRNA).

[0124] The pharmaceutical compositions of the inventions can be administered to any animal that can experience the beneficial effects of the agents of the invention. Such animals include humans and non-humans such as primates, pets and farm animals.

[0125] The present invention also comprises pharmaceutical compositions comprising the agents disclosed herein. Routes of administration and dosages of effective amounts of the pharmaceutical compositions comprising the agents are also disclosed. The agents of the

present invention can be administered in combination with other pharmaceutical agents in a variety of protocols for effective treatment of disease.

[0126] Pharmaceutical compositions of the present invention are administered to a subject in a manner known in the art. The dosage administered will be dependent upon the age, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired. One may administer the viral vectors, siRNA, RNAi, shRNA or other inhibitors, or related compound in a local rather than systemic manner, for example, via injection of directly into the desired target site, often in a depot or sustained release formulation. Furthermore, one may administer the composition in a targeted drug delivery system, for example, in a liposome coated with a tissue-specific antibody, targeting, for example, the liver, and more specifically hepatocytes. The liposomes will be targeted to and taken up selectively by the desired tissue. Also included in a targeted drug delivery system is nanoparticle specific liver delivery of the viral vectors, siRNA, RNAi, shRNA or other inhibitors, or compound, alone or in combination with similar compounds. A summary of various delivery methods and techniques of siRNA administration in ongoing clinical trials is provided in Zuckerman and Davis 2015; Nature Rev. Drug Discovery, Vol. 14: 843-856, Dec. 2015 the contents of which is hereby incorporated by reference in its entirety.

[0127] One of ordinary skill in the art will appreciate that a method of administering pharmaceutically effective amounts of the pharmaceutical compositions of the invention to a patient in need thereof, can be determined empirically, or by standards currently recognized in the medical arts. The agents can be administered to a patient as pharmaceutical compositions in combination with one or more pharmaceutically acceptable excipients. It will be understood that, when administered to a human patient, the total daily usage of the agents of the pharmaceutical compositions of the present invention will be decided within the scope of sound medical judgment by the attending physician. The specific therapeutically effective dose level for any particular patient will depend upon a variety of factors: the type and degree of the cellular response to be achieved; activity of the specific agent or composition employed; the specific agents or composition employed; the age, body weight, general health, gender and diet of the patient; the time of administration, route of administration, and rate of excretion of the agent; the duration of the treatment; drugs used in combination or coincidental with the specific agent; and like factors well known in the

medical arts. It is well within the skill of the art to start doses of the agents at levels lower than those required to achieve the desired therapeutic effect and to gradually increase the dosages until the desired effect is achieved.

[0128] Dosaging can also be administered in a patient-specific manner to provide a predetermined concentration of the agents in the blood, as determined by techniques accepted and routine in the art.

[0129] In various embodiments, the present application discloses compositions for regulating the CASP8 through a non-apoptotic pathway in MASH, in particular a composition that inhibits METRN expression or function. In various embodiments, the present application discloses a composition that reduces CASP8 expression or function, in particular inhibition of METRN expression or function. In various embodiments, the present application discloses a composition that inhibits METRN expression or function. In various embodiments, the present application discloses a composition that inhibits Kit expression or function. In various embodiments, the present application discloses a composition that inhibits signal transducer and activator of transcription 3 (Stat3) expression or function. In various embodiments, the present application discloses a composition comprising CASP8 siRNA. In various embodiments, the present application discloses a composition comprising METRN siRNA. In various embodiments, the present application discloses a composition comprising Kit siRNA. In various embodiments, the present application discloses a composition comprising Stat3 siRNA. In various embodiments, the siRNA is at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identical to the siRNA designs listed in Table 1. In some embodiments, the siRNA consists of a siRNA nucleic acid sequence of Table 1. In various embodiments, the present application discloses a composition comprising CASP8 shRNA. In various embodiments, the present application discloses a composition comprising METRN shRNA. In various embodiments, the present application discloses a composition comprising Kit shRNA. In various embodiments, the present application discloses a composition comprising Stat3 shRNA. For example, in various embodiments the composition is a vector encoding a shRNA wherein the shRNA comprises a nucleic acid sequence encoding the nucleic acid sequences provided in Table 1. In various embodiments, the shRNA comprises a nucleic acid sequence at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identical to the nucleic acid sequence as provided in Table 1. In some embodiments, the shRNA consists of a nucleic acid sequence of Table 1. In various

embodiments, the vector is a viral vector comprising a nucleic acid encoding a CASP8 or METERN short-hairpin RNA (shRNA). In various embodiments, the viral vector is an AAV vector. In various embodiments, the viral vector is a vector that preferentially targets the liver or liver cells. In various embodiments the AAV is AAV 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 or variants thereof. In various embodiments, the AAV is AAV8 or a variant thereof. In some embodiments, the AAV, including the AAV8, is a hepatocyte-targeted AAV. In some embodiments, the composition comprises hepatocyte-targeted AAV8 comprising a nucleic acid encoding CASP8 or METERN short-hairpin RNA (shRNA).

[0130] In certain aspects, described herein is a nucleic acid comprising a sequence of any one of the small interfering ribonucleic acid (siRNA) designs from Table 1. In certain aspects, described herein is a nucleic acid consisting of a sequence of any one of the small interfering ribonucleic acid (siRNA) designs from Table 1.

[0131] In certain aspects, described herein is a nucleic acid comprising a sequence encoding a short-hairpin RNA (shRNA), wherein the shRNA comprises a nucleic acid sequence provided in Table 1. In certain aspects, described herein is a nucleic acid comprising a sequence encoding a short-hairpin RNA (shRNA), wherein the shRNA consists of a nucleic acid sequence provided in Table 1.

[0132] In some embodiments, the nucleic acid comprises a hepatocyte-targeting motif. In some embodiments, the hepatocyte-targeting motif is N-acetyl galactosamine (GalNac).

[0133] In certain aspects, described herein is a vector comprising the nucleic acid sequence disclosed above.

[0134] In certain aspects, described herein is a viral vector comprising the nucleic acid disclosed above. In certain aspects, described herein is a viral vector encapsulating the nucleic acid disclosed above. In some embodiments, the viral vector is an AAV vector. In some embodiments, the AAV vector is AAV8. In some embodiments, the viral vector is a hepatocyte-targeted AAV.

Methods of diagnosis

[0135] In certain aspects, described herein is a method of diagnosing metabolic dysfunction-associated steatohepatitis (MASH) in a subject comprising: determining the level

of meteorin (METRN) in a sample from the subject; and diagnosing the subject with MASH if the level of METRN expression in the sample is increased compared to a sample from a healthy subject.

[0136] In some embodiments, the subject is diagnosed with fibrotic MASH.

[0137] In some embodiments, the sample is blood or blood plasma or liver cells.

[0138] In some embodiments, the level of METRN is determined using an antibody. In some embodiments, the level of METRN is determined by ELISA. In some embodiments, the level of mRNA is determined using RT-qPCR or RNA-seq.

[0139] In some embodiments, the invention comprises detecting in a biological sample whether there is an increase in an mRNA encoding METRN. Methods for detecting and quantifying METRN molecules in biological samples are known the art. For example, protocols for detecting and measuring a METRN protein molecule using either polyclonal or monoclonal antibodies specific for the polypeptide are well established. Non-limiting examples include Western blot, enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), and fluorescence activated cell sorting (FACS).

[0140] In one aspect, the invention provides a device for determining whether a sample from a subject contains METRN protein, or a combination thereof, the device comprising at least one antibody that specifically binds to METRN protein, or a fragment thereof. In another aspect, the invention provides a device for determining whether a sample from a subject contains METRN nucleic acid, or a combination thereof, the device comprising at least one primer, primer pair, or nucleic acid probe, that specifically binds to METRN nucleic acid, or a fragment thereof.

[0141] In one embodiment, a biological sample comprises, a blood sample, serum, cells (including whole cells, cell fractions, cell extracts, and cultured cells or cell lines), tissues (including tissues obtained by biopsy), body fluids (e.g., urine, sputum, amniotic fluid, synovial fluid), or from media (from cultured cells or cell lines). In one embodiment, a biological sample comprises, liver cells. The methods of detecting or quantifying a METRN molecule include, but are not limited to, amplification-based assays with (signal amplification) hybridization based assays and combination amplification-hybridization

assays. For detecting and quantifying a METRN molecule, an exemplary method is an immunoassay that utilizes an antibody or other binding agents that specifically bind to an METRN protein or epitope of such, for example, Western blot or ELISA assays. In some embodiments, the level of mRNA is determined using RT-qPCR or RNA-seq.

EXAMPLES

[0142] Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to one of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims. The following examples are provided by way of illustration only and not by way of limitation. Those of skill in the art will readily recognize a variety of noncritical parameters that could be changed or modified to yield similar results.

Example 1 – Deletion of hepatocyte CASP8 in vivo lowers liver fibrosis

[0143] CASP8 inhibitors fail in MASH clinical trials, but CASP8 knockout mice show a protective effect in mouse MASH models. Without being bound by theory, whether CASP8 induces liver fibrosis through a non-apoptotic pathway in MASH is tested.

[0144] **FIGS. 1A-H** show deletion of hepatocyte CASP8 in vivo lowers liver fibrosis. Male Casp8^{f/f} mice were fed a diet with high fructose, palmitate, and cholesterol (FPC) for 8 weeks to induce steatosis. The mice were then injected with adeno-associated viral (AAV) vector 8-TBG-Cre or AAV8-TBG-LacZ control and continued on the FPC diet for an additional 8 wks. **FIG. 1A** shows the experimental scheme. **FIG. 1B** shows deletion of hepatocyte CASP8 in MASH mice does not affect body weight and liver/body ratio. **FIG. 1C** shows the Casp8 is deleted by immunoblot in the livers of AAV8-TBG-Cre vs. AAV8-TBG-LacZ treated mice. **FIG. 1D** shows histochemical H & E stains and histochemical Sirius red stains of liver sections, and the bar charts indicating inflammatory cells and Sirius red % area are decrease in AAV8-TBG-Cre treated livers. **FIG. 1E** shows immunohistochemistry α -smooth muscle actin (α SMA) stains of liver sections and a bar chart indicating decreased α SMA % area in hepatocyte-Casp8 knockout livers. **FIG. 1F** is bar charts showing decreased mRNA markers of hepatic stellate cell activation for AAV8-TBG-Cre-treated mice compared with AAV8-TBG-LacZ controls. **FIG. 1G** shows immunohistochemistry F4/80 stains of liver

sections and a bar chart indicating decreased F4/80 % area in hepatocyte-Casp8 knockout livers. **FIG. 1H** shows immunohistochemistry cl-Casp3 or TUNEL stain of liver sections and the bar charts indicating cl-Casp3 % cells and TUNEL % cells. These data indicate that deletion of hepatocyte CASP8 lower liver fibrosis and liver inflammation without altering cell death, and suggest CASP8 promotes liver fibrosis through a non-apoptotic role in MASH. Liver fibrosis is due to hepatic stellate cell activation, and CASP8 was deleted in hepatocytes in **FIG. 1**.

Example 2 — Method of activating a hepatic-stellate cell (HSC) through a non-cell death pathway

[0145] Described herein is a method of activating a hepatic-stellate cell (HSC) through a non-cell death pathway. The method uses a hepatocyte-stellate cell co-culture system in vitro. Culture medium from a hepatocyte is collected and transferred to a quiescent HSC. Then, the HSC is harvested to determine the activity by measuring fibrotic gene expression. This method avoids cell-cell direct interaction and has the advantage of studying a hepatocyte-secreted protein that activates hepatic stellate cells.

[0146] **FIG. 2** shows deletion of Casp8 in hepatocytes lowers hepatic stellate cells (HSC) activation by conditioned medium (CM) from the hepatocytes. **FIG. 2A** shows that AML12 mouse hepatocytes were transfected with scrambled RNA (Scr) or siCasp8. After 24 hours (h), the medium was changed to DMEM/0.1% FBS. After another 24 h, CM was transferred to quiescent HSCs. After 72 h, the HSCs were assayed for *Timp1*, *Spp1*, and *Colla1* mRNA and showed significantly decreased in siCasp8-CM transferred HSCs. Mean $\pm$ SEM; n=4; *p < 0.05. *Inset*, Casp8 immunoblot. **FIG. 2B** shows that THLE2 human hepatocytes were transfected with scrambled RNA (Scr) or siCasp8. After 24 h, the medium was changed to DMEM/0.1% FBS. After another 24 h, CASP8 mRNA was measured in THLE2 cells (left), and CM from THLE2 cells was transferred to quiescent HSCs. After 72 h, the HSCs were assayed for *Timp1*, *Spp1*, and *Colla1* mRNA (right) and showed significantly decreased in siCASP8-CM transferred HSCs. Mean $\pm$ SEM; n=4; *p < 0.05. These data suggest that hepatocyte-secreted molecules promote hepatic stellate activation.

Example 3 – Characterization of molecules in the CM

[0147] To characterize the molecules in the CM, a heat-inactivation assay was performed in **FIG. 3**, which will inactivate all proteins in CM.

[0148] **FIG. 3** shows heat-inactivation of hepatocyte (HC) conditioned medium (CM) blocks hepatic stellate cell (HSC) activation and negates the difference between Scr-HC and Casp8-silenced-HC CM. Control or heat-inactivated (HI; 100°C x 5 min) CM from Scr- or siCasp8-treated HCs was added to HSCs as in **FIG. 2A**. After 72 h, the HSCs were assayed for the indicated mRNAs. Mean $\pm$ SEM; n=4; *p < 0.05; ns, non-significant. There are no difference about HSC genes (3rd and 4th bars) between Scr- and siCasp8-CM treated HSCs after heat inactivation. These data suggest that the molecules in the CM are proteins that contribute to HSC activation.

Example 4 – Identifying proteins which promote HSC gene expression in the CM

[0149] To identify the proteins which promote HSC gene expression in the CM, a RNAseq and LC-MS/MS experiment was combined to screen the candidates.

[0150] **FIG. 4** shows RNAseq and LC-MS/MS data indicating that *Metrn* is downregulated in Casp8-silenced AML12 hepatocytes and culture medium. **FIG. 4A** shows the screening scheme. **FIG. 4B** shows 11 candidates were selected from the overlap of the RNAseq and LC-MS/MS data. **FIG. 4C** shows qPCR verification of the candidate genes from (B) in Casp8 hepatocyte-KO livers. Mean $\pm$ SEM; n=5 mice; *p < 0.05; **p < 0.01. **FIG. 4D** shows *Metrn* protein was decreased in the samples from **FIG. 1C** and the quantification result. Mean $\pm$ SEM; n=4; ***p < 0.001. It suggests a candidate gene-*Metrn* after bioinformatics analysis of the screening data.

Example 5 – Exploring METRN expression in experimental and human MASH to study Metern function in MASH

[0151] METRN expression was explored in experimental and human MASH to study Metern function in MASH.

[0152] **FIG. 5** shows *Metrn* expression is increased in human and mouse MASH livers. **FIG. 5A** shows *METRN* mRNA and protein were increased MASH human livers. **FIG. 5B**

shows *METRN* mRNA is increased based on RNAseq GEO dataset 126848, comparing livers from healthy and non-alcoholic fatty liver disease (NAFLD) subjects. **FIG. 5C** shows *METRN* mRNA expression in liver single cells (normal liver) from the Human Protein Atlas database. The data indicates *METRN* expresses in hepatocytes. **FIG. 5D-E** shows Meteorin protein was increased in the livers of mice fed chow or the MASH-inducing FPC diet for 16 wks (D), HF-CDAA diet for 8 wks (E). For (A) and (B), mean $\pm$ SEM; * $p < 0.05$. These data suggest *METRN* is increased in human and mouse MASH livers, and *METRN* expresses in hepatocytes.

Example 6 – Blocking CASP8 expression in hepatocyte to study whether METRN is the downstream gene of CASP8

[0153] CASP8 expression was blocked in hepatocytes to study whether *METRN* is the downstream gene of CASP8 in **FIG. 6**.

[0154] **FIG. 6** shows siCasp8 decreases *Metrn* expression in mouse and human hepatocytes. **FIG. 6A** shows Scr- and siCasp8-treated AML12 cells were assayed for Caspase 8 (Casp8) and Meteorin (*Metrn*) by immunoblot. **FIG. 6B** shows Scr- and siCasp8-treated THLE2 cells were assayed for *Casp8* and *Metrn* mRNA and protein. **FIG. 6C** shows Scr- and siCasp8-treated primary human hepatocytes were assayed for *Casp8* and *Metrn* mRNA and protein. Mean $\pm$ SEM; $n=3$; * $p < 0.05$; ***, $P < 0.001$. *METRN* is decreased in mouse hepatocyte cell line, human hepatocyte cell line, and primary cultured human hepatocytes. These data suggest *METRN* is positively regulated by CASP8 in hepatocytes.

Example 7 – Examining fibrotic gene expression to study METRN function on HSC

[0155] HSC was incubated with r*METRN* and fibrotic gene expression was checked to study *METRN* function on HSC in **FIG. 7**.

[0156] **FIG. 7** shows *METRN* induces pro-fibrotic gene expression in hepatic stellate cells (HSCs). Primary mouse HSCs were treated with 400 nM recombinant (r) Meteorin or vehicle for 48 h. The HSCs were then assayed for *Timp1*, *Colla1* and *Acta2* mRNA by qPCR. Mean $\pm$ SEM; $n=4$; * $p < 0.05$, ** $p < 0.01$. These data suggest *METRN* is able to induce fibrotic gene expression in hepatic stellate cells.

Example 8 – Rescue experiment in siCASP8-CM-treated HSCs

[0157] Rescue experiments were performed in siCasp8-CM-treated HSCs to examine whether METRN is sufficient to induce HSC activation.

[0158] **FIG. 8** shows HSC activation by the conditioned medium (CM) of Casp8-silenced AML 12 hepatocytes is enhanced by genetically restoring Metrn. **FIG. 8A** shows AML12 cells were transfected with scrambled (Scr) or siCasp8 siRNA and GFP or Metrn plasmid, as indicated. After 24 h, the medium was changed to DMEM/0.1% FBS. After another 24 h, Meteorin protein was measured in the AML12 cells. The data indicate overexpressed Metrn is similar with Src-treated group in hepatocytes. **FIG. 8B** shows CM from the AML12 cells in (A) was transferred to quiescent HSCs. After 72 h, the HSCs were assayed for *Timp1* and *Spp1* mRNA. Mean $\pm$ SEM; n=4; ***p < 0.001. The result indicates Metrn contained CM increase silencing Casp8-decreased HSC gene expression. These data suggest genetic restoration of Metrn is sufficient to induce HSC activation when CASP8 is inactivated.

Example 9 – Necessity of Metrn to induce HSC activation

[0159] METRN was blocked in hepatocytes and the CM was transferred to HSC to check whether Metrn is necessary to induce HCS activation and to examine HSC activity in FIG.9.

[0160] **FIG. 9** shows deletion of METRN in THLE-2 human hepatocytes lowers hepatic stellate cell (HSC) activation by THLE-2 conditioned medium (CM). **FIG. 9A** shows THLE2 cells were transfected with scrambled RNA (Scr) or siMetrn. After 24 h, the medium was changed to DMEM/0.1% FBS. After another 24 h, METRN mRNA and protein were measured in THLE2 cells. **FIG. 9B** shows CM from THLE2 cells from (A) was transferred to quiescent HSCs. After 72 h, the HSCs were assayed for *Timp1* and *Spp1*, mRNA. Mean $\pm$ SEM; n=4; *p < 0.05, ***p<0.001. HSC genes are down-regulated in silencing Metrn CM-treated HSCs. These results indicated that blocking METRN in hepatocytes also reduced HSC activity in the CM condition, and METRN is necessary to induce HSC activation.

Example 10 – Examining how Metrn induces HSC gene expression

[0161] How Metrn induces HSC gene expression in HSCs was explored. In view of the literature about METRN, another family protein of METRN, STAT3 signaling was tested.

[0162] **FIG. 10** shows METRN activates Stat3 in hepatic stellate cells (HSCs). **FIG. 10A** shows Primary mouse HSCs were treated with 400 ng/ml recombinant (r) Meteorin or vehicle for 48 h. The HSCs were then assayed for phospho (p-Y705) and total Stat3 by immunoblot. The result indicates that Metrn induces p-Stat3 in HSCs. **FIG. 10B** shows primary mouse HSCs were treated with 100 ng/ml recombinant (r) Meteorin, vehicle, or both Meteorin and Stat3 for 2 h. The HSCs were then assayed for phospho (p-Y705) Stat3. Metrn induced p-Stat3 is blocked by STAT3 inhibitor. These data suggest METRN activates HSC through the STAT3 pathway.

Example 11 – Identifying METRN receptor on the HSC cell surface

[0163] The identity of METRN receptor on the HSC cell surface was examined. According to a recent discovery in cardiomyocytes, C-Kit is a candidate. This was tested by genetic deletion of Kit in HSCs.

[0164] **FIG. 11** shows Hepatic stellate cell activation is reduced by silencing Kit/Stat3 in primary mouse hepatic stellate cells. **FIG. 11A** shows primary mouse HSCs were transfected with scrambled (Scr) or siKit/siStat3 siRNA as indicated. After 48 h, Kit/Stat3 mRNA was measured in the HSC cells. N=3-4. *P<0.05. **FIG. 11B** shows primary mouse HSCs were transfected with scrambled (Scr) or siKit/Stat3 siRNA, as indicated. After 24 h, the cells were treated with vehicle or rMetrn. After another 48 h, Timp1 and Spp1 mRNA was measured in the HSC cells. N=4. **P<0.01, ***P<0.001, ****P<0.0001. Both silencing Kit and Stat3 significantly blocked HSC gene expression. These data suggest METRN induces HSC gene expression through the Kit/Stat3 pathway. All of the data from **FIG. 2** to **FIG. 11** described how hepatocyte CASP8 activates HSC activation. Back to the original discovery in this invention, CASP8 promotes HSC independent of apoptotic function in vivo.

Example 12 – Requirement of METRN expression and HSC activation in caspase activity of CASP8 in vitro

[0165] The requirement of METRN expression and HSC activation in caspase activity of caspase 8 in vitro was examined. CASP8 inhibitors was used to block apoptosis and METRN and HSC gene expressions were checked simultaneously in **FIG. 12**.

[0166] **FIG. 12** shows Metrn expression in AML12 hepatocytes does not require the caspase activity of caspase 8. **FIG. 12A** shows AML12 cells were treated for 48 h with 20 mM of the pan-caspase inhibitor ZVAD or the caspase 8 inhibitor ITED, or DMSO vehicle control, and then Metrn mRNA was measured by qPCR. Both ITED and ZVAD did not alter CASP8 target gene-METRN expression. **FIG. 12B** shows AML12 mouse hepatocytes were treated with IETD or Veh in DMEM/0.1% FBS. After 24 h, the conditioned medium (CM) was transferred to quiescent HSCs. After 72 h, the HSCs were assayed for Timp1 and Col1a1 mRNA. IETD did not increase HSC gene expression in CM transfer experiment. **FIG. 12C** indicates as a positive control for the inhibitors, AML12 cells were treated for 24 h with or without 5 mg/ml Jo2 to induce apoptosis together with DMSO or 20 mM ZVAD or ITED. The active form of Casp3 (cl-Casp3), as a measure of pan-caspase or upstream caspase 8 activity, was then assayed by immunoblot. n.s., not significant. These data suggest METRN expression is independent of the caspase activity of CASP8.

Example 13 – Other genetic evidence

[0167] Other genetic evidence was provided using the CM transfer experiment in **FIG. 13**.

[0168] **FIG. 13** shows the protective effect of silencing hepatocyte Casp8 on hepatic stellate cell activation was lost when hepatocytes were transfected with the CASP8 C360A mutant. AML12 cells were transfected with scrambled (Scr) or siCasp8 siRNA and GFP, Casp8 WT or C360A protease inactive mutation plasmid, as indicated. After 24 h, the medium was changed to DMEM/0.1% FBS. After another 24 h, the conditioned medium (CM) from hepatocytes was transferred to quiescent HSCs. After 72 h, the HSCs were assayed for Col1a1 and Timp1 mRNA. In the graph, both CASP8 WT and CASP8 C360A mutation increased siCasp8 decreased HSC gene expression. These data also suggest that CASP8-induced HSC activation is independent of its protease activity.

[0169] In summary, CASP8 promotes liver fibrosis through the METRN-KIT-STAT3 pathway in MASH. This pathway is relevant to human MASH, and METRN is a plausible therapeutic target using a hepatocyte-targeted GalNAc-small interfering RNA platform.

Example 14- Deletion of hepatocyte CASP8 in mice with established diet-induced hepatosteatosis suppresses the progression to early MASH fibrosis without blocking hepatocyte apoptosis

[0170] A previous human genome-wide association study (GWAS) study documented a link between *CASP8* loci and disease progression (Vujkovic et al., A multi-ancestry genome-wide association study of unexplained chronic ALT elevation as a proxy for nonalcoholic fatty liver disease with histological and radiological validation. *Nat Genet.* 2022 Jun;54(6):761-771. PMID: 35654975). Although directionality could not be assessed from this study, germline deletion of hepatocyte caspase-8 in a weight-loss model of fatty liver injury in mice decreased liver steatosis, cell death, inflammation, and oxidative stress (Hatting M, Zhao G, Schumacher F, Sellge G, Al Masaoudi M, Gaßler N, Boekschoten M, Müller M, Liedtke C, Cubero FJ, Trautwein C. Hepatocyte caspase-8 is an essential modulator of steatohepatitis in rodents. *Hepatology.* 2013 Jun;57(6):2189-201. PMID: 23339067). To assess if higher *CASP8* expression is linked to worsening MASH in humans, an analysis of liver *CASP8* expression was conducted using datasets from the Gene Expression Omnibus. Notably, the average level of *CASP8* mRNA expression was significantly higher in the livers of individuals with histologically confirmed MASH compared with livers of lean, healthy control individuals (**FIG. 14A**). To complement these findings, it was shown here that the expression of full-length caspase-8 protein was elevated in the livers of individuals with MASH versus normal healthy control individuals (**FIG. 14B**). Furthermore, it was confirmed that liver *CASP8* is increased following the development of MASH in mice fed a human-relevant MASLD/MASH-inducing diet containing fructose, palmitate, and cholesterol (FPC) (PMID: 28068223) or a high-fat, choline-deficient L-amino acid-defined diet (HF-CDAA) (Wei G, An P, Vaid KA, Nasser I, Huang P, Tan L, Zhao S, Schuppan D, Popov YV. Comparison of murine steatohepatitis models identifies a dietary intervention with robust fibrosis, ductular reaction, and rapid progression to cirrhosis and cancer. *Am J Physiol Gastrointest Liver Physiol.* 2020 Jan 1;318(1):G174-G188. PMID: 31630534) (**FIGS. 14C-D**).

[0171] To determine if this increase in *CASP8* played a role in steatosis-to-MASH progression, AAV8-TBG-Cre was injected into *Casp8^{fl/fl}* mice after 8 weeks of FPC diet feeding to delete caspase-8 specifically in hepatocytes in mice with pre-MASH hepatosteatosis (Wang X, Zheng Z, Caviglia JM, Corey KE, Herfel TM, Cai B, Masia R,

Chung RT, Lefkowitz JH, Schwabe RF, Tabas I. Hepatocyte TAZ/WWTR1 Promotes Inflammation and Fibrosis in Nonalcoholic Steatohepatitis. *Cell Metab.* 2016 Dec 13;24(6):848-862. PMID: 28068223). The mice were then maintained on the FPC diet for an additional 8 weeks to allow for the development of MASH and early fibrosis (**FIG. 15A**). Successful caspase-8 knockout in the livers of the AAV8-TBG-Cre-treated mice was confirmed (**FIG. 15B**), and there were no significant changes in body weight, liver-to-body weight ratio, or FBG between the two groups (**FIG. 15C-E**). Analyses of Sirius red-positive area and monocytic cells in the livers of the two groups of mice revealed decreases in liver fibrosis and inflammation, respectively, in the livers of the knockout group (**FIG. 15F**). Immunofluorescence staining showed reduced α -smooth muscle actin (α -SMA)-positive area and F4/80-positive macrophages in the knockout livers, providing further evidence decreased fibrosis and inflammation, respectively (**FIG. 15G-H**). Furthermore, the livers of the knockout mice had lower expression of mRNAs associated with hepatic stellate cell (HSC) activation, fibrosis, and inflammation (**FIG. 15I**), and plasma ALT concentration was also lower in the knockout mice (**FIG. 15J**). Notably, the percentages of caspase-3-positive (apoptotic) and TUNEL-positive hepatocytes were not significantly different between the 2 groups of mice (**FIGS. 15K-L**), which raised the possibility hepatocyte caspase-8 promoted NASH progression via a non-apoptotic mechanism (below).

[0172] Turning to HF-CDAA-fed mice, where MASH development is more rapid and associated with a higher incidence of hepatocyte apoptosis compared with the FPC model (Wei G, An P, Vaid KA, Nasser I, Huang P, Tan L, Zhao S, Schuppan D, Popov YV. Comparison of murine steatohepatitis models identifies a dietary intervention with robust fibrosis, ductular reaction, and rapid progression to cirrhosis and cancer. *Am J Physiol Gastrointest Liver Physiol.* 2020 Jan 1;318(1):G174-G188. PMID: 31630534). *Casp8^{fl/fl}* mice were fed the HF-CDAA diet for 4 weeks, followed by AAV8-TBG-Cre virus injection and then continuance on the diet for an additional 4 weeks (**FIG. 16A**). Caspase-8 deletion in the Cre-treated livers was confirmed (**FIG. 16B**), and the two cohorts had similar body and liver weights, and fasting blood glucose (**FIGS. 16C-E**). As with the FPC model, hepatocyte caspase-8 knockout led to decreases in liver fibrosis and inflammation (**FIGS. 16F-H**). Despite the higher level of hepatocyte apoptosis in this model, there was no difference in the percentage of apoptotic hepatocytes between the two groups of mice (**FIG. 16I**). These data further support a non-apoptotic role of caspase-8 in MASH fibrosis progression and bring to

mind a clinical trial showing that inhibiting hepatocyte apoptosis using the caspase inhibitor emricasan did not ameliorate MASH-related fibrosis (Harrison SA, Goodman Z, Jabbar A, Vemulapalli R, Younes ZH, Freilich B, Sheikh MY, Schattenberg JM, Kayali Z, Zivony A, Sheikh A, Garcia-Samaniego J, Satapathy SK, Therapondos G, Mena E, Schuppan D, Robinson J, Chan JL, Hagerty DT, Sanyal AJ. A randomized, placebo-controlled trial of emricasan in patients with NASH and F1-F3 fibrosis. *J Hepatol.* 2020 May;72(5):816-827. PMID: 31887369).

Example 15- A caspase-8—meteorin pathway in hepatocytes activates HSCs

[0173] Seeking to explore potential mechanisms linking hepatocyte caspase-8 to liver fibrosis progression in MASH. Activated HSCs are the main source of collagen-producing myofibroblasts in fibrotic liver disease and play a key role in MASH fibrosis (PMID: 24264436). An *ex-vivo* model (Wang X, Zheng Z, Caviglia JM, Corey KE, Herfel TM, Cai B, Masia R, Chung RT, Lefkowitz JH, Schwabe RF, Tabas I. Hepatocyte TAZ/WWTR1 Promotes Inflammation and Fibrosis in Nonalcoholic Steatohepatitis. *Cell Metab.* 2016 Dec 13;24(6):848-862. PMID: 28068223) was used in which primary murine or human HSCs were incubated with conditioned media from siCasp8-treated or control human or murine primary hepatocytes. Consistent with the *in-vivo* data, markers of HSC activation were reduced in HSCs incubated with conditioned media from Casp8-silenced mouse and human primary hepatocytes compared with control conditioned media (**FIGS. 17A-B, 17C-D**). Further, heat treatment of the CM prior to incubation with the HSCs blocked CM-induced HSC activation and abrogated the difference between siCasp8 and control conditions, suggesting that one or more secreted proteins were responsible for hepatocyte caspase-8-dependent activation of HSCs (**FIG. 17E**).

[0174] Next RNA-seq was conducted to identify genes encoding secretory proteins that were diminished in Casp8-silenced AML12 mouse hepatocytes. Additionally, LC-MS/MS was employed to identify potential caspase-8-dependent hepatocyte secretory proteins that activate HSCs. Candidate genes were then identified by integrating the RNA-seq and LC-MS/MS analyses with a secreted protein database (**FIGS. 18A-D**), of which 11 genes were selected for qPCR confirmation in our hepatocyte-Casp8-knockout FPC-MASH livers. Notably, *Metrn*, encoding the protein meteorin, emerged as a significant candidate (**FIG. 18E**). Further investigation revealed a decrease in meteorin protein in primary mouse

hepatocytes upon Casp8 silencing (**FIG. 18F**). *METRN* mRNA and meteorin protein were also reduced by CASP8-silencing in the THLE2 human hepatocyte cell line (**FIG. 18G**) and in primary human hepatocytes (**FIG. 18H**). Meteorin protein was also decreased in the livers of hepatocyte-Casp8 knockout versus control MASH mice (**FIGS. 18I-J**). Pol II ChIP of AML12 hepatocytes indicated that Casp8 silencing reduced Pol II binding to the *Metrn* promoter (**FIG. 18K**), suggesting transcriptional induction of *Metrn* by caspase-8. Further in silicon analysis revealed the presence of a YY1 binding site on the *Metrn* promoter. YY1 ChIP of AML12 hepatocytes indicated that Casp8 silencing reduced YY1 binding to the *Metrn* promoter (**FIG. 18L**), suggesting transcriptional induction of *Metrn* by YY1. In addition, *METRN* mRNA was reduced by CASP8-silencing in the AML12 hepatocyte cell line (**FIG. 18M**) and in primary human hepatocytes (**FIG. 18N**). Moreover, the pan-caspase inhibitor ZVAD and the caspase-8 inhibitor IETD, both of which inhibited Fas-induced apoptosis as expected, did not lower the expression of *Metrn* in AML12 cells (**FIGS. 18O-P**), further implying a non-apoptotic role of caspase-8 in this pathway.

[0175] Based on the above data and the previous experimental MASH results, disclosed herein is that caspase-8-induced meteorin contributes to liver fibrosis in MASH. First primary murine HSCs were incubated with recombinant (r-) meteorin and found this treatment led to the induction of fibrotic gene expression in the HSCs (**FIG. 19A**). Similarly, incubation of normal or MASH human HSCs with r-meteorin also led to an increase in fibrotic gene expression (**FIGS. 19B-C**). Returning to the above conditioned medium transfer experiment above, *Metrn* was transfected into Casp8-silenced hepatocytes to restore *Metrn* to Scr control levels (**FIG. 19D**), followed by transfer of the conditioned medium to HSCs. The data show that that HSC activation by conditioned medium from Casp8-silenced hepatocytes was restored partially (*Timp1*) or fully (*Spp1*) when these hepatocytes were transfected with *Metrn* (**FIG. 19E**). Conversely, the conditioned medium of *METRN*-silenced human hepatocytes showed less HSC activation compared with the medium of control hepatocytes (**FIGS. 19F-G**). Finally, building upon the previous data suggesting that Casp8 promotes HSC activation independently of its apoptotic function, it was found that treatment of hepatocytes with the Casp8 inhibitor IETD did not impede hepatocyte conditioned medium from activating HSCs (**FIG. 19H**). Furthermore, the protective effect of silencing hepatocyte Casp8 on conditioned medium-induced HSC activation was lost similarly when hepatocytes were transfected with wild-type *Casp8* WT versus a plasmid encoding catalytically inactive

C360A-caspase-8 (**FIG. 19I**). Thus, caspase-8-induced HSC activation is independent of its protease activity, which is required for caspase-8-mediated apoptosis (PIMD: 19924290, PMID: 11002417).

[0176] It was next sought to elucidate the signaling pathway involved in meteorin-induced HSC activation. Meteorin-like protein, which shares similar homology to meteorin, has been shown to activate profibrotic STAT3 in the setting of pulmonary fibrosis (PMID: 26324850). Activation of STAT3 has been shown to correspond with liver fibrosis in patients with MASH (PMID: 36687470), and there are data linking STAT3 activation to HSC activation and liver fibrosis in MASH (PMID: 36687470). In this context, it was found that treatment of primary mouse HSCs with r-meteorin protein markedly increased Y705 phosphorylation of STAT3 (**FIG. 19J**), which is a measure of STAT3 activation. Moreover, silencing either the STAT3-activating receptor, Kit, or Stat3 itself in HSCs partially blocked HSC gene expression (**FIGS. 19K-L**). These findings suggest that meteorin induces HSC gene expression through the Kit/Stat3 pathway.

[0177] Example 16- Meteorin is increased in human and mouse MASH liver, and genetic restoration of hepatocyte meteorin blocks the protective effect of hepatocyte caspase-8 knockout on MASH fibrosis

[0178] Both *METRN* mRNA and meteorin protein are increased in human versus normal liver (**FIG. 20A**), aligning with findings with *METRN* mRNA from a public bulk RNA-seq dataset (**FIG. 20B**). Meteorin protein elevation was also observed in the livers of FPC-fed and HF-CDAA-fed mice with MASH (**FIGS. 20C-D**) but not in the livers of mice with simple steatosis (**FIGS. 20E-F**). As the transcription factor of *Metrn*, YY1 protein was increased in human and mouse NASH livers (**FIGS. 20G-I**). Given these observations, it was asked whether genetic restoration of meteorin in hepatocytes could block the protective effect of hepatocyte Casp8 knockout on liver fibrosis in MASH. To address this question, AAV8-TBG-Cre ± AAV8-TBG-*Metrn* was administered to *Casp8^{fl/fl}* mice, with AAV8-TBG GFP as control, after 8 weeks of FPC diet and then analyzed the mice after 16 weeks of FPC feeding (**FIG. 21A**). As before, robust caspase-8 deletion was achieved with AAV8-TBG-Cre, with concomitant decrease in meteorin, and a level of meteorin restoration was achieved with AAV8-TBG-*Metrn* that was similar to the control liver level (**FIG. 17B**). There were no significant differences in body weight, liver-to-body ratio, or fasting blood glucose among the

three groups of mice (**FIGS. 21C-E**). As before, Sirius red positive area and mononuclear cells were decreased by hepatocyte caspase-8 knockout, but these changes were abrogated in the capase-8 knockout mice treated with AAV8-TBG-Metrn (**FIG. 21F**). Analyses of fibrotic and inflammatory mRNA expression, COLA1- and F4/80-positive areas, and plasma ALT further confirmed that restoration of meteorin in hepatocytes abrogated the beneficial effects of hepatocyte caspase-8 KO on MASH (**FIGS. 21G-I**). These findings provide *in-vivo* causation evidence that hepatocyte caspase-8 promotes MASH by inducing meteorin.

[0179] Example 17- Silencing of hepatocyte meteorin after the development of diet-induced MASH reverses liver fibrosis

[0180] Given that therapies that silence genes specifically in hepatocytes, *e.g.*, using GalNAc-siRNA, are now in use in humans to treat various diseases (Gardin A, Remih K, Gonzales E, Andersson ER, Strnad P. Modern therapeutic approaches to liver-related disorders. *J Hepatol.* 2022 Jun;76(6):1392-1409. PMID: 35589258), it was sought to conduct a proof-of-concept study in experimental MASH using hepatocyte-specific deletion of meteorin. In general, the strategy for MASH therapy is to be administered to individuals with early MASH, not simple steatosis, with the primary goal of reversing liver fibrosis (REF). In this context, mice were subjected to FPC-feeding for 16 weeks (basal), followed by treatment with hepatocyte-specific AAV8-H1-shMetrn or a control vector, followed by continuance on the diet for an additional 12 weeks (**FIG. 22A**). As designed, meteorin levels were reduced in the livers of mice treated with shMetrn (**FIG. 22B**). The three groups of mice had similar body weights, liver-to-body weight ratios, and fasting blood glucose levels (**FIGS. 22C-E**). After 28 weeks of FPC feeding, liver fibrosis and inflammation were increased compared to the 16-week basal livers, but shMetrn treatment during the progression period resulted in regression to the 16-week level (**FIG. 22F**). A similar pattern was observed for the endpoints of fibrosis- and inflammation-related mRNAs, and plasma ALT. (**FIGS. 22G-H**). These data complement the findings from meteorin-restoration model in the previous section by showing the pro-fibrotic nature of hepatocyte meteorin in MASH and provide proof-of-concept evidence that silencing hepatocyte meteorin in early MASH can reverse MASH, particularly MASH fibrosis.

[0181] As will be apparent to one of ordinary skill in the art from a reading of this disclosure, the present disclosure can be embodied in forms other than those specifically

disclosed above. The particular embodiments described above are, therefore, to be considered as illustrative and not restrictive. Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, numerous equivalents to the specific embodiments described herein.

[0182] The scope of the invention is as set forth in the appended claims and equivalents thereof, rather than being limited to the examples contained in the foregoing description. The contents of all of the references disclosed herein are incorporated by reference in their entirety.

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CLAIMS

What is claimed is:

1. A method for treating or preventing metabolic dysfunction-associated steatohepatitis (MASH) in a subject in need thereof, the method comprising administering to the subject a composition targeting a caspase 8 (CASP8) pathway in hepatocytes.
2. The method of claim 1, wherein MASH is diagnosed in the subject by determining *METRN* mRNA levels, meteorin protein levels, or both *METRN* mRNA levels and meteorin protein levels in a sample from the subject.
3. The method of claims 1-2, wherein the subject has increased levels of *METRN* mRNA, meteorin protein, or both *METRN* mRNA and meteorin protein as compared to a sample from a healthy subject.
4. The method of claims 2-3, wherein the subject is determined to have fibrotic MASH if the sample levels of *METRN* mRNA levels, meteorin protein levels, or both *METRN* mRNA levels and meteorin protein levels of the subject are increased by a 3 to 4 fold compared to the expression of *METRN* mRNA and/or meteorin protein of a subject not suffering from MASH.
5. The methods of claims 2-4, wherein the sample comprises blood or blood plasma, or liver cells.
6. The method of claims 1-5, wherein the composition reduces CASP8 expression in the subject when compared to untreated subjects or to expression level of CASP8 in the subject pre-treatment.
7. The method of claims 1-6, wherein the composition comprises a CASP8 small interfering ribonucleic acid (siCASP8).
8. The method of claim 7, wherein the siCASP8 comprises at least one of the sequences encoding a small interfering ribonucleic acid (siCASP8) of Table 1.
9. The method of claim 7 or 8, wherein the siCASP8 is a hepatocyte-targeted siRNA.
10. The method of claims 7-9, wherein the siCASP8 comprises a ligand-based targeting

molecule.

11. The method of claim 10, wherein the ligand-based targeting molecule comprises an aptamer.
12. The method of claims 8-11, wherein the siCASP8 comprises N-acetyl galactosamine (GalNac).
13. The method of claims 7 or 8, wherein the siCASP8 comprises a naked siRNA.
14. The method of claims 7 or 8, wherein the siCASP8 comprises an antibody-protamine.
15. The method of claim 14, wherein the antibody-protamine is conjugated to siCASP8.
16. The method of claim 1-6, the composition comprises an anti-sense oligonucleotide.
17. The method of claims 1-6, wherein the composition comprises a CASP8 short-hairpin ribonucleic acid (shCASP8).
18. The method of claim 17, wherein the shCASP8 comprises at least one of the nucleic acid sequences of Table 1.
19. The method of claims 17-18, wherein the composition comprises a viral vector comprising a nucleic acid sequence encoding a shCASP8.
20. The method of claim 19, wherein the viral vector is an adeno-associated vector (AAV).
21. The method of claim 20, wherein the viral vector is AAV8.
22. The method of claims 20 or 21, wherein the viral vector is a hepatocyte-targeted AAV.
23. The method of any one of claims 1-22, wherein the subject is a mammal.
24. The method of claim 23, wherein the mammal is a human.
25. The method of claims 1-24, wherein the composition reduces or inhibits *METRN* mRNA expression, meteorin protein expression, or both *METRN* mRNA expression and meteorin protein expression when compared to untreated subjects or to expression level of *METRN* mRNA, level of meteorin protein, or both levels *METRN* mRNA and

meteorin protein in the subject pre-treatment.

26. The method of claims 1-5, wherein the composition comprises a METRN small interfering ribonucleic acid (siMETRN), a Signal transducer and activator of transcription 3 (STAT3) siRNA (siSTAT3), or a STAT3-activating receptor (Kit) siRNA (siKit).
27. The method of claim 26, wherein the composition comprises at least one of the sequences encoding the small interfering ribonucleic acid (siMETRN, siSTAT3, or siKit) of Table 1.
28. The method of claims 26-27, wherein the siMETRN, siSTAT3, or siKit is a hepatocyte-targeted siRNA.
29. The method of claim 28, wherein the siMETRN, siSTAT3, or siKit comprises a ligand-based targeting molecule.
30. The method of claim 29, wherein the ligand-based targeting molecule comprises an aptamer.
31. The method of claims 26-30, wherein the siMETRN, siSTAT3, or siKit siRNA comprises N-acetyl galactosamine (GalNac).
32. The method of claim 7 or 8, wherein the siMETRN, siSTAT3, or siKit comprises a naked siRNA.
33. The method of claim 7 or 8, wherein the siMETRN, siSTAT3, or siKit comprises an antibody-protamine.
34. The method of claim 14, wherein the antibody-protamine is conjugated to siMETRN, siSTAT3, or siKit.
35. The method of claims 1-5, wherein the composition comprises a METRN short-hairpin ribonucleic acid (shMETRN), a STAT3 shRNA (shSTAT3), or Kit shRNA (shKit).
36. The method of any one of claim 35, wherein the shMETRN, shSTAT3, or shKit comprises at least one of the nucleic acid sequences of Table 1.

37. The method of claims 35-36, wherein the composition comprises a viral vector comprising a nucleic acid sequence encoding a shRNA.
38. The method of claim 37, wherein the viral vector is an adeno-associated vector (AAV).
39. The method of claim 38, wherein the viral vector is AAV8.
40. The method of claim 38 or 39, wherein the viral vector is a hepatocyte-targeted AAV.
41. The method of claims 26-40, wherein the composition reduces or inhibits *METRN*, *STAT3*, or *Kit* expression when compared to untreated subjects or to expression level of *METRN*, *STAT3*, or *Kit* in the subject pre-treatment.
42. The method of claims 1-5, wherein the composition comprises a guide RNA (gRNA) or a single-molecule guide RNA (sgRNA) comprising a spacer sequence that is complementary to a portion of a nucleic acid sequence encoding CASP8, METRN, STAT3, or KIT.
43. The method of claim 42, wherein the gRNA or the sgRNA is pre-complexed with a DNA endonuclease.
44. The method of claim 43, wherein the DNA endonuclease is a Cas9 endonuclease.
45. The method of claims 42-44, wherein the composition reduces or inhibits expression of one or more of CASP8, *METRN*, *STAT3*, *Kit* when compared to untreated subjects or to expression level of CASP8, *METRN*, *STAT3*, or *Kit* in the subject pre-treatment.
46. The method of any one of claims 25-45, wherein the subject is a mammal.
47. The method of claim 46, wherein the mammal is a human.
48. Method of claims 1-47, wherein the composition is delivered systemically.
49. A composition for treating or preventing MASH comprising a hepatocyte-targeted nucleic acid capable of targeting the caspase 8 (CASP8) pathway.
50. The composition of claim 49, wherein the composition comprises a viral vector comprising the nucleic acid.

51. The composition of claim 50 or 49, wherein the composition reduces or inhibits expression of one or more of CASP8, METRN, STAT3, or Kit.
52. The composition of claims 50-51, wherein the viral vector is an AAV vector.
53. The composition of claim 52, wherein the viral vector is AAV8.
54. The composition of claim 49, wherein the hepatocyte-targeted nucleic acid comprises N- acetyl galactosamine (GalNac).
55. The composition of claim 50, wherein the viral vector is a hepatocyte-targeted AAV.
56. The composition of claim 49, wherein the composition comprises a guide ribonucleic acid (gRNA) or a single-molecule guide RNA (sgRNA) comprising a spacer sequence that is complementary to a portion of a nucleic acid sequence encoding CASP8, METRN, STAT3, or Kit.
57. The composition of claim 56, wherein the gRNA or the sgRNA is pre-complexed with a DNA endonuclease.
58. The composition of claim 57, wherein the DNA endonuclease is a Cas9 endonuclease.
59. The composition of claim 49, wherein the composition comprises at least one of the small interfering ribonucleic acid (siRNA) sequences from Table 1.
60. The composition of claim 54, wherein the hepatocyte-targeted nucleic acid comprises any one of the small interfering ribonucleic acid (siRNA) sequences from Table 1.
61. The composition of claim 49-53 or 55, wherein the composition comprises an expression vector targeting the caspase 8 (CASP8) pathway.
62. The composition of any one of claims 49-53 or 55, wherein the expression vector targeting the caspase 8 (CASP8) pathway encodes a CASP8, METERN, STAT3, or Kit short-hairpin RNA (shRNA).
63. A nucleic acid comprising a sequence of any one of the small interfering ribonucleic acid (siRNA) designs from Table 1.

64. A nucleic acid comprising a sequence encoding a short-hairpin RNA (shRNA), wherein the shRNA comprises a nucleic acid sequence provided in Table 1.
65. The nucleic acid of claim 64, wherein the nucleic acid comprises a hepatocyte-targeting motif.
66. The nucleic acid of claim 65, wherein the hepatocyte-targeting motif comprises N-acetyl galactosamine (GalNac).
67. A vector comprising the nucleic acid sequence of claim 64.
68. A viral vector comprising the nucleic acid of claim 64.
69. The viral vector of claim 68, wherein the viral vector is an AAV vector.
70. The viral vector of claim 69, wherein the AAV vector is AAV8.
71. The viral vector of claims 68 or 69, wherein the viral vector is a hepatocyte-targeted AAV.
72. A method of diagnosing metabolic dysfunction-associated steatohepatitis (MASH) in a subject comprising:
- determining the level of meteorin (METRN) in a sample from the subject; and
- diagnosing the subject with MASH if the level of METRN expression in the sample is increased compared to a sample from a healthy subject.
73. The method of claim 72, wherein the subject is diagnosed with fibrotic MASH.
74. The method of claim 72 or 73, wherein the sample is blood or blood plasma or liver cells.
75. The method of any one of claims 72-74, wherein the level of METRN is determined using an antibody.
76. The method of any one of claims 72-74, wherein the level of METRN is determined by ELISA.

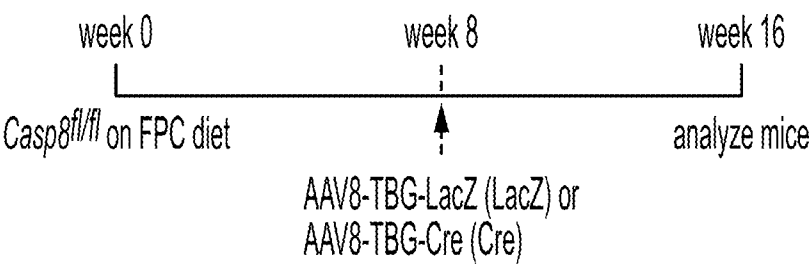


FIG. 1A

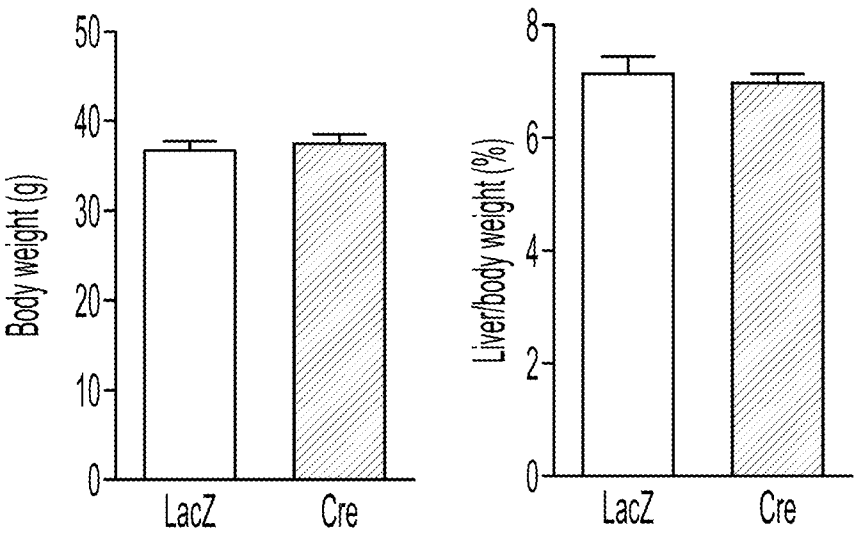


FIG. 1B

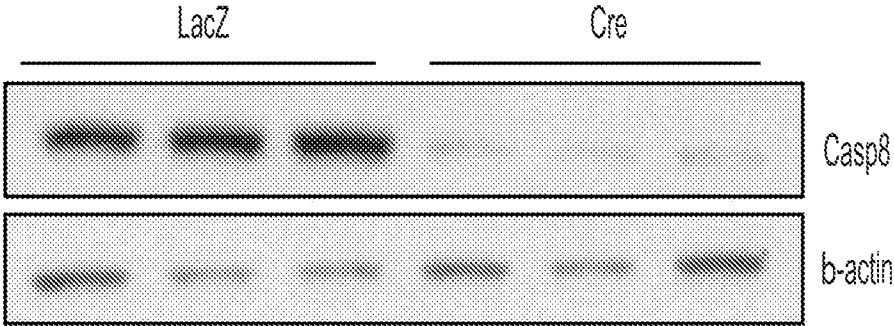


FIG. 1C

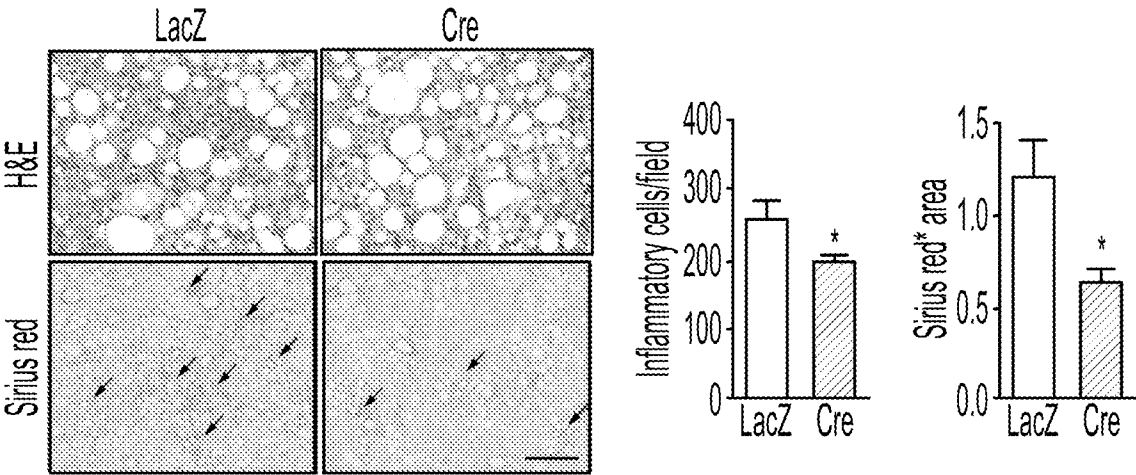


FIG. 1D

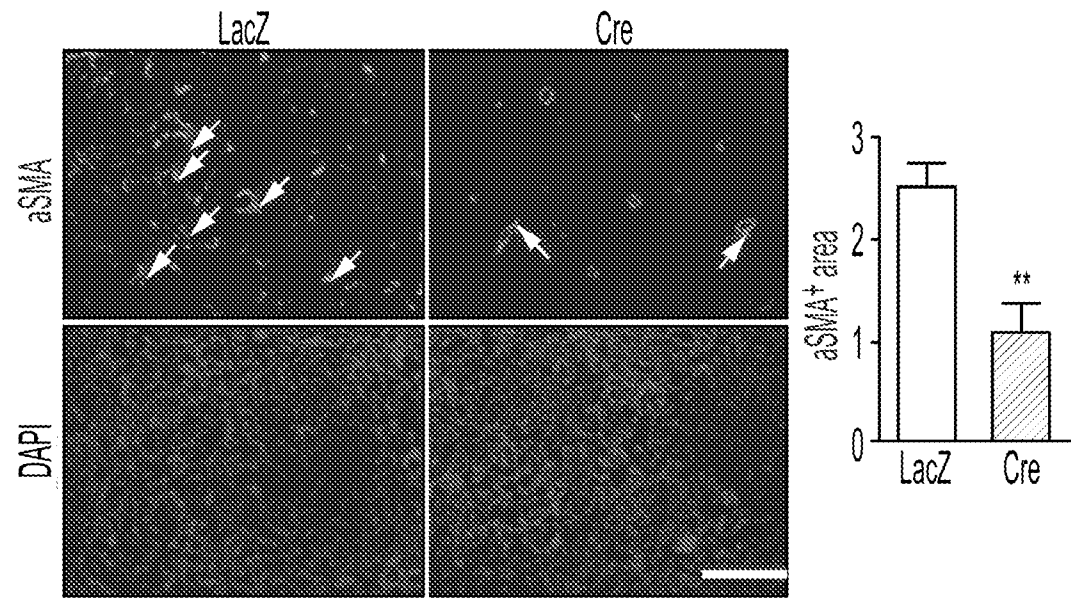


FIG. 1E

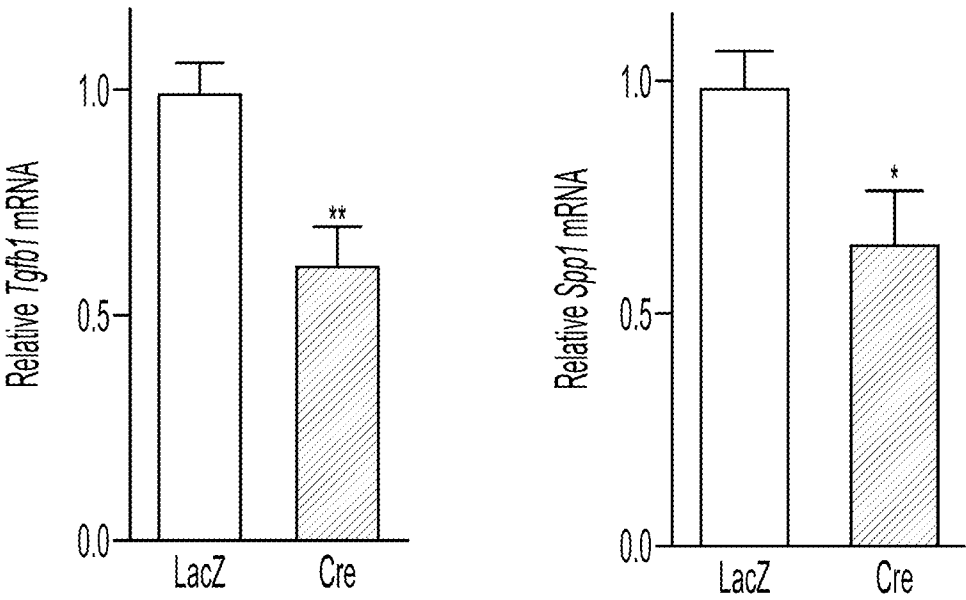


FIG. 1F

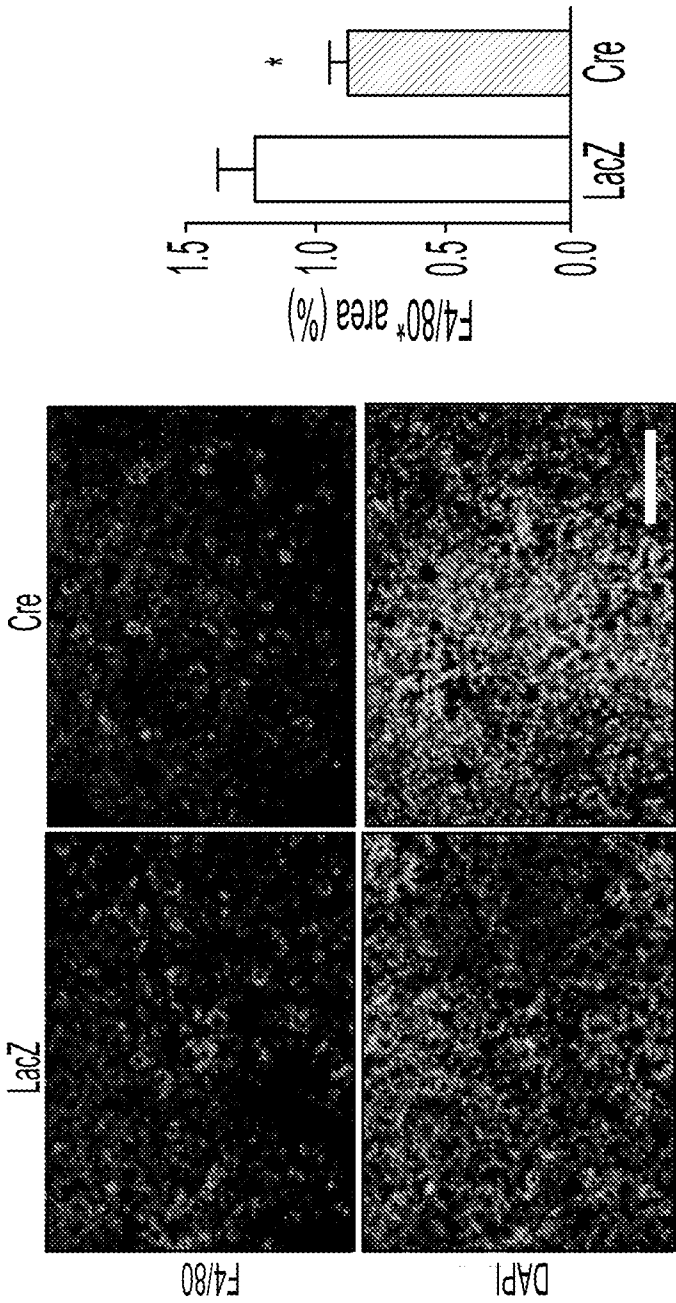


FIG. 1G

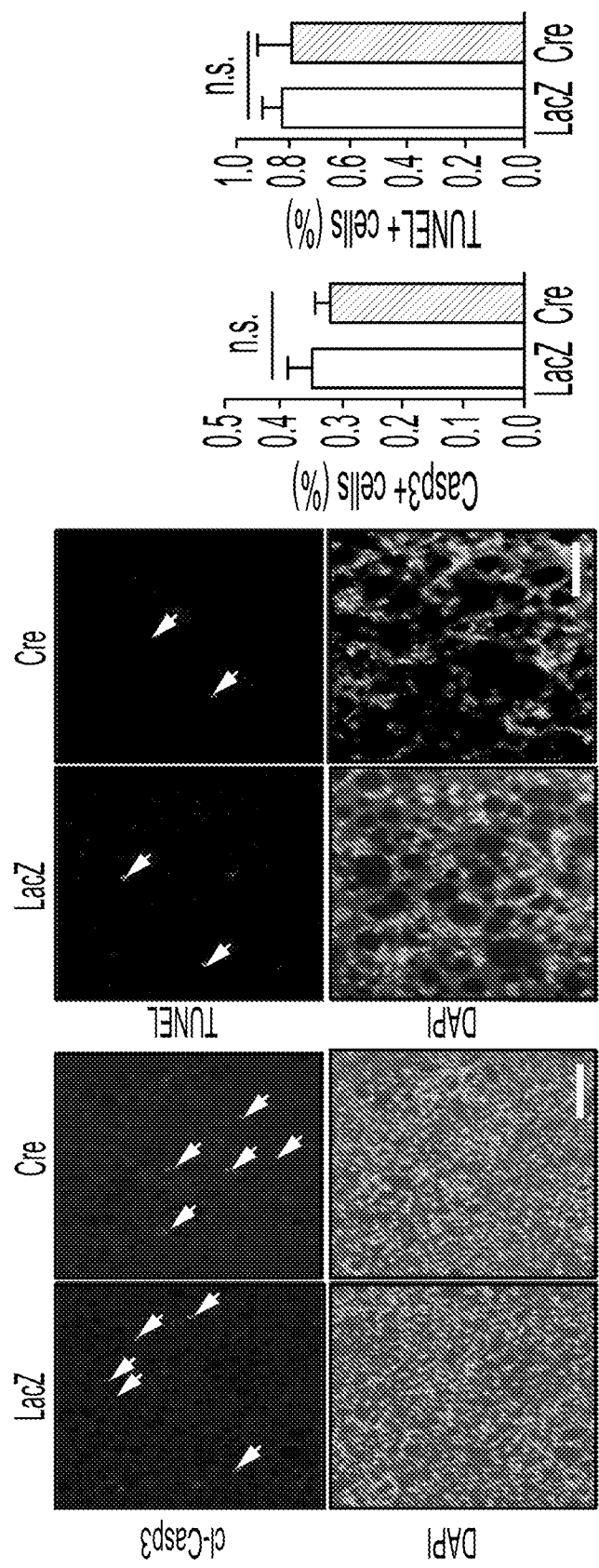


FIG. 1H

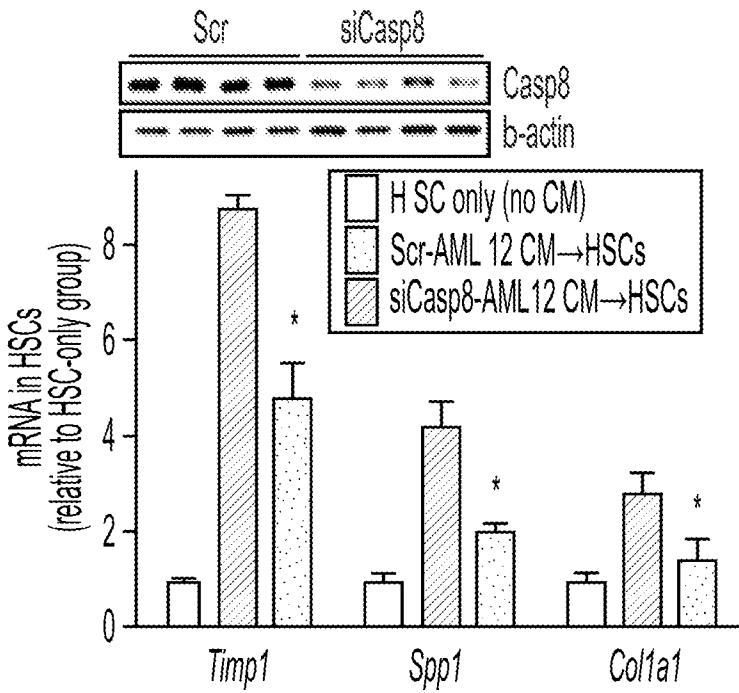


FIG. 2A

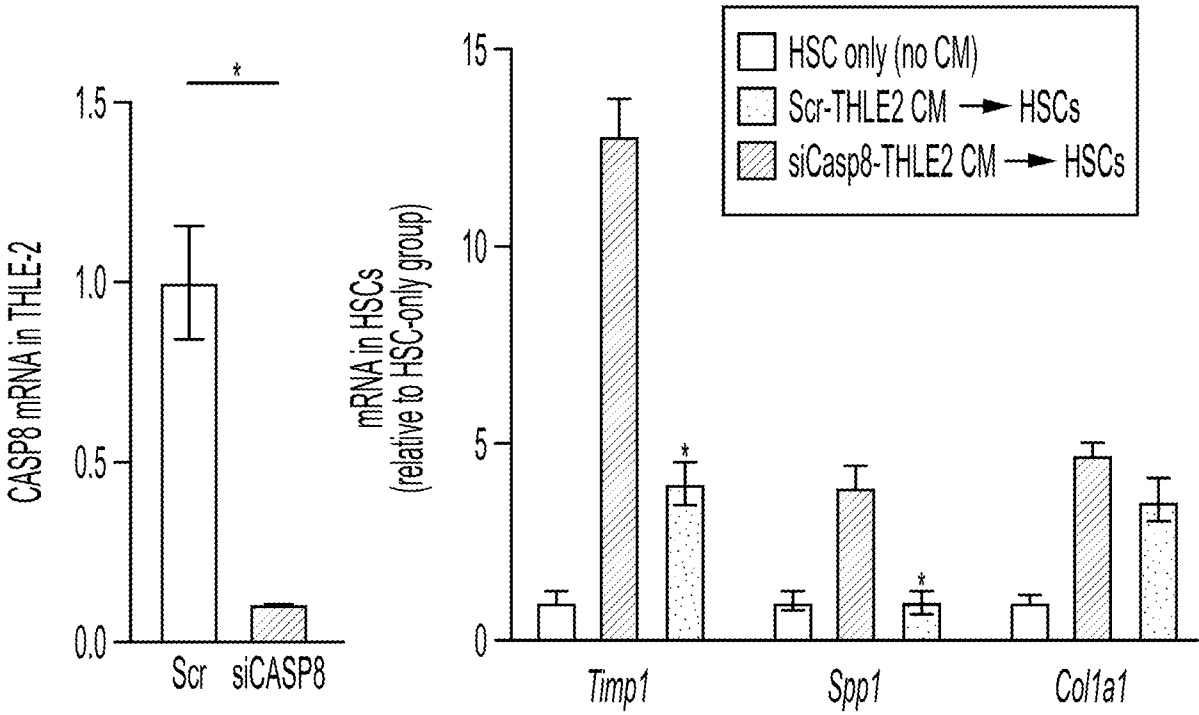


FIG. 2B

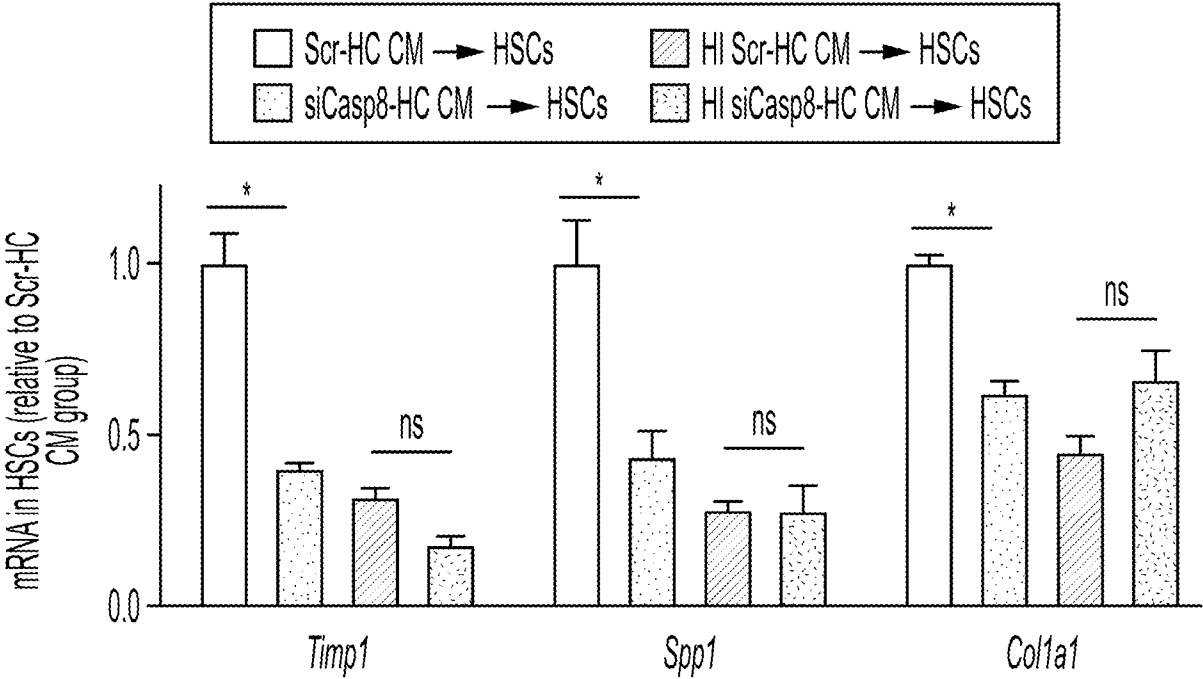


FIG. 3

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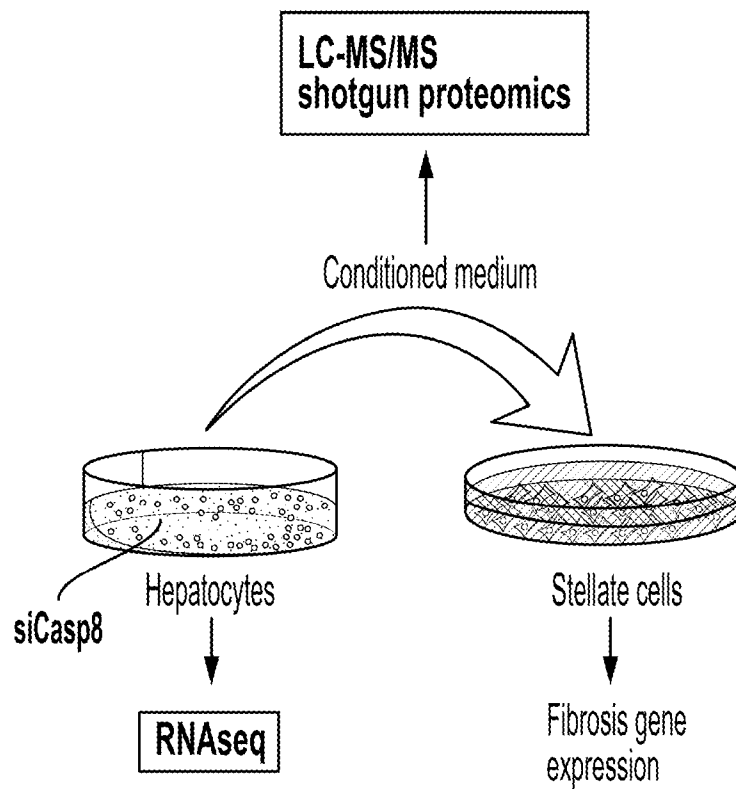


FIG. 4A

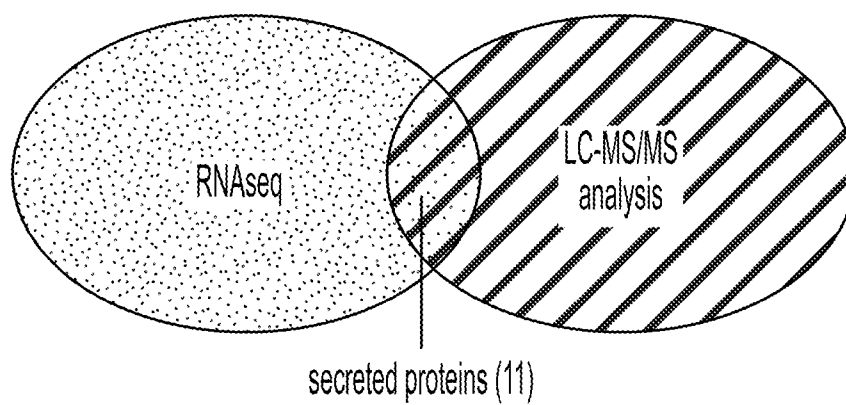


FIG. 4B

9/66

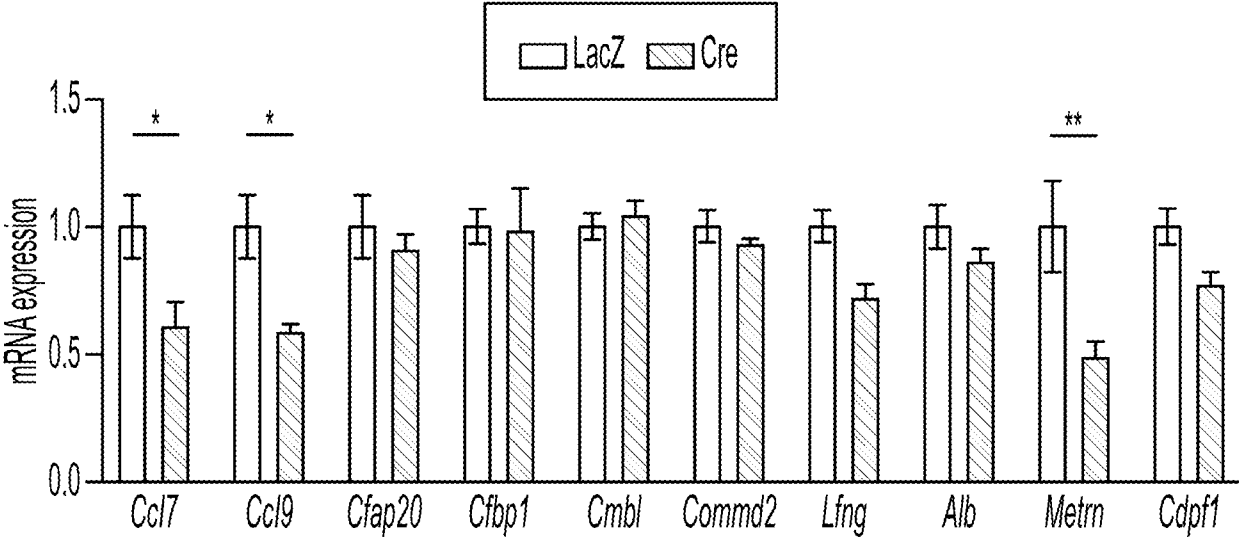


FIG. 4C

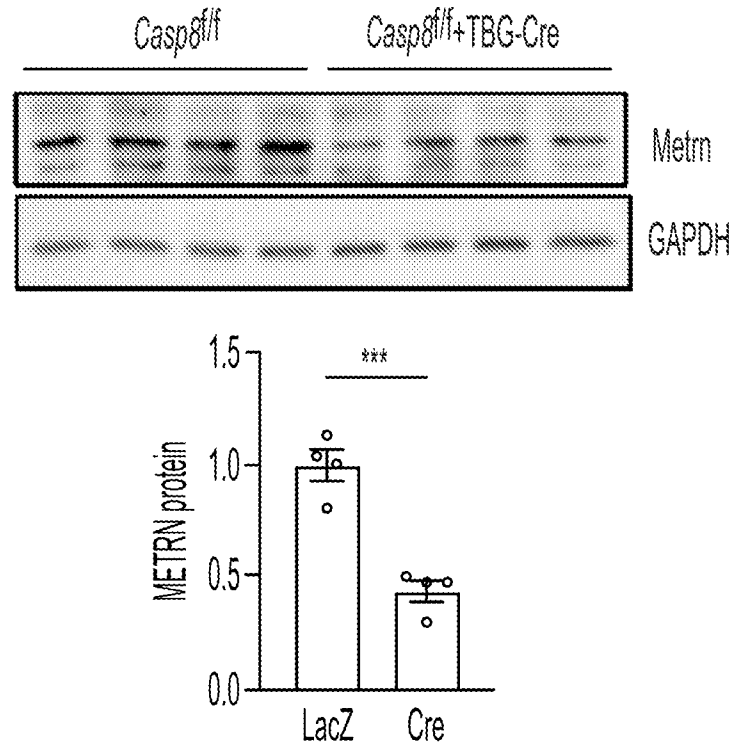


FIG. 4D

10/66

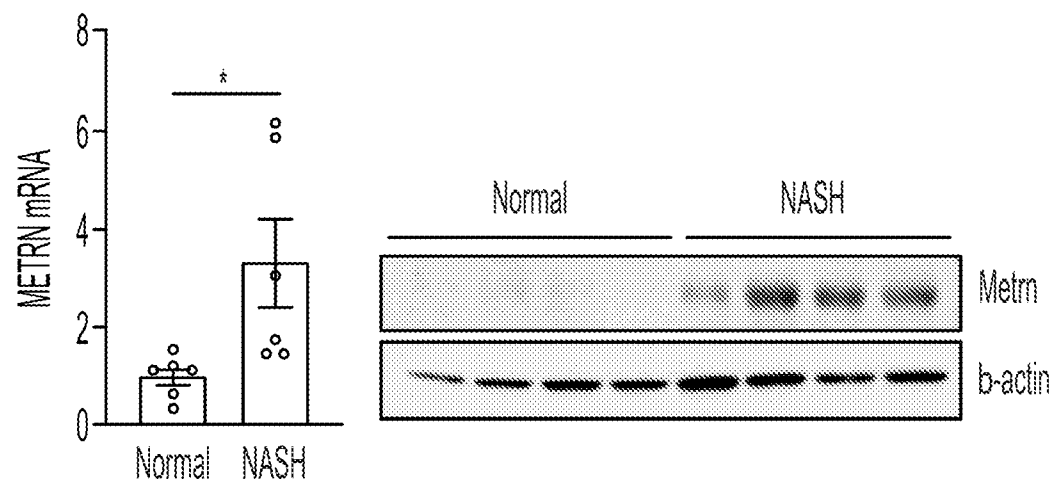


FIG. 5A

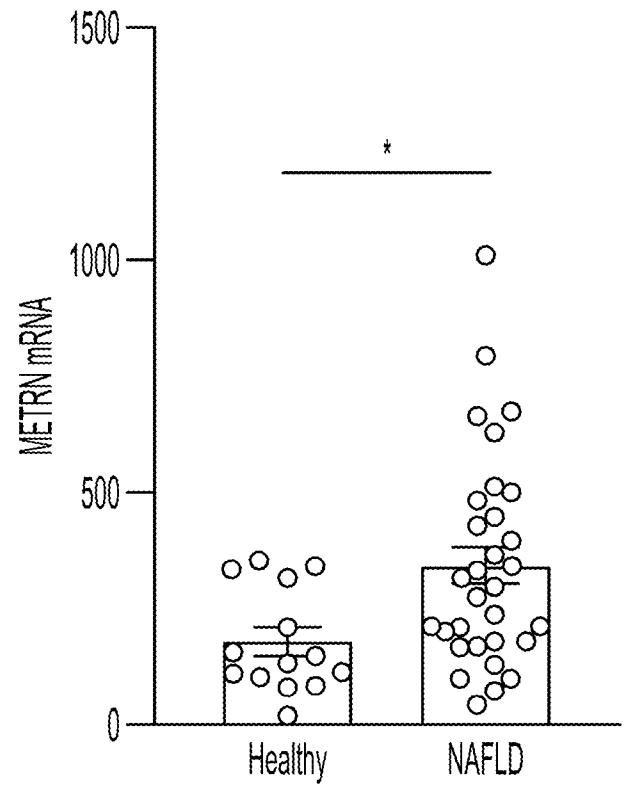


FIG. 5B

11/66

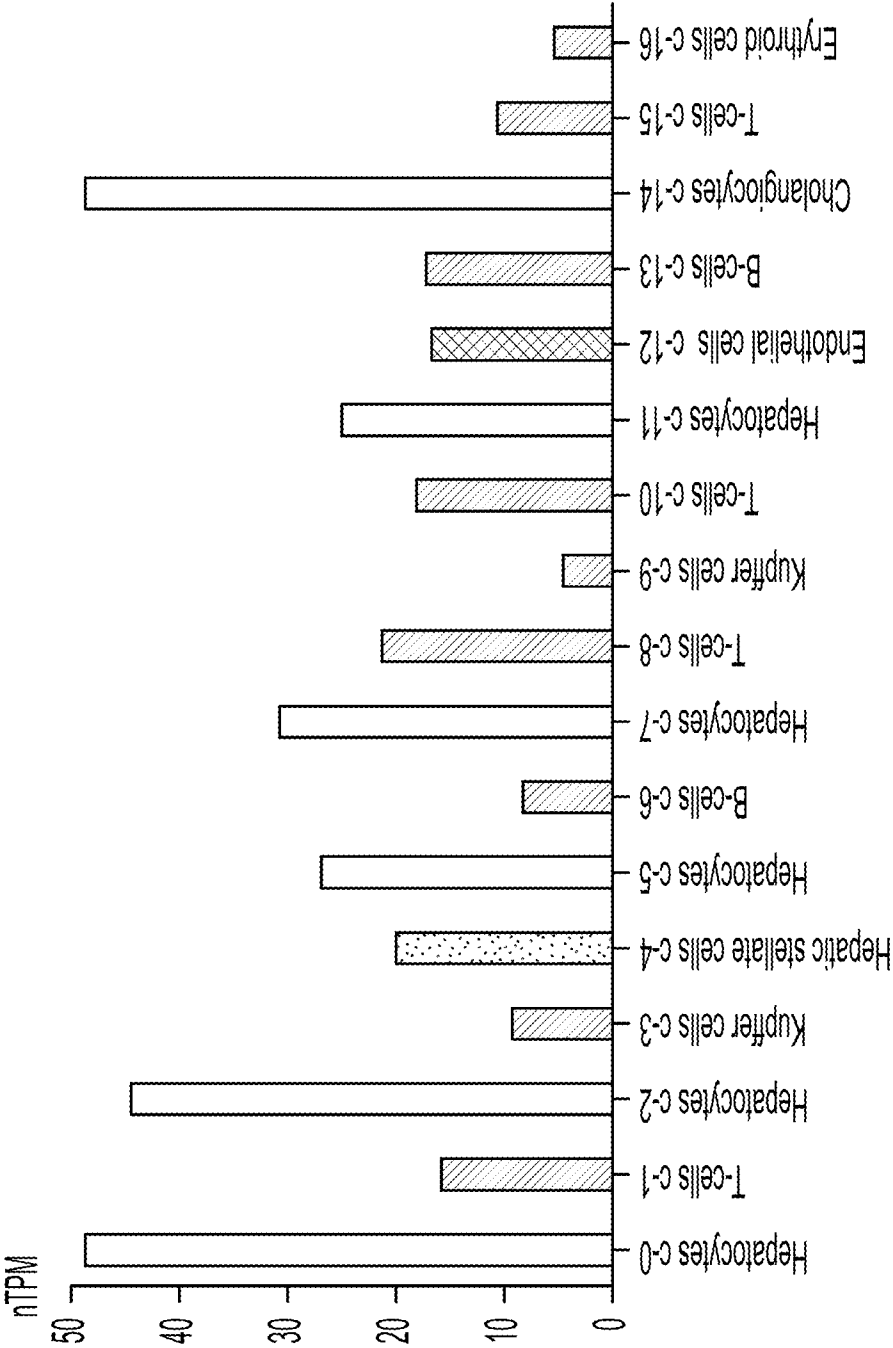


FIG. 5C

12/66

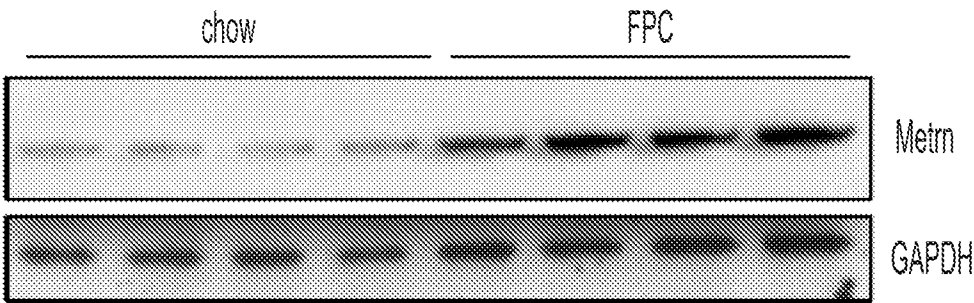


FIG. 5D

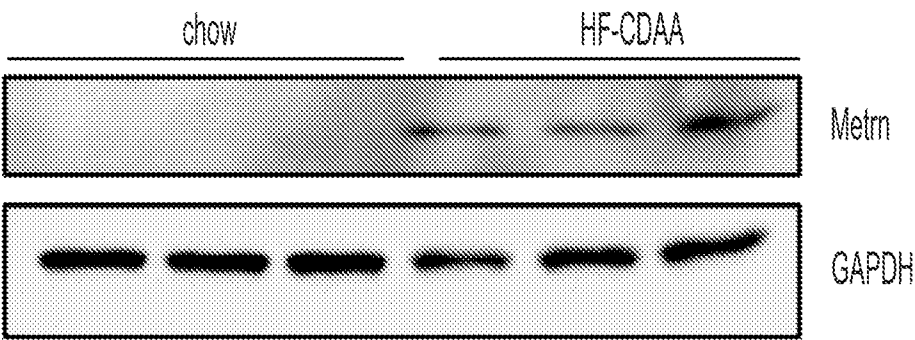


FIG. 5E

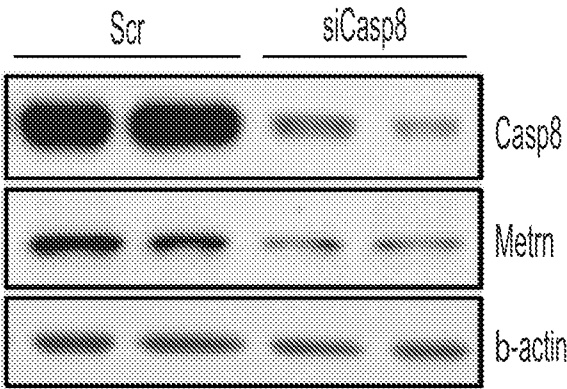


FIG. 6A

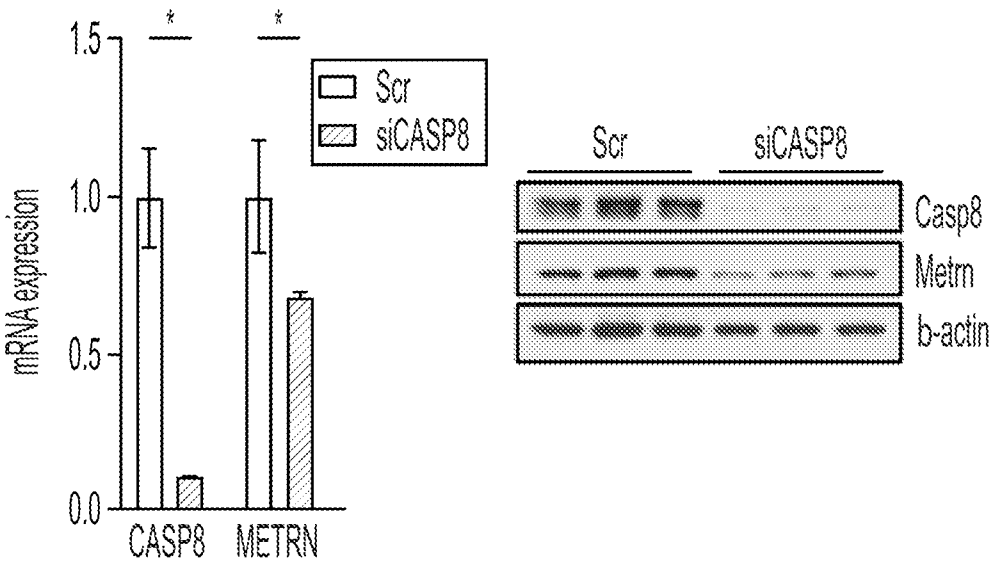


FIG. 6B

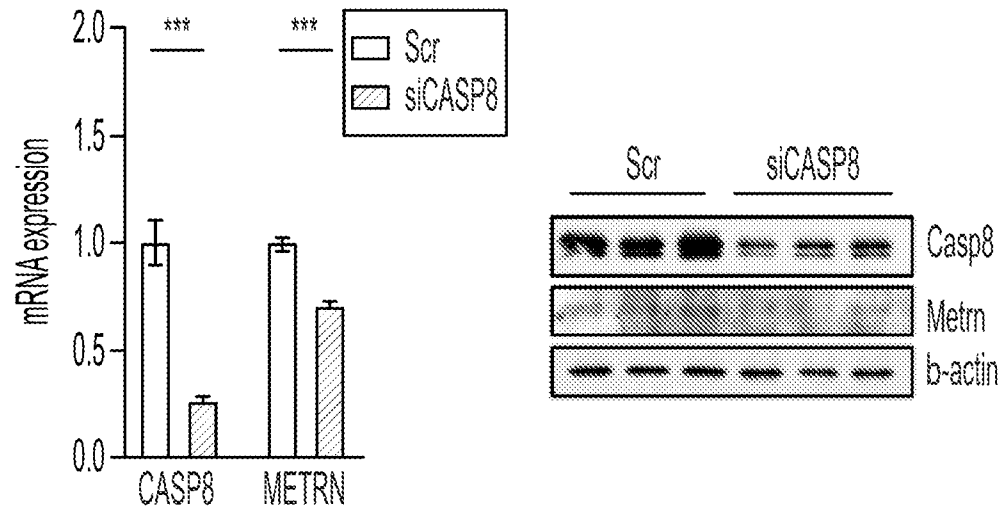


FIG. 6C

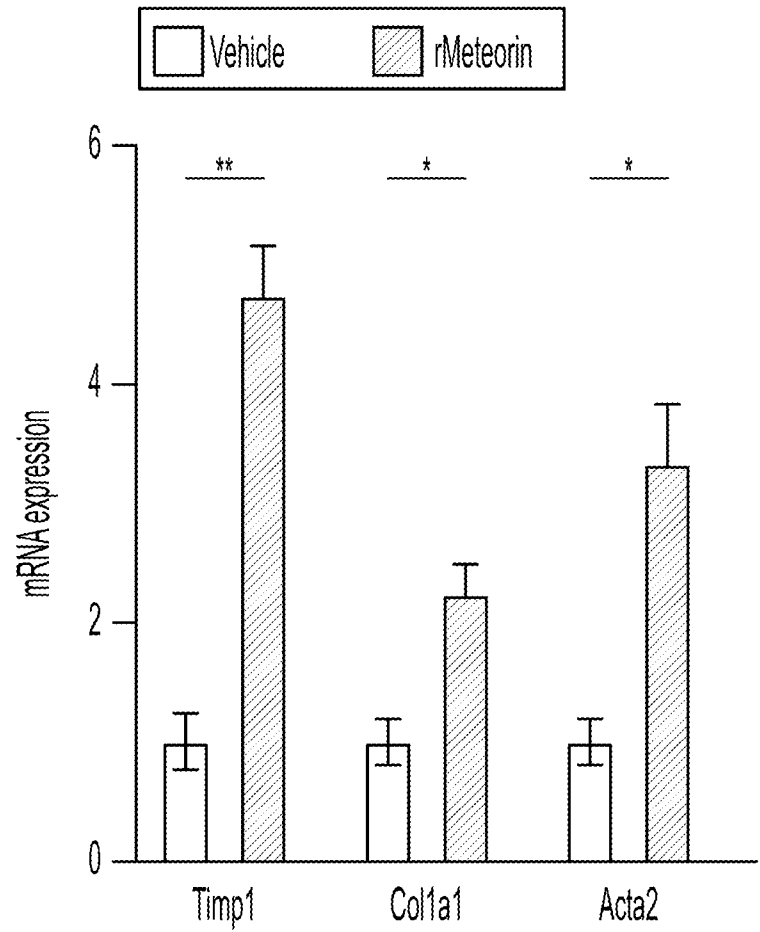


FIG. 7

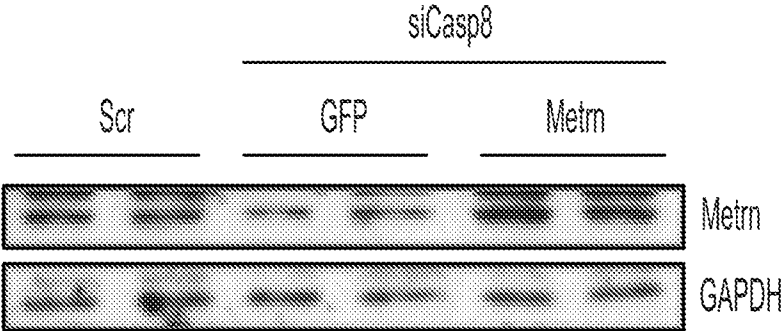


FIG. 8A

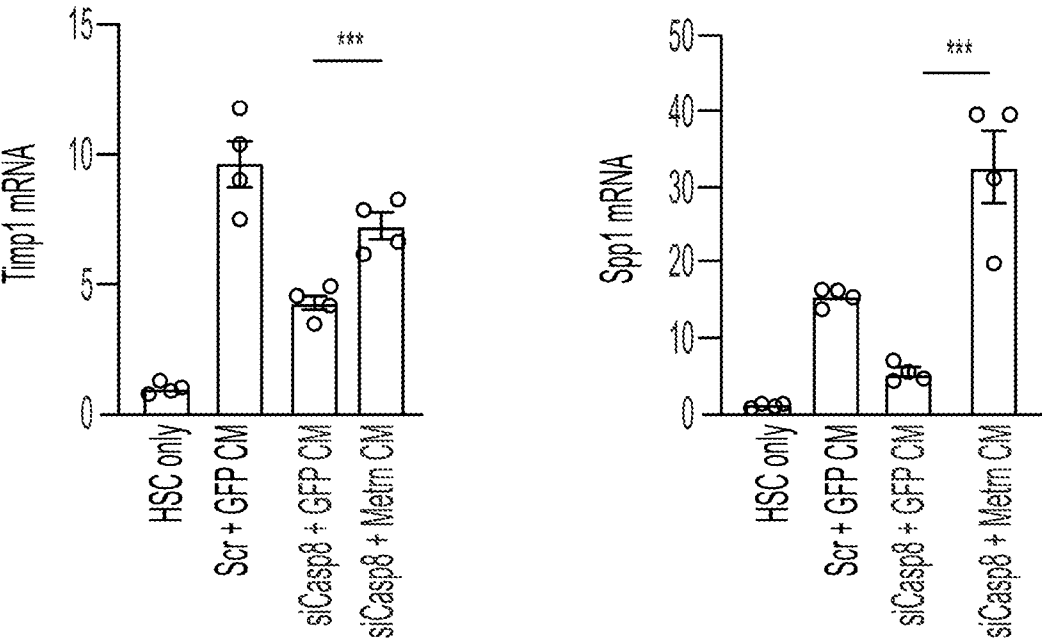


FIG. 8B

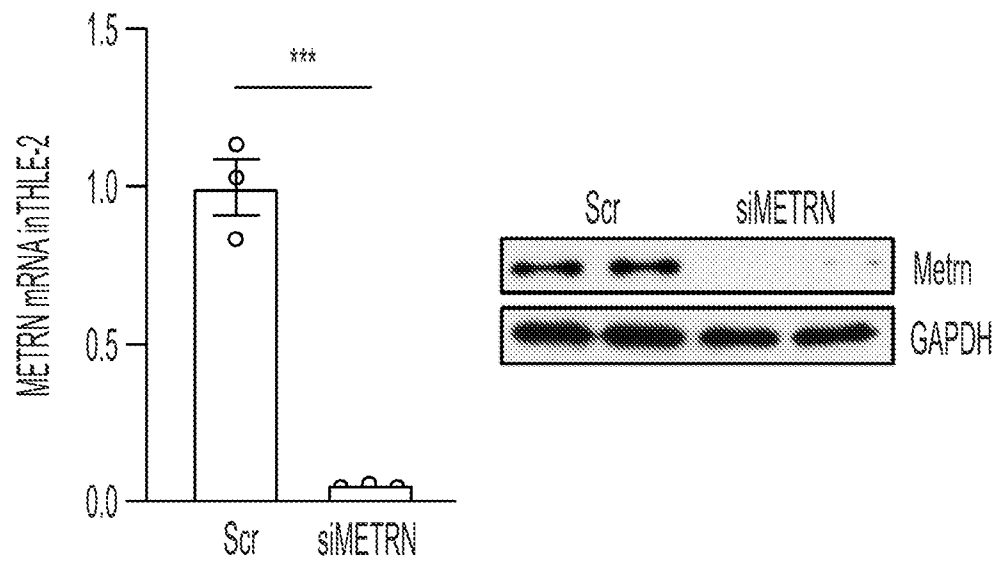


FIG. 9A

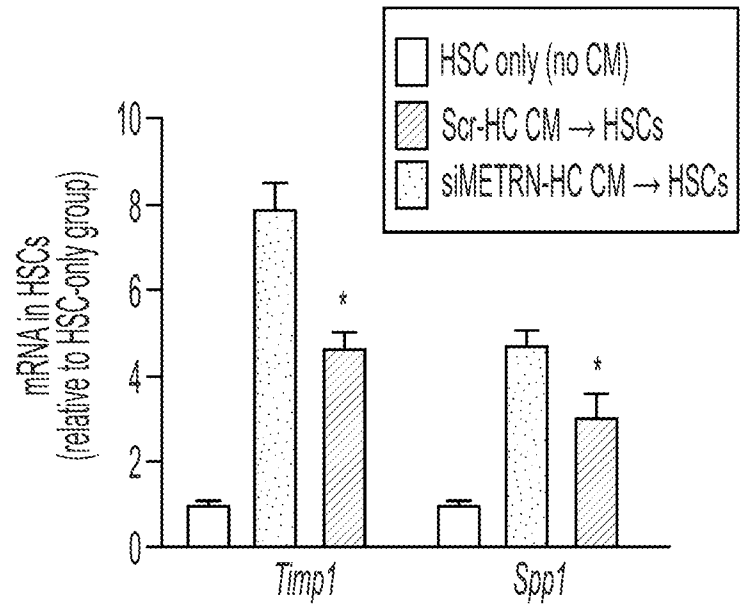


FIG. 9B

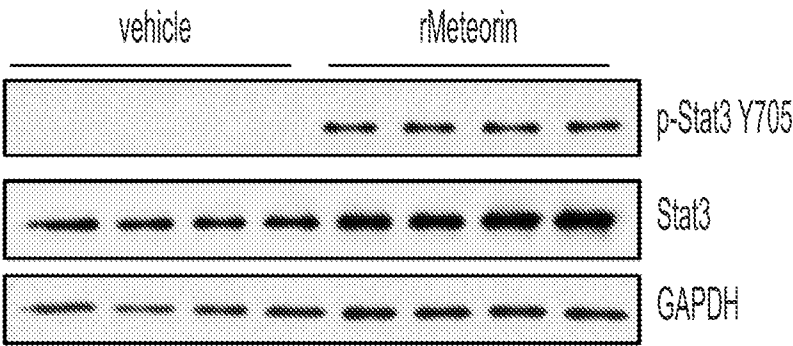


FIG. 10A

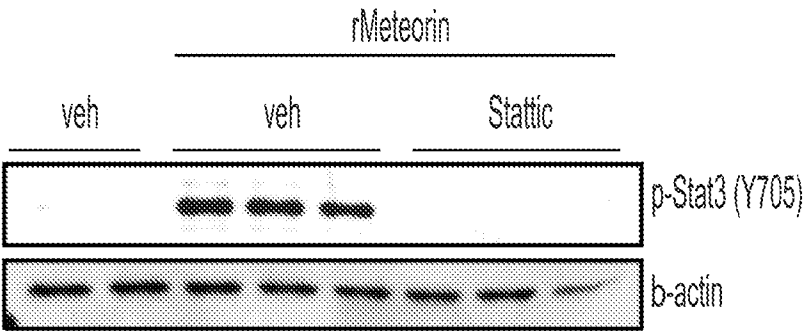


FIG. 10B

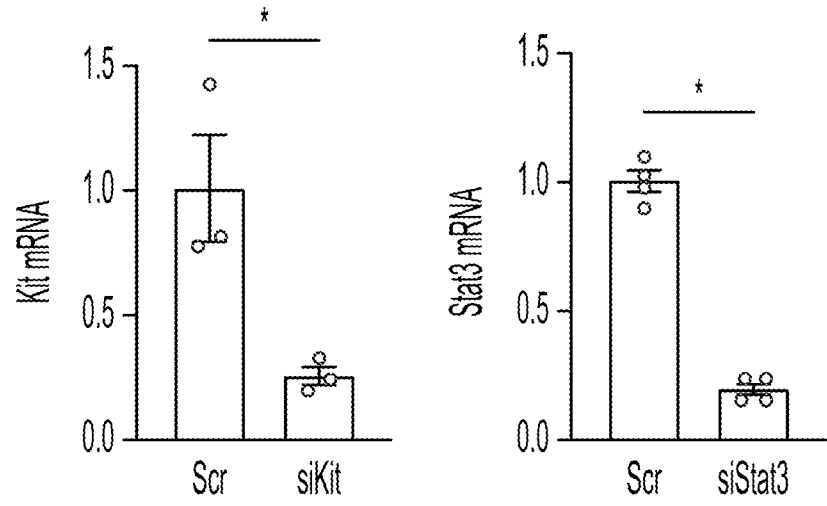


FIG. 11A

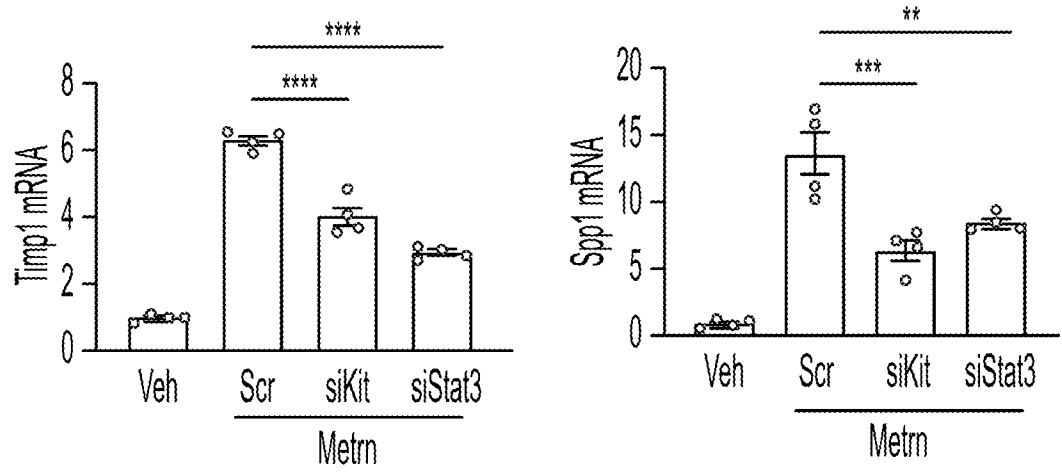


FIG. 11B

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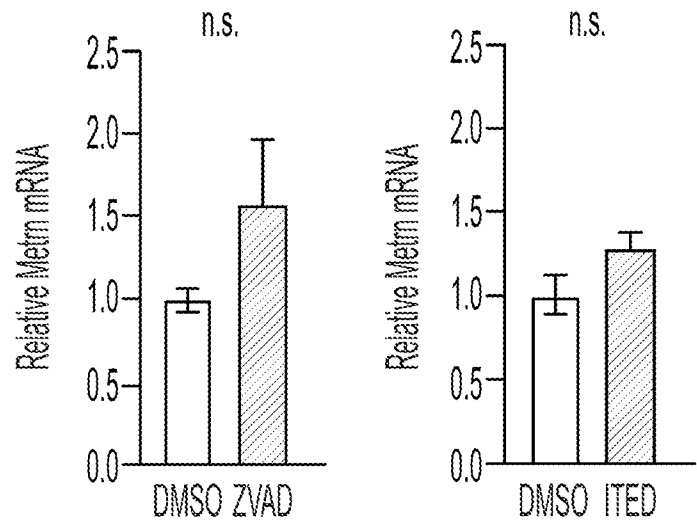


FIG. 12A

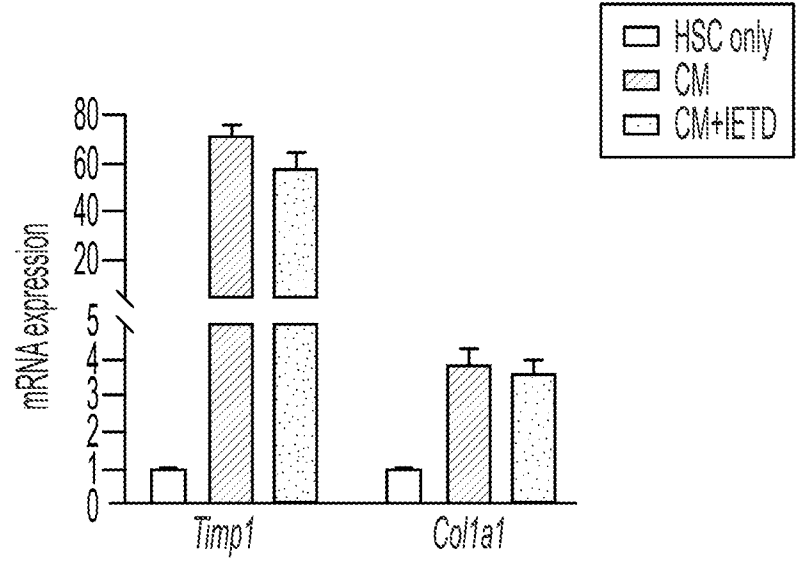


FIG. 12B

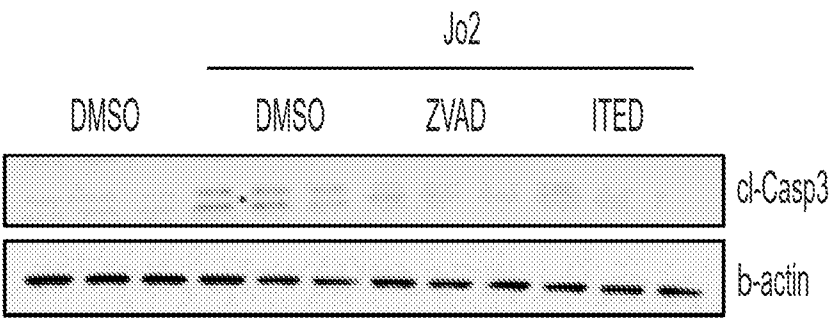


FIG. 12C

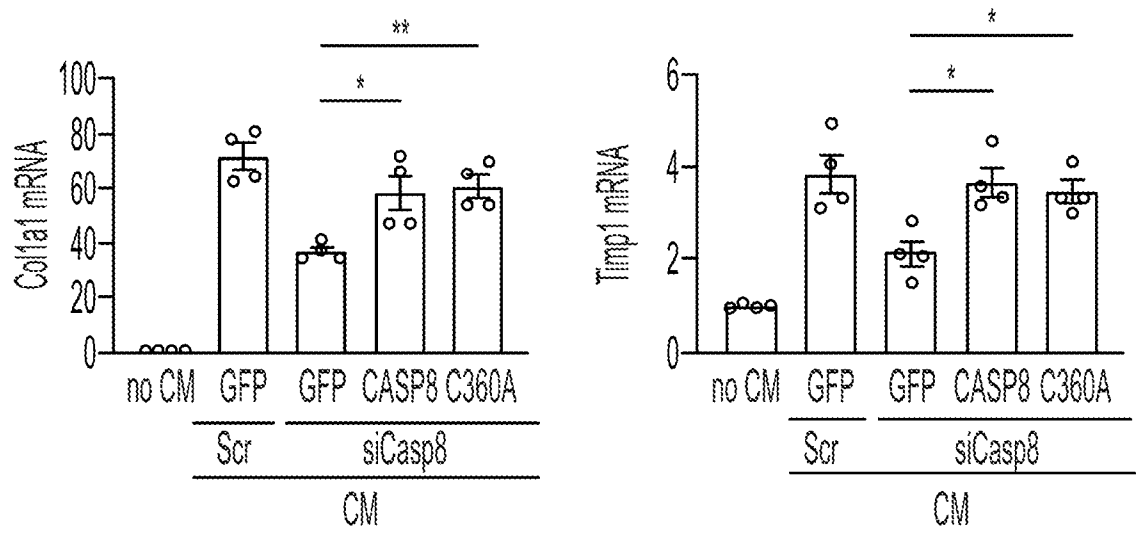


FIG. 13

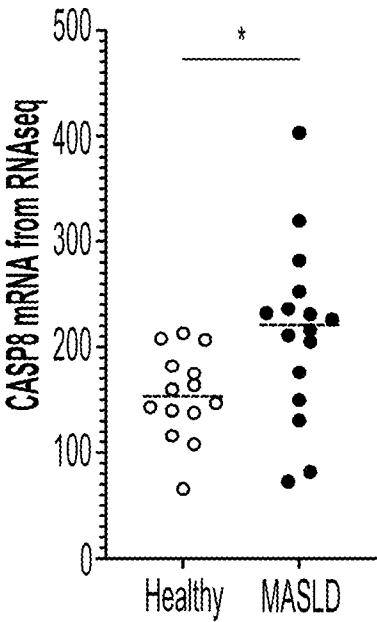


FIG. 14A

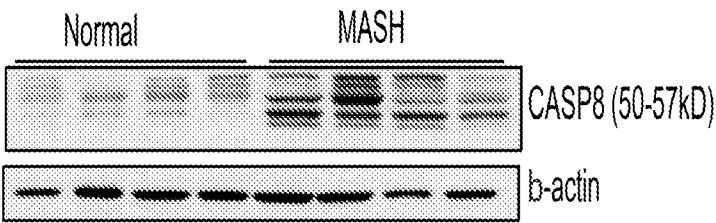


FIG. 14B

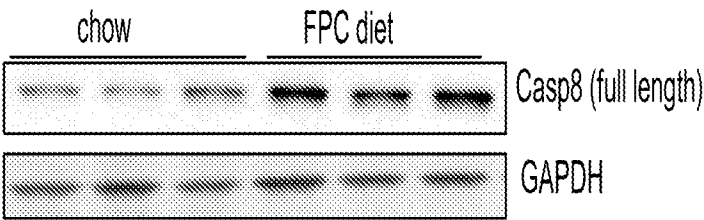


FIG. 14C

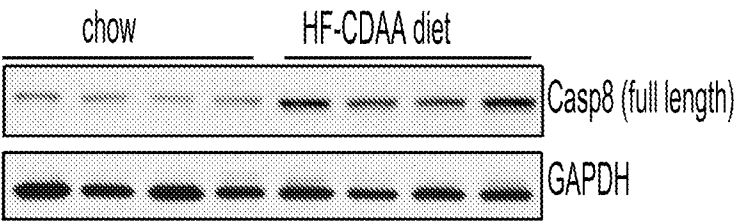


FIG. 14D

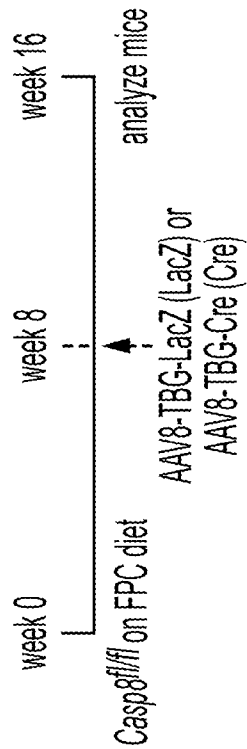


FIG. 15A

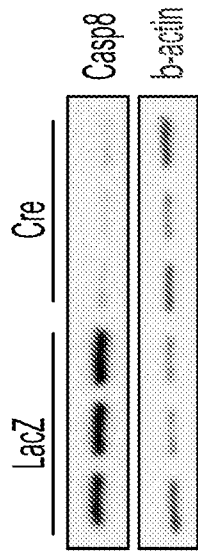


FIG. 15B

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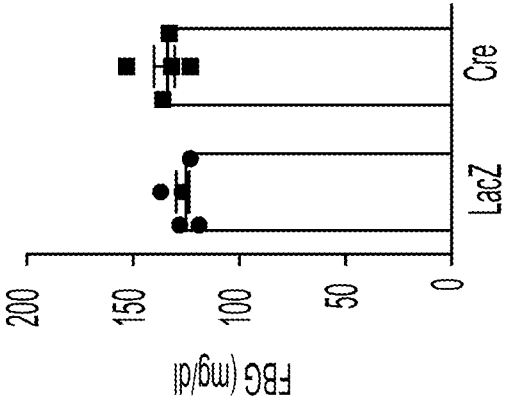


FIG. 15E

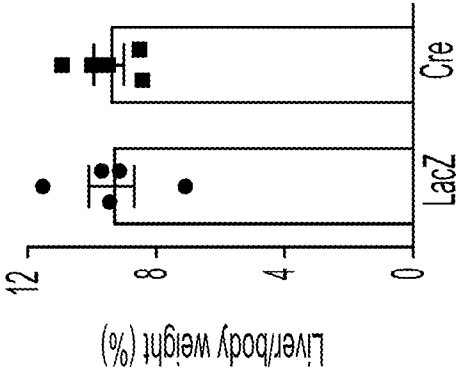


FIG. 15D

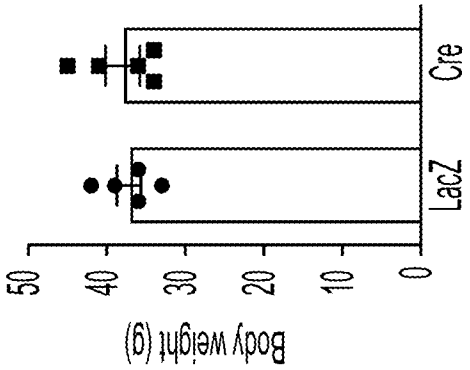


FIG. 15C

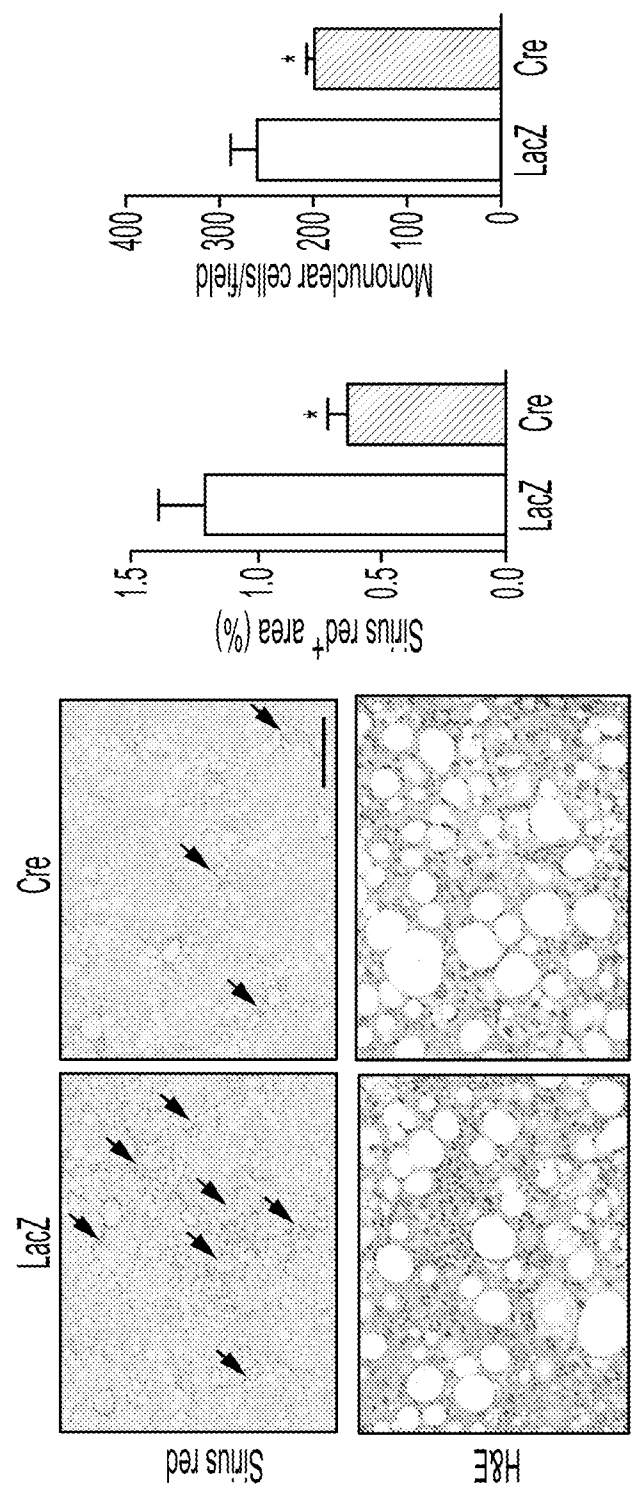


FIG. 15F

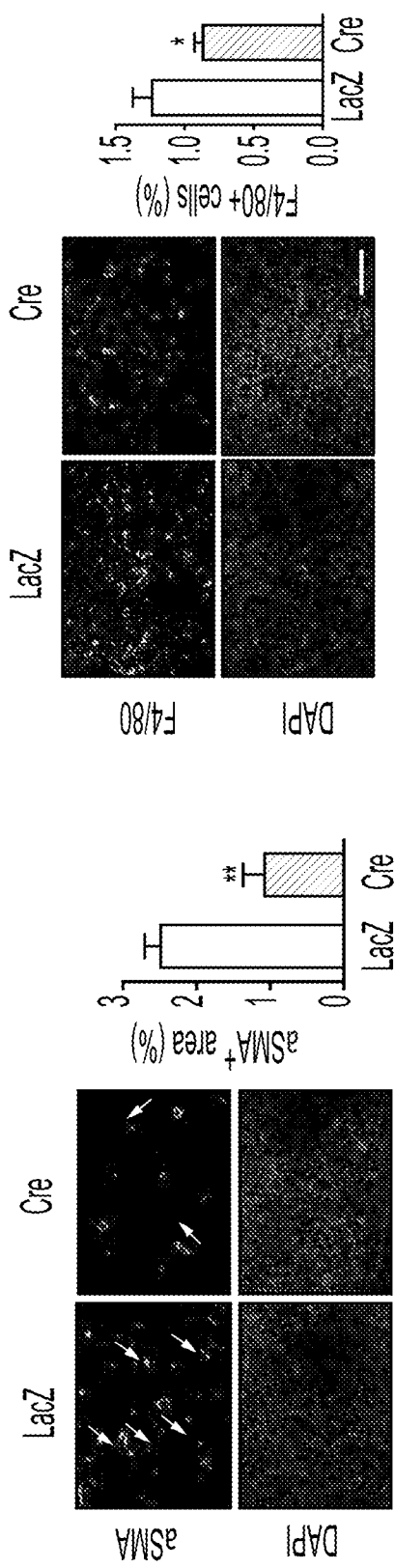


FIG. 15G

FIG. 15H

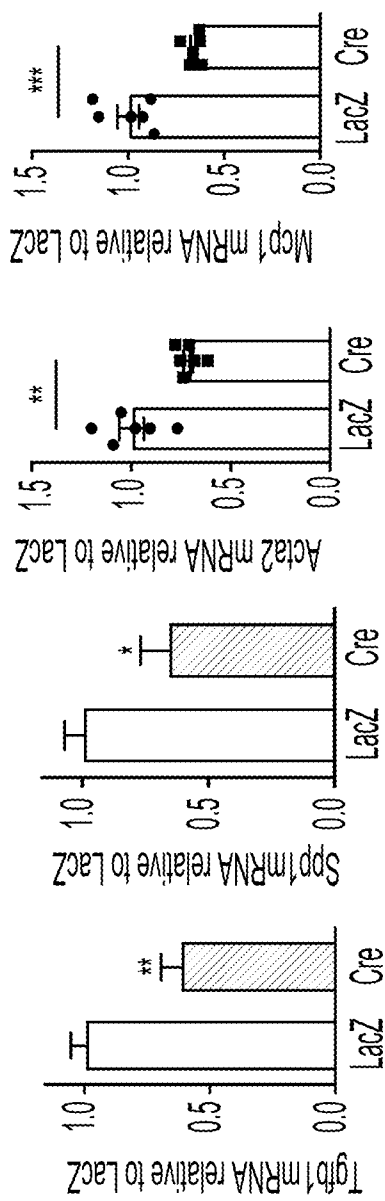


FIG. 15I

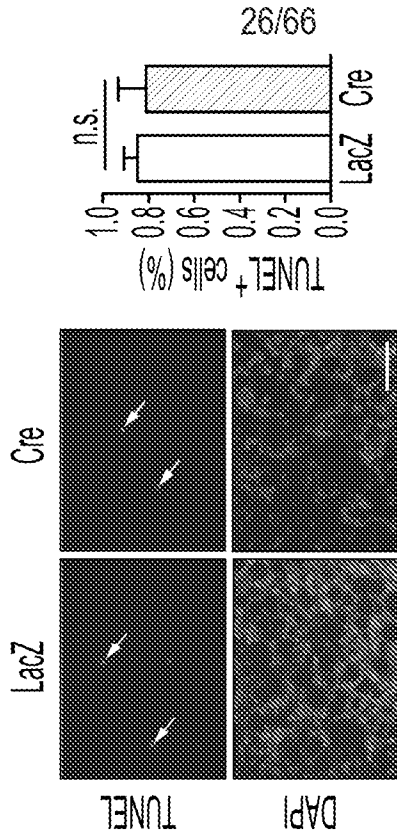


FIG. 15L

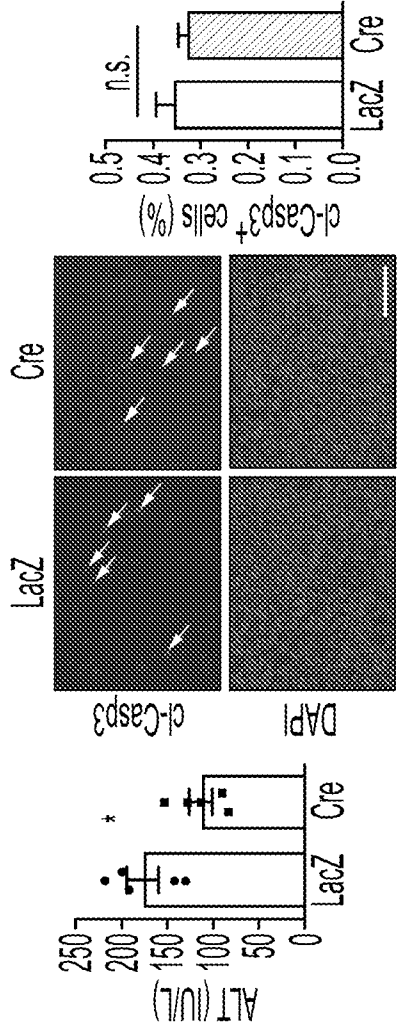


FIG. 15K

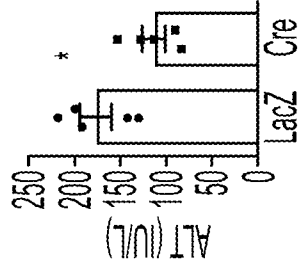


FIG. 15J

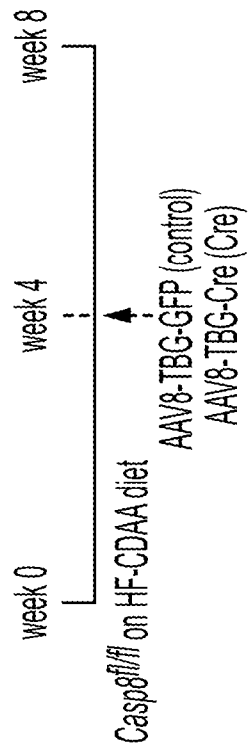


FIG. 16A

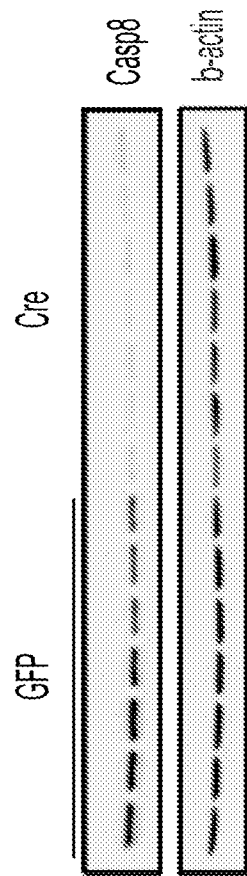


FIG. 16B

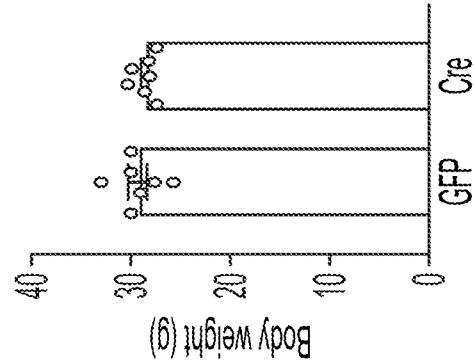


FIG. 16C

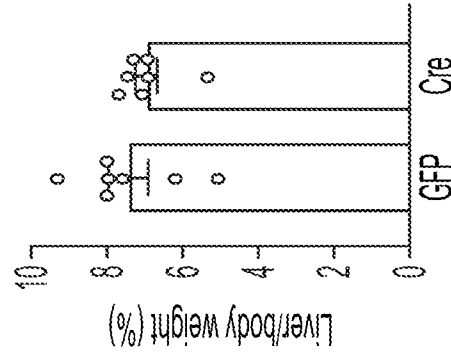


FIG. 16D

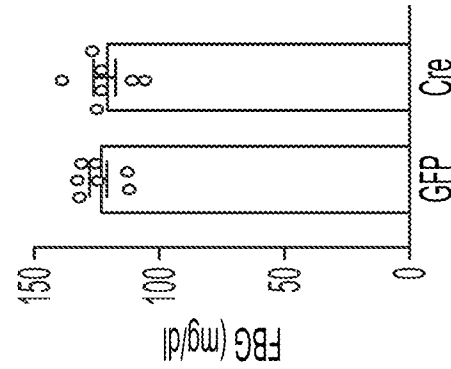


FIG. 16E

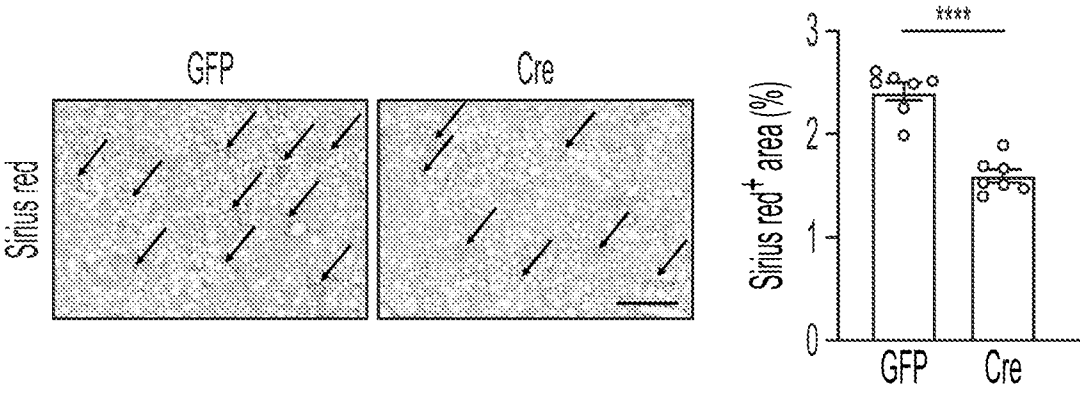


FIG. 16F

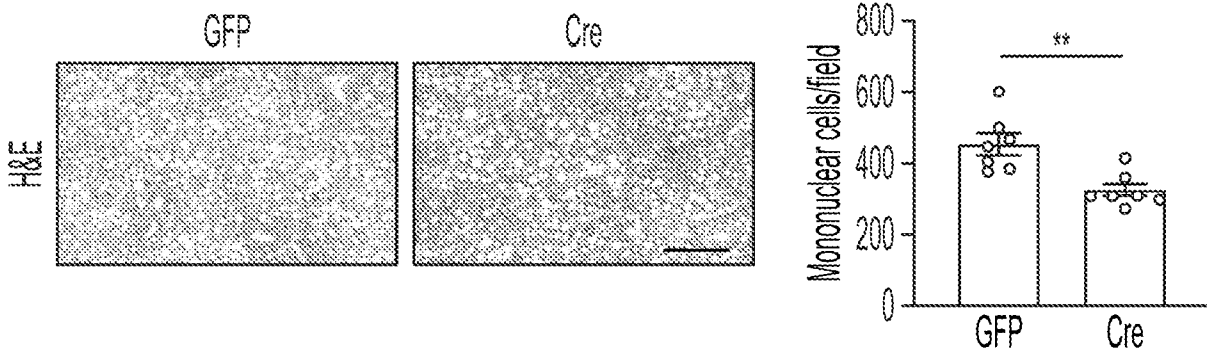


FIG. 16G

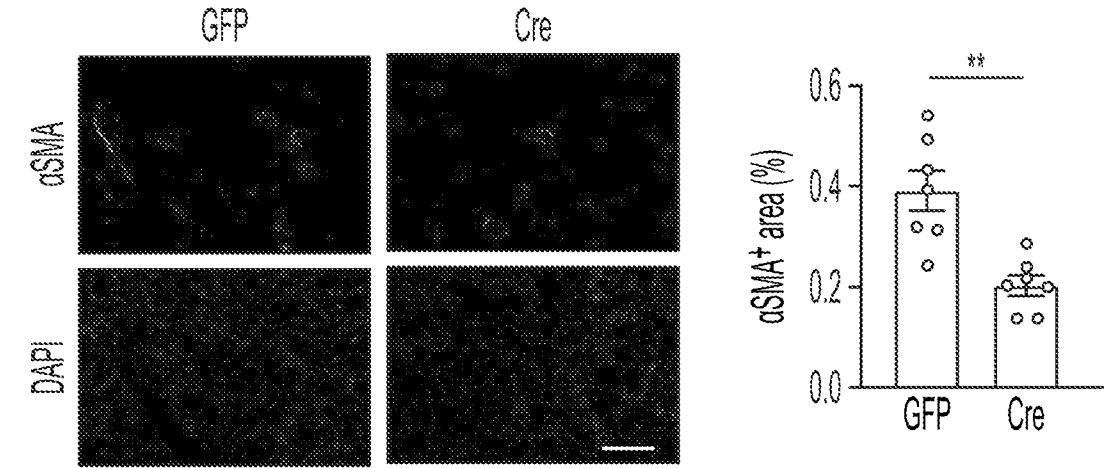


FIG. 16H

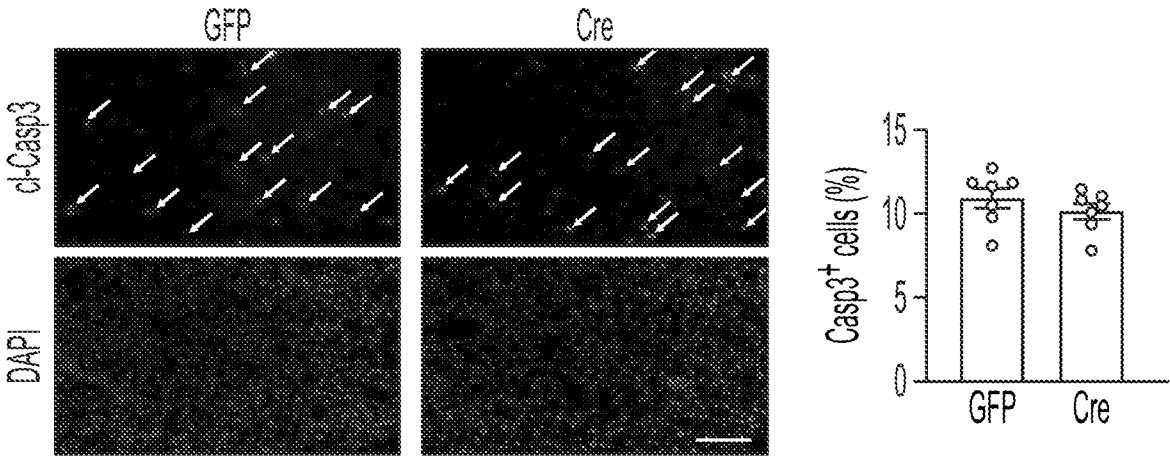


FIG. 16I

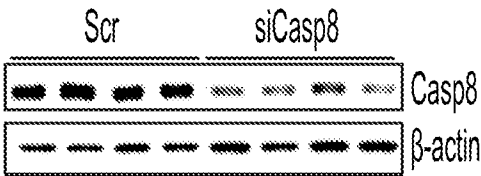


FIG. 17A

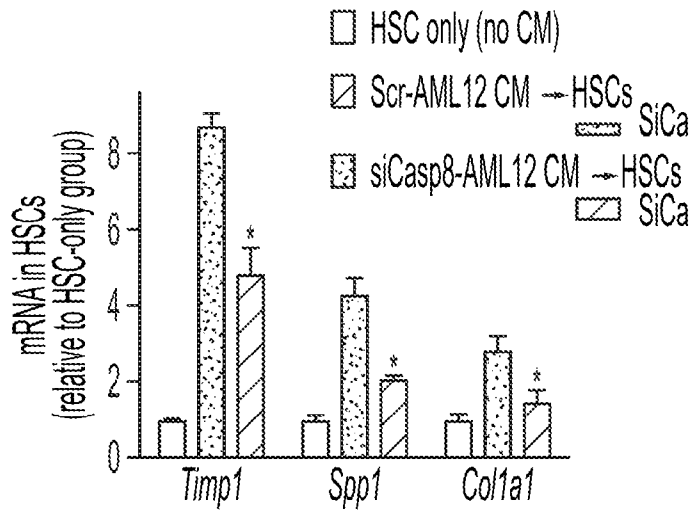


FIG. 17B

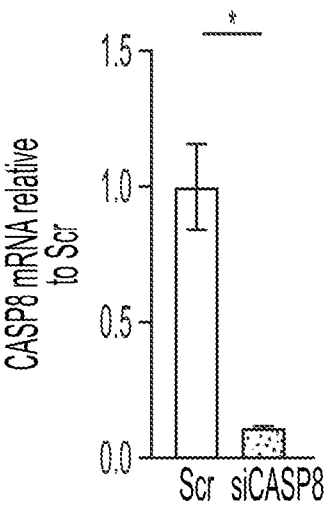


FIG. 17C

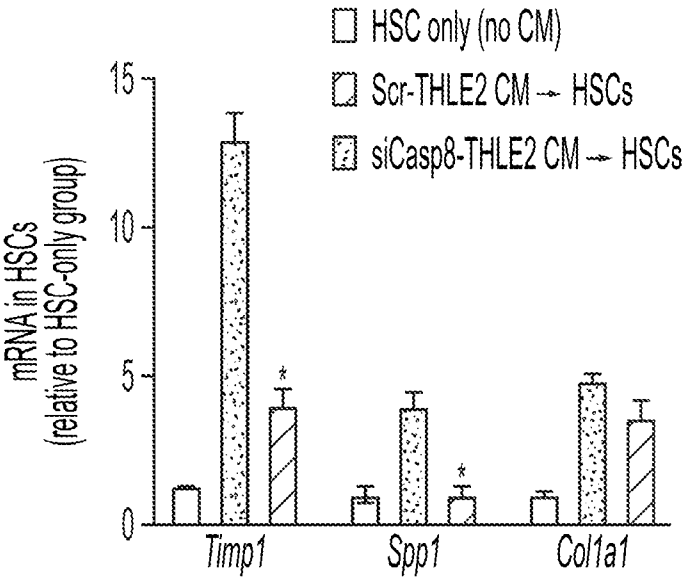


FIG. 17D

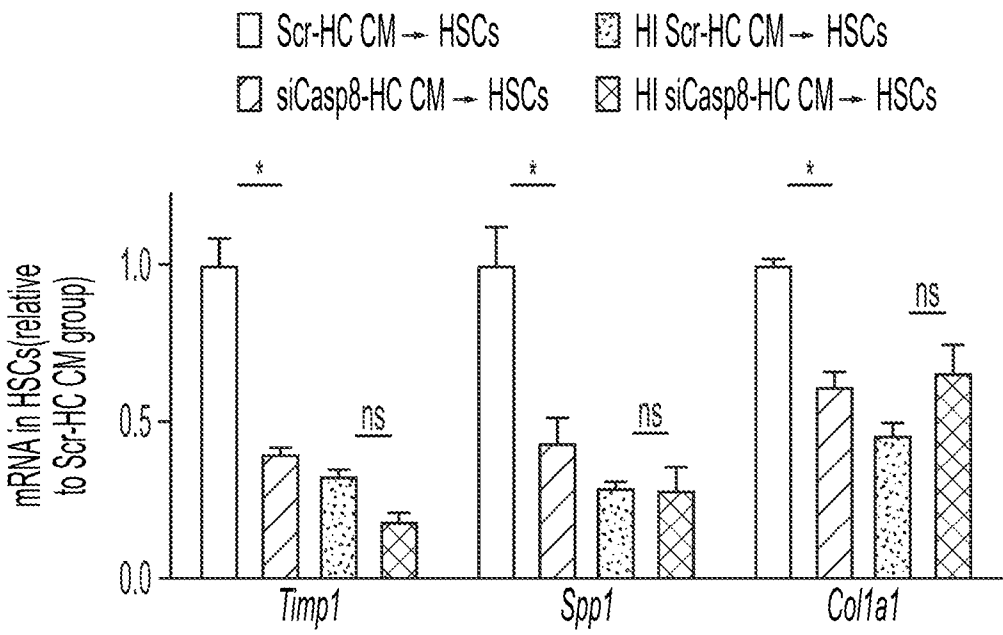


FIG. 17E

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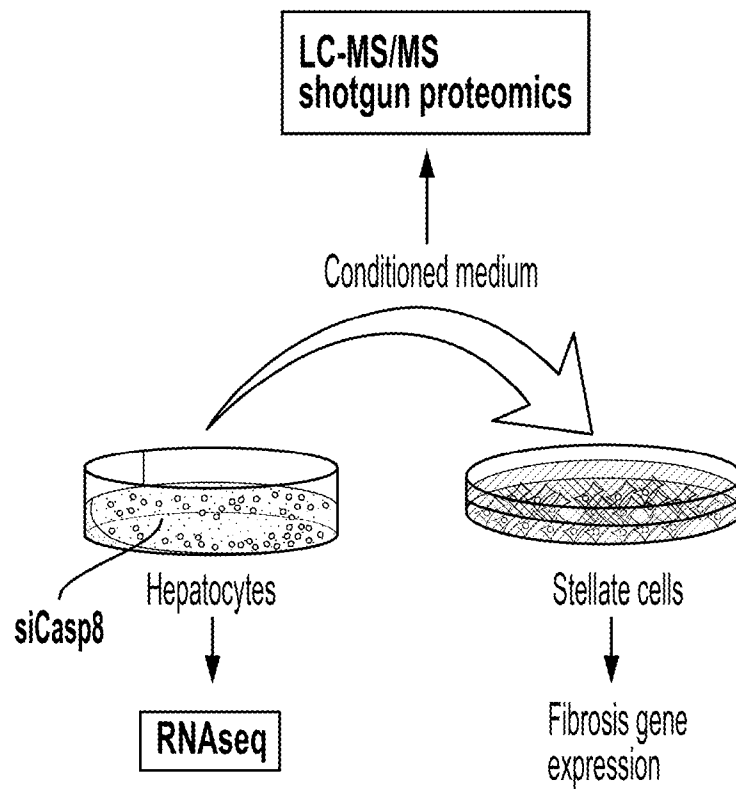


FIG. 18A

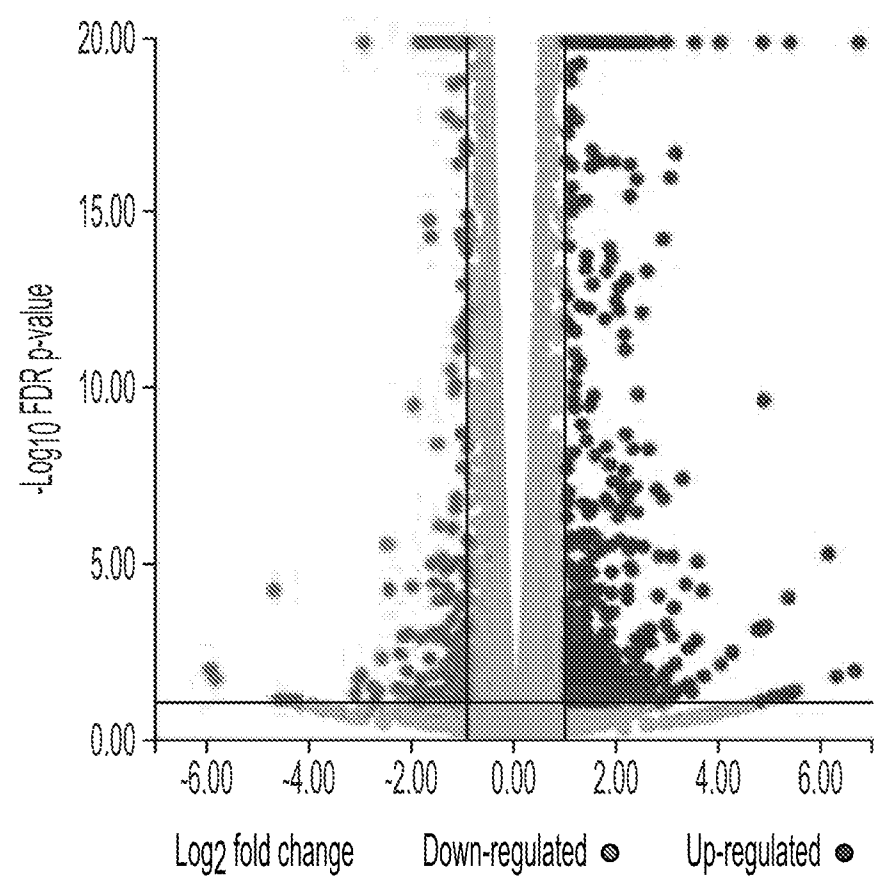


FIG. 18B

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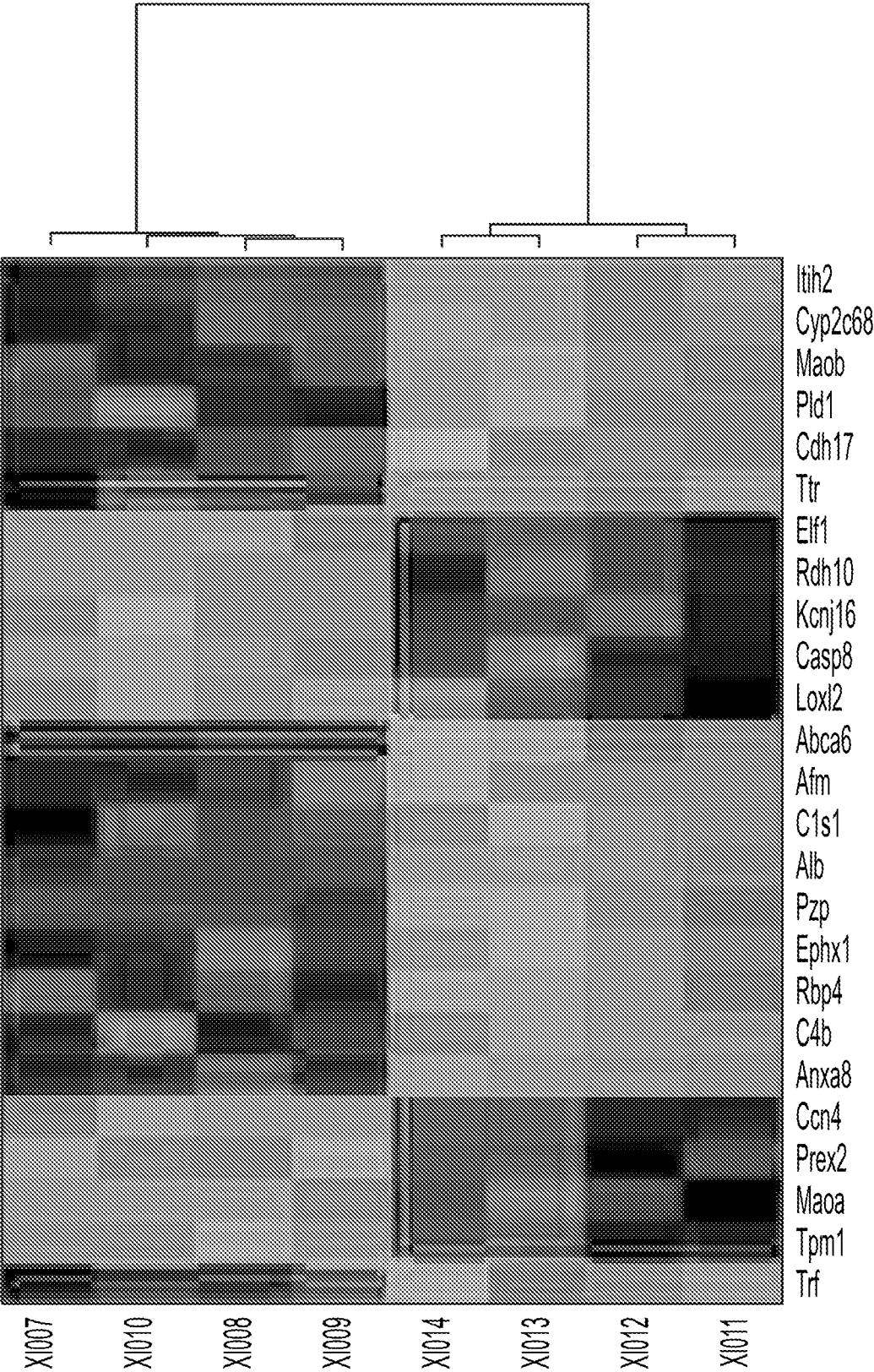


FIG. 18C

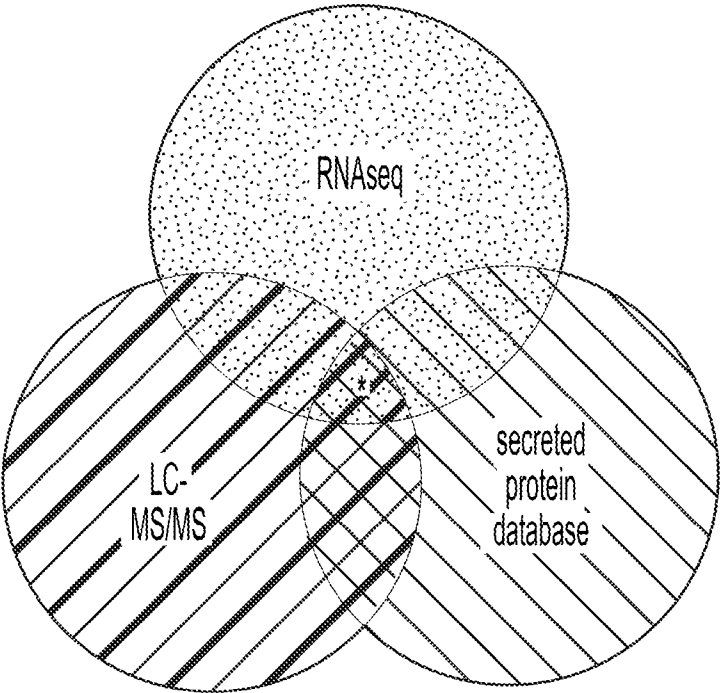


FIG. 18D

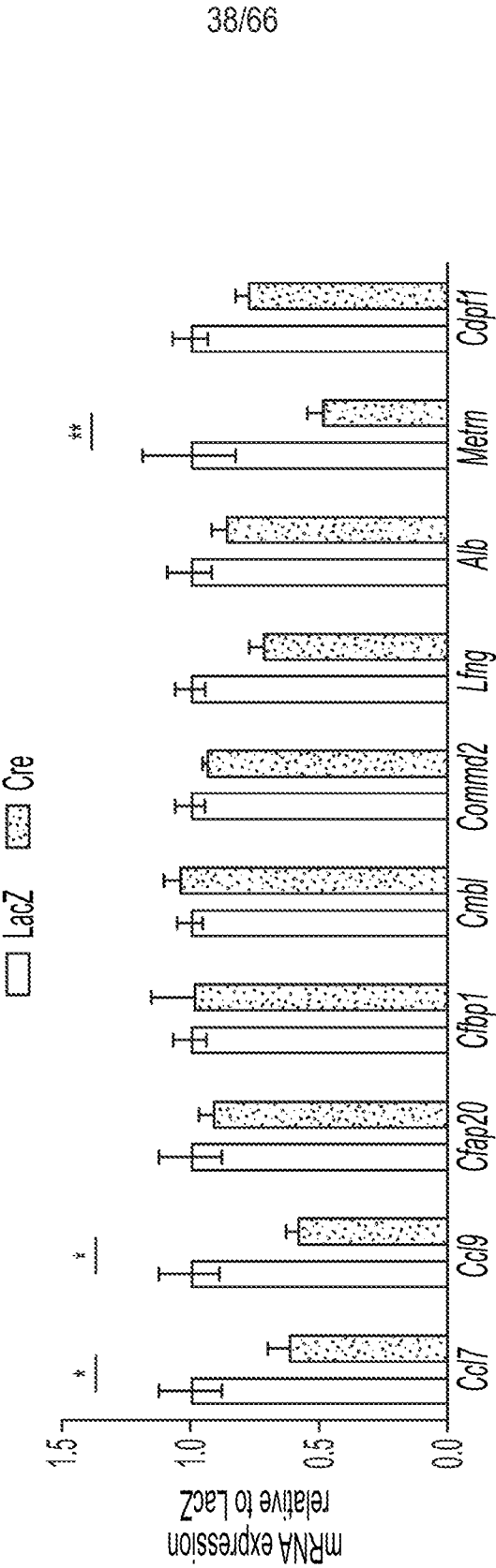


FIG. 18E

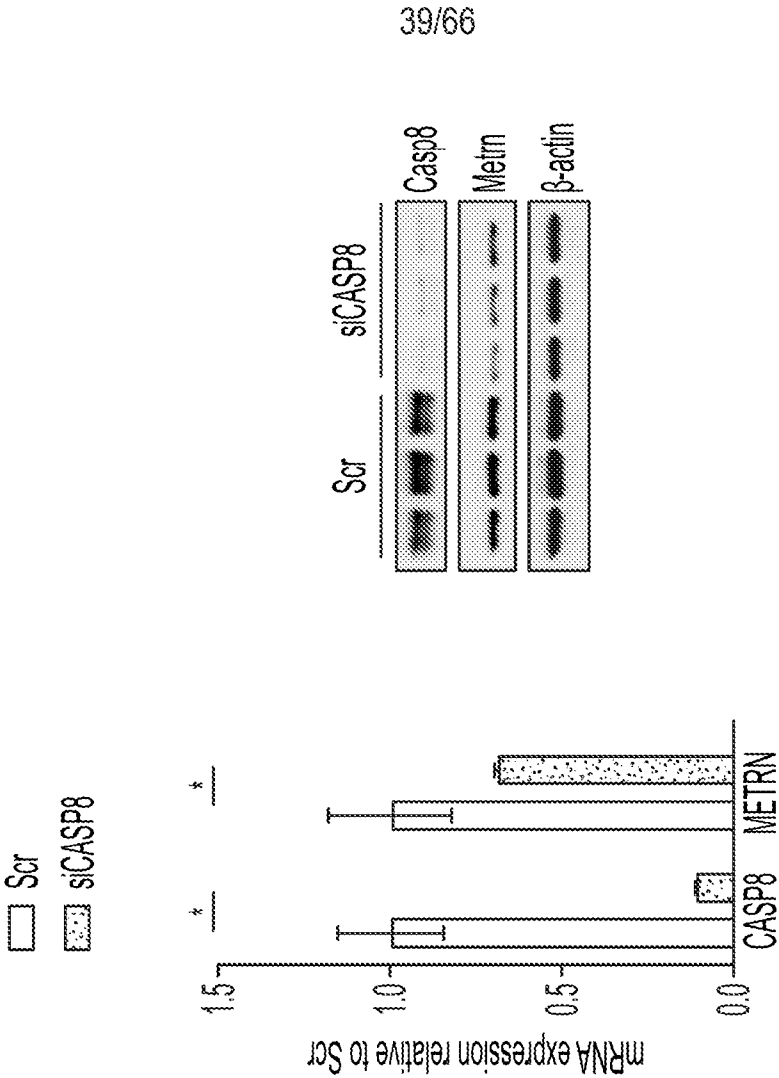


FIG. 18G

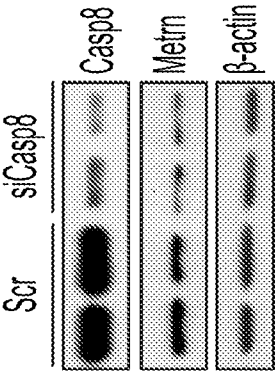


FIG. 18F

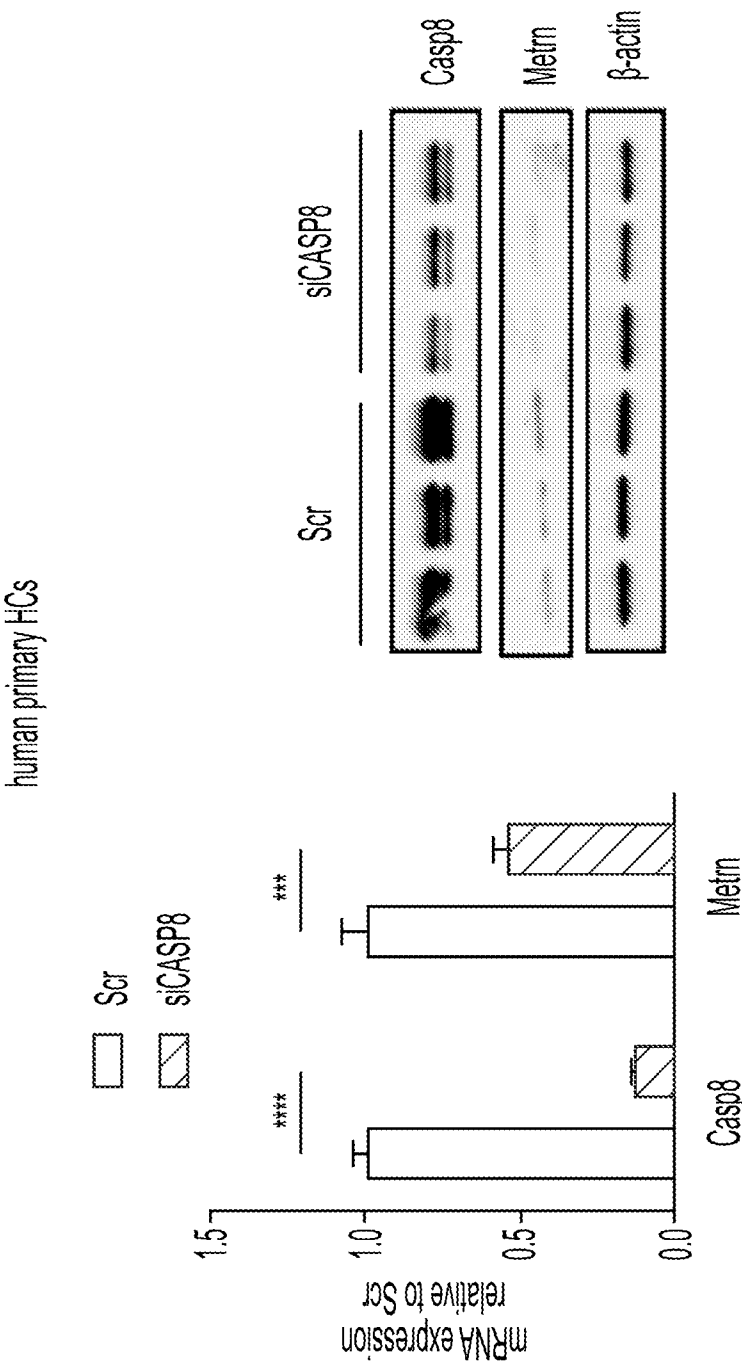


FIG. 18H

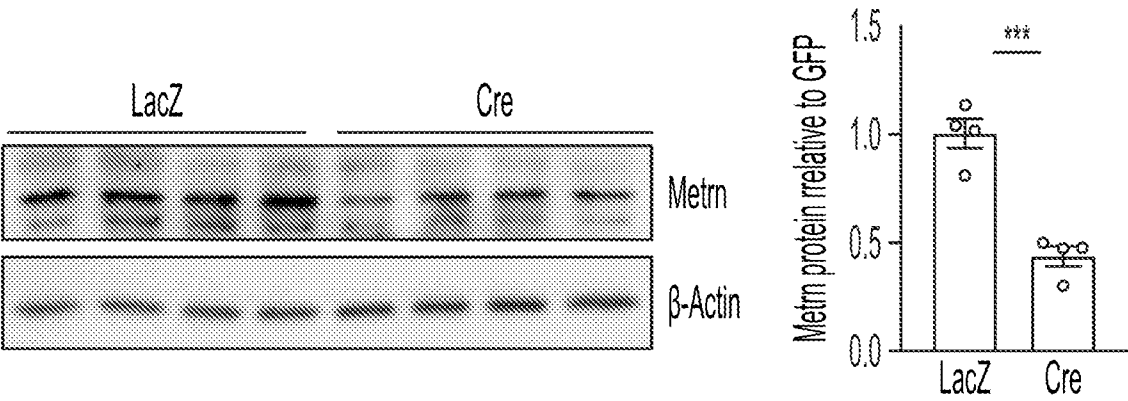


FIG. 18I

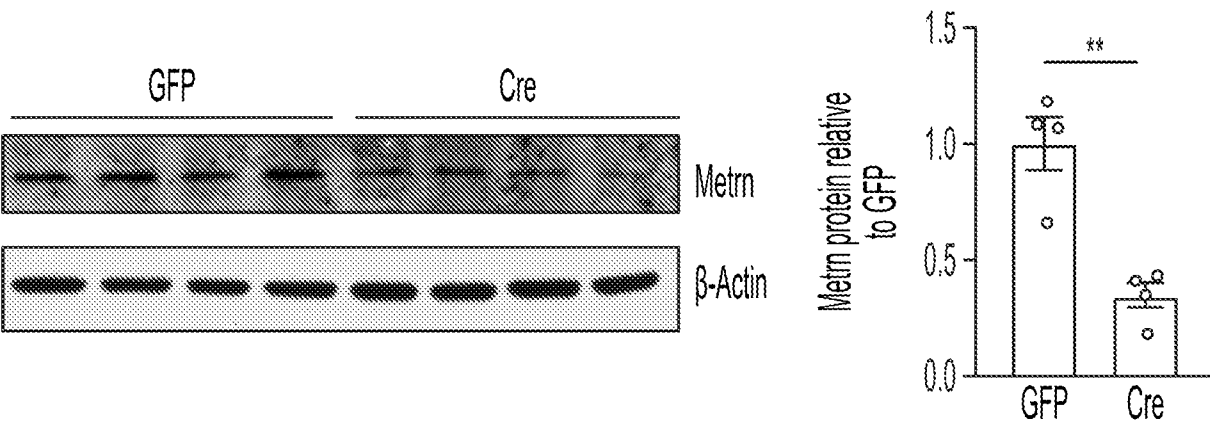


FIG. 18J

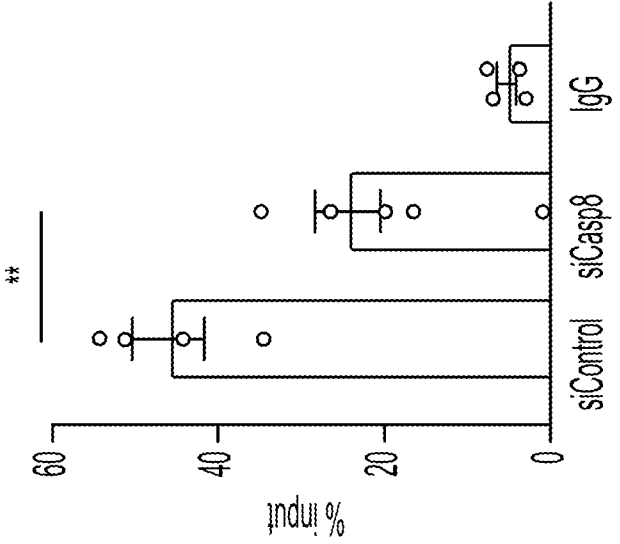


FIG. 18L

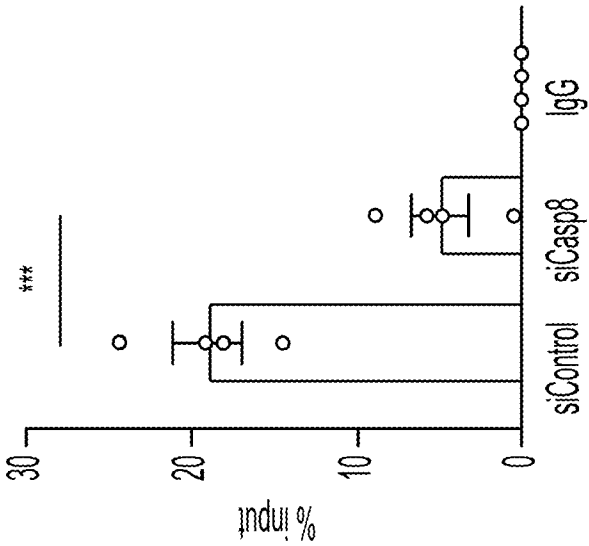


FIG. 18K

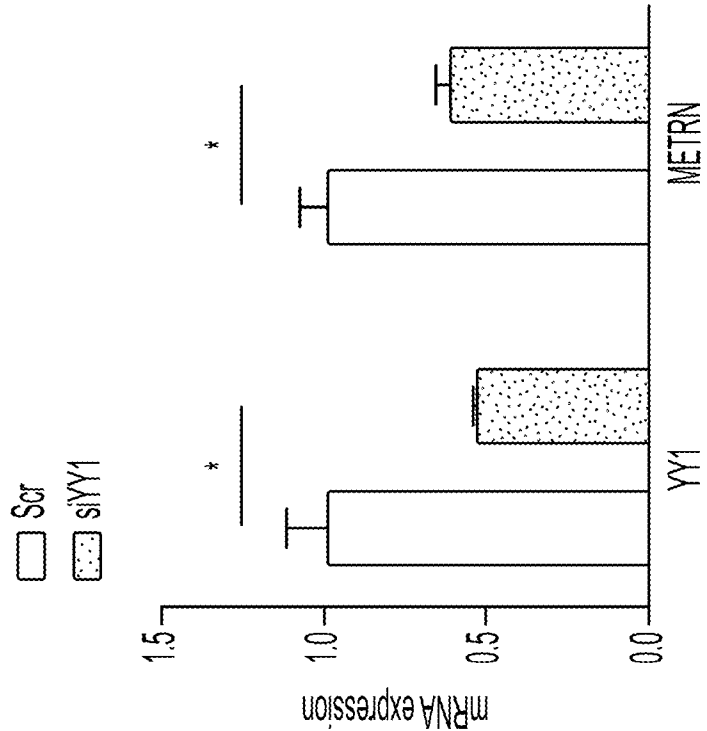


FIG. 18N

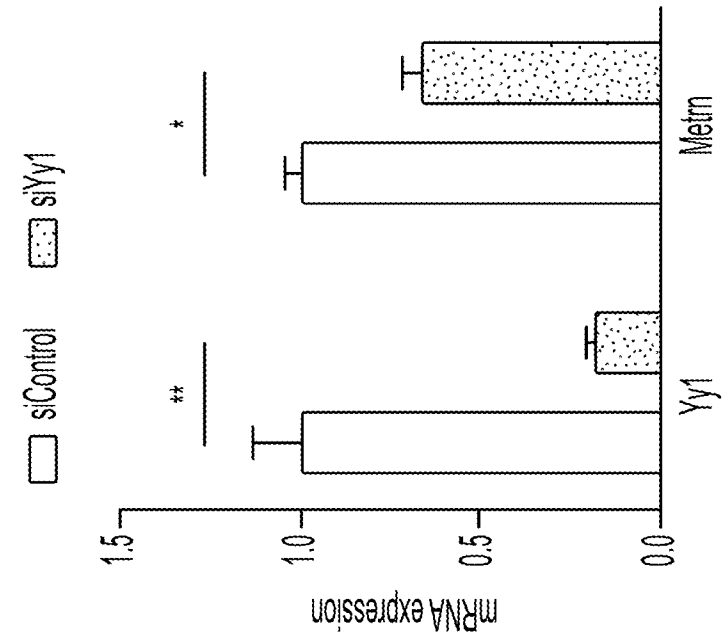


FIG. 18M

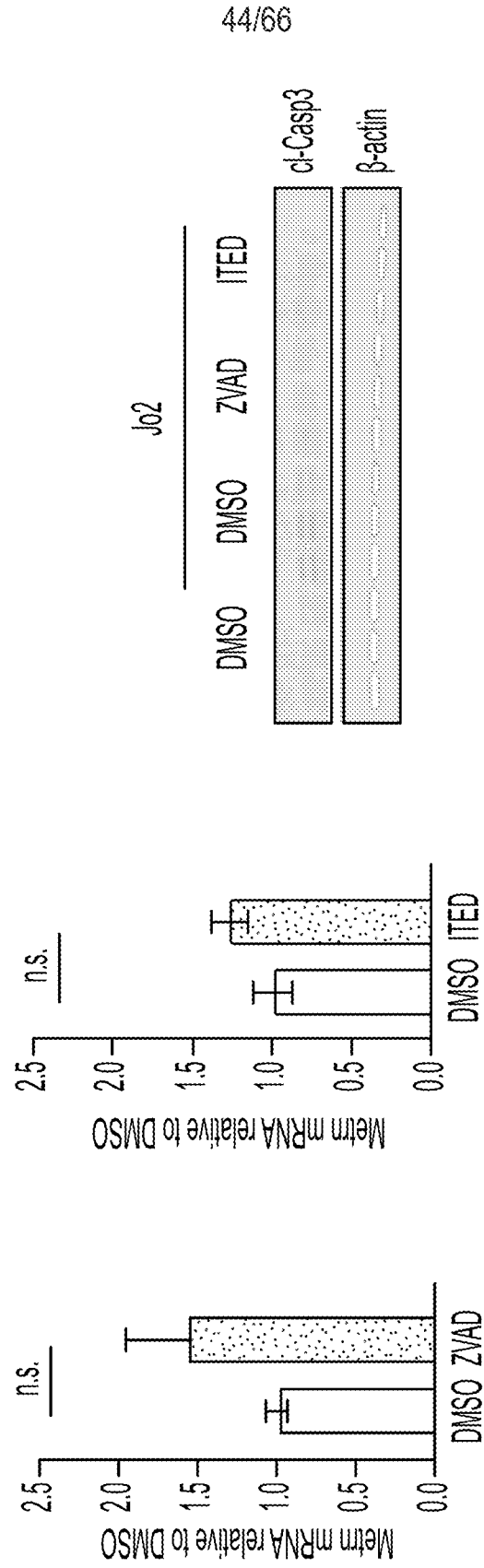


FIG. 18P

FIG. 18O

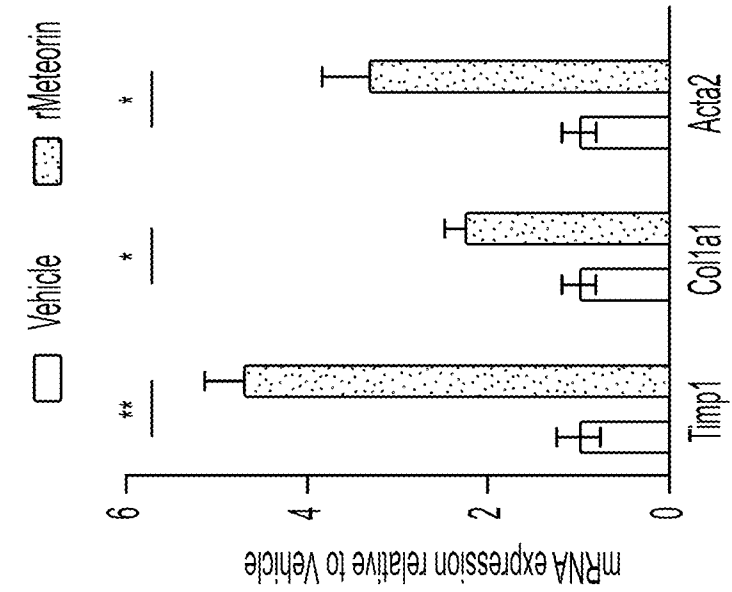


FIG. 19A

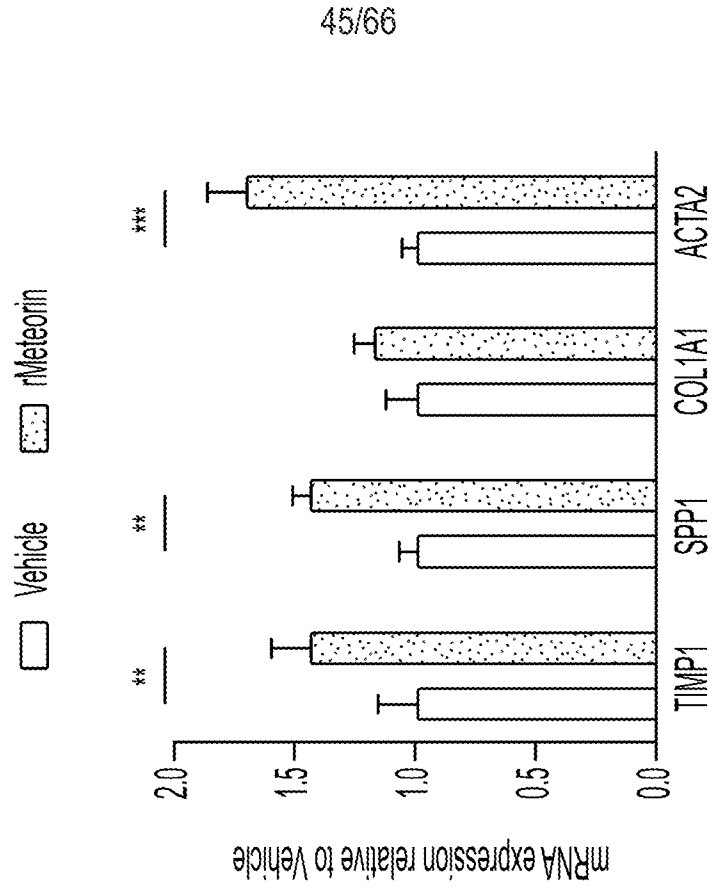


FIG. 19B

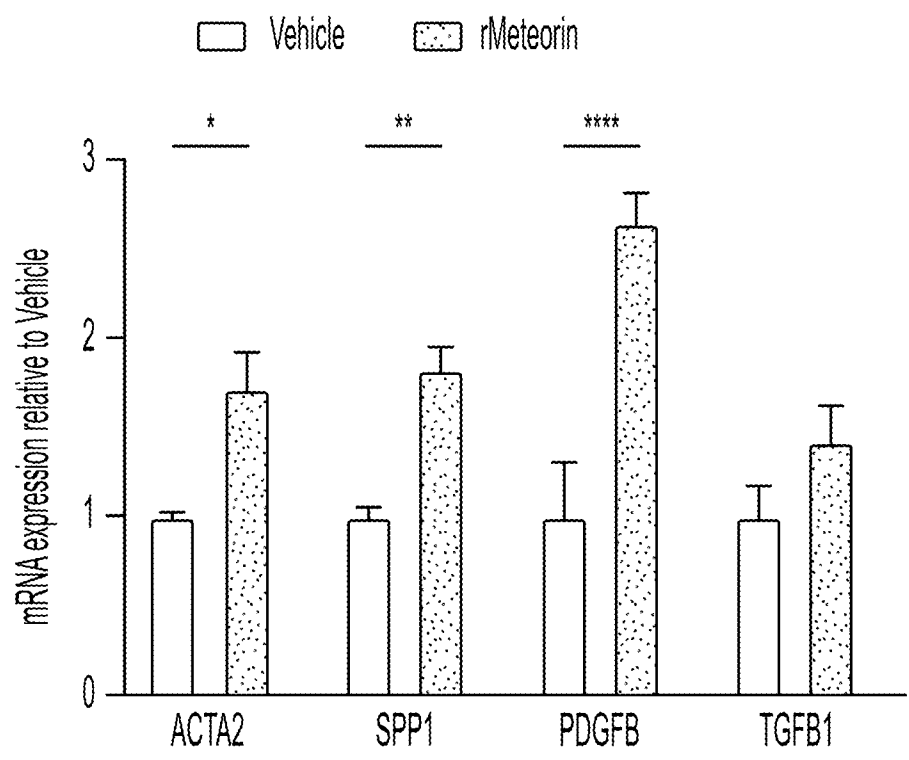


FIG. 19C

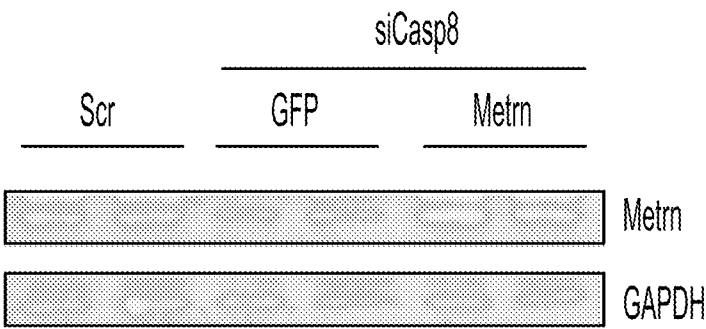


FIG. 19D

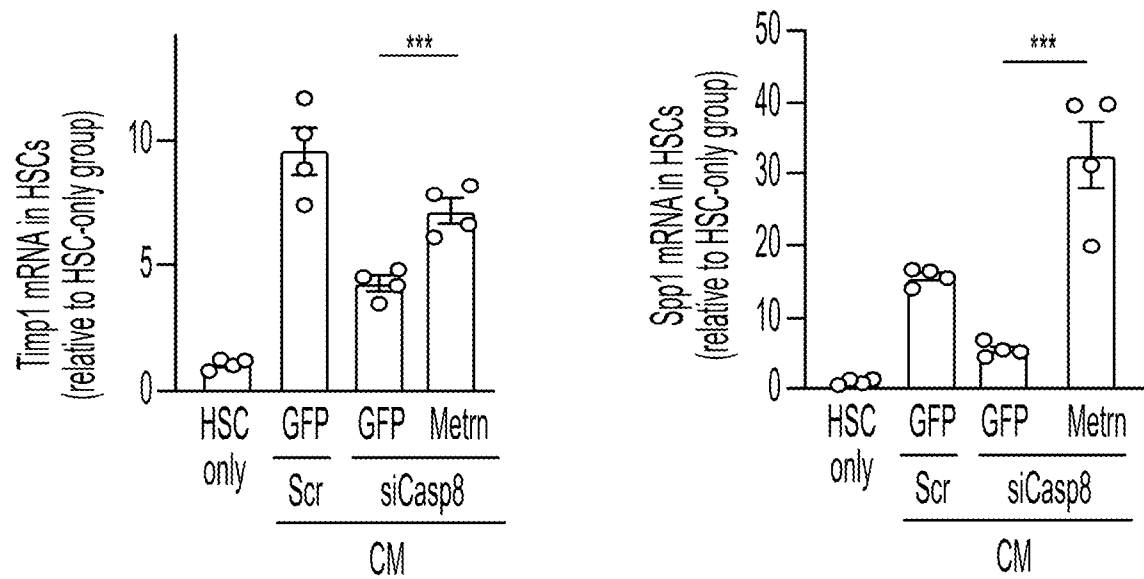


FIG. 19E

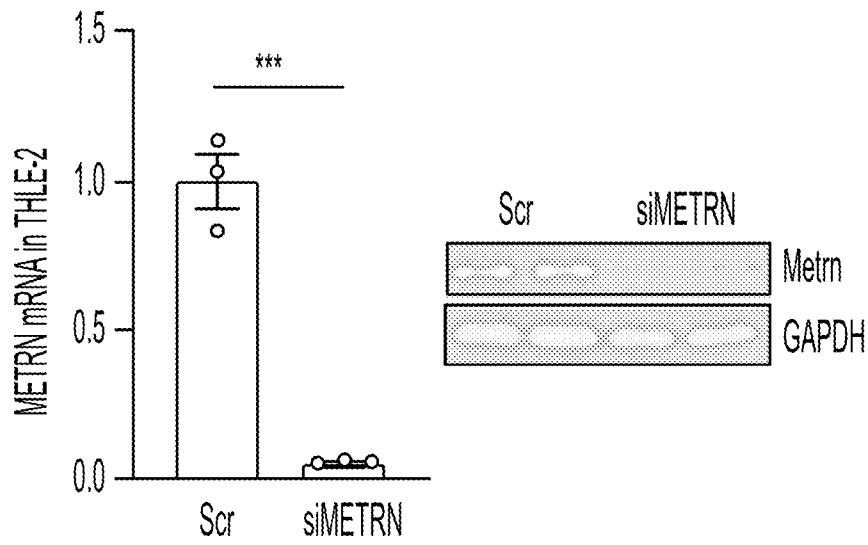


FIG. 19F

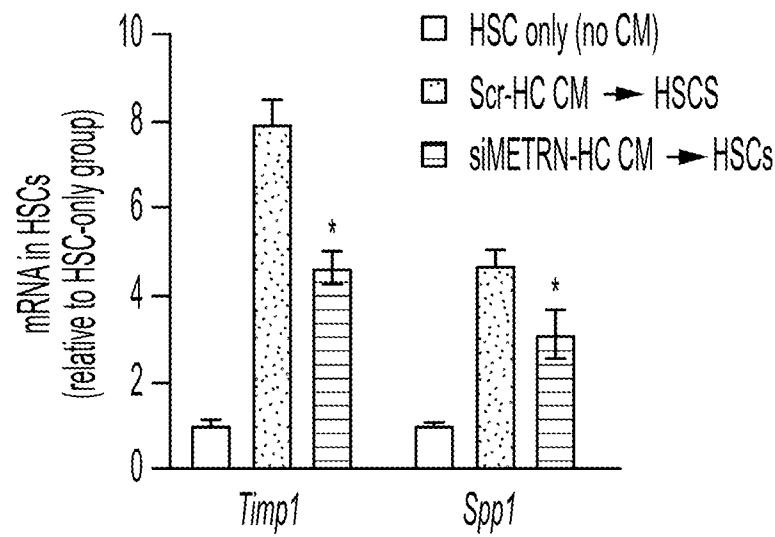


FIG. 19G

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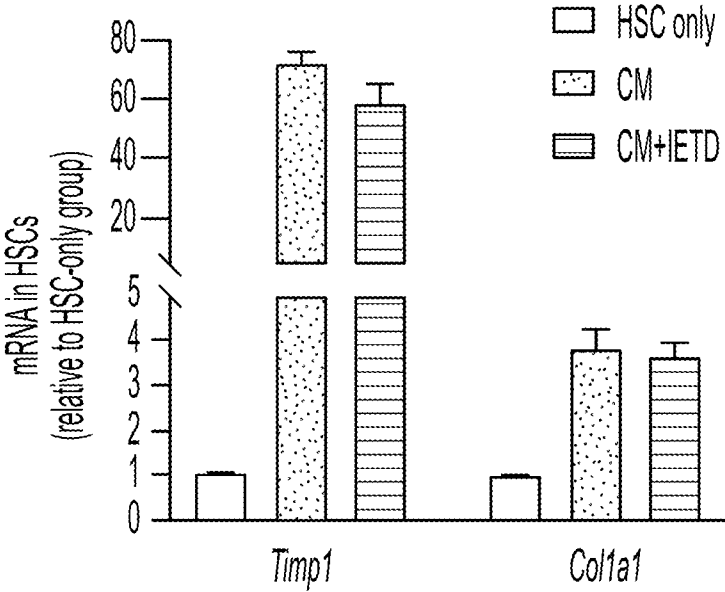


FIG. 19H

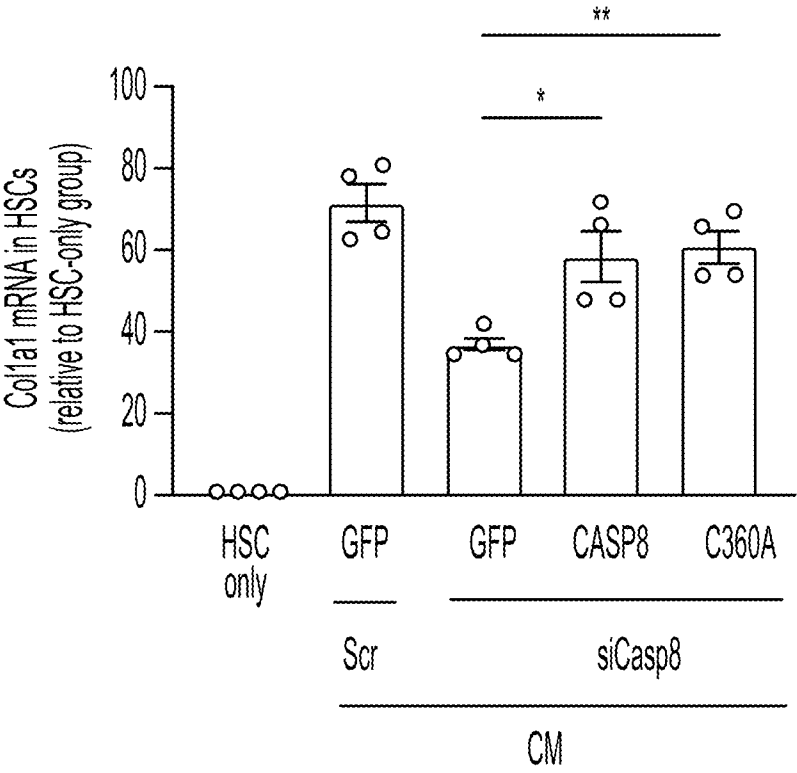


FIG. 19I

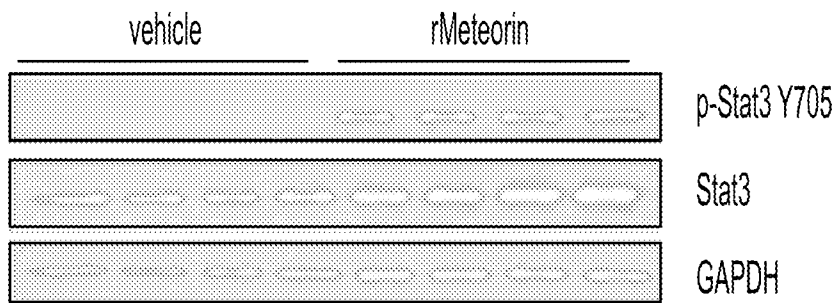


FIG. 19J

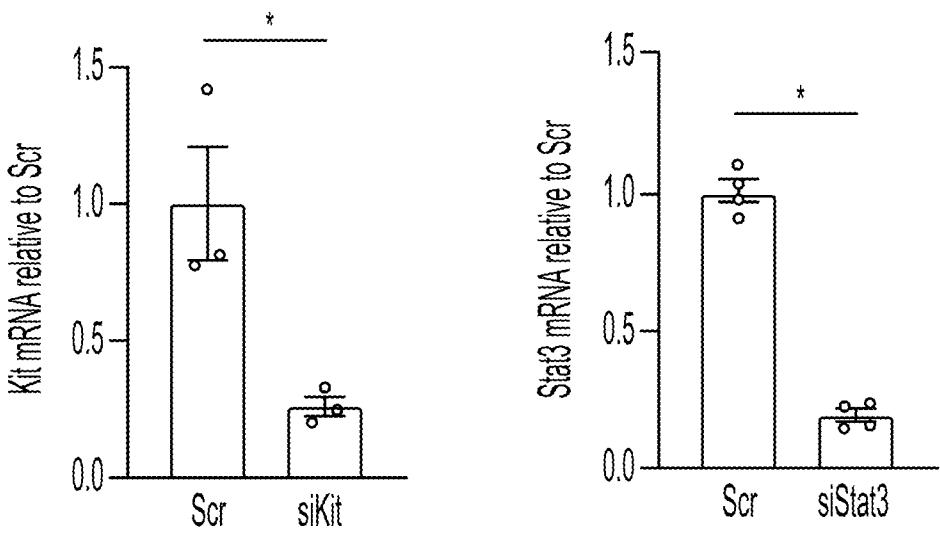


FIG. 19K

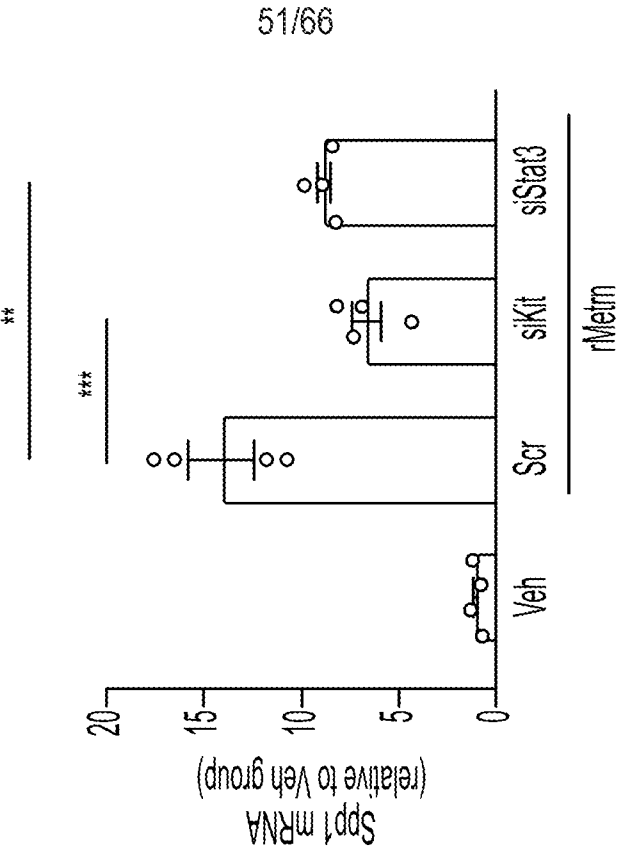
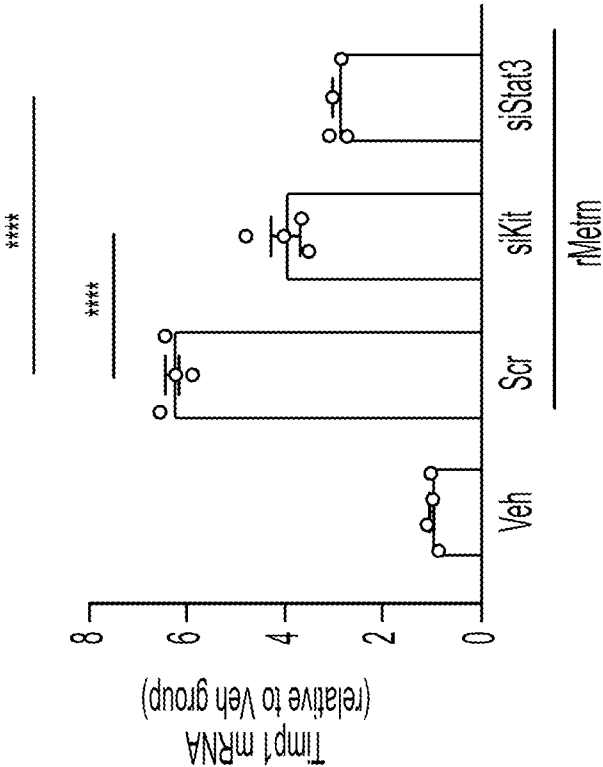


FIG. 19L



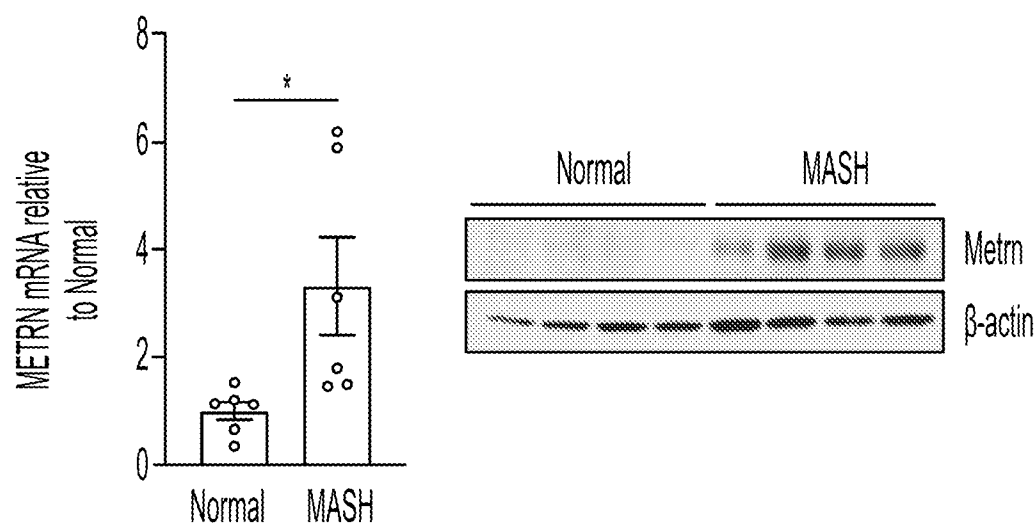


FIG. 20A

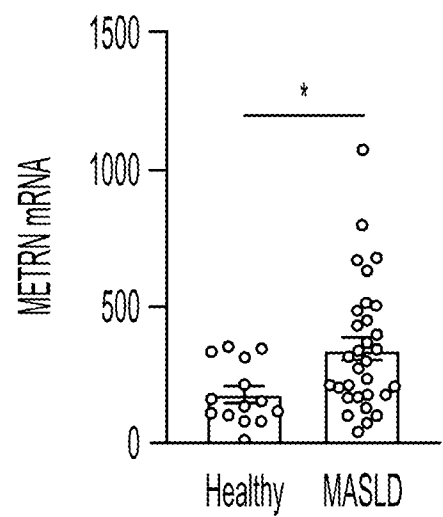


FIG. 20B

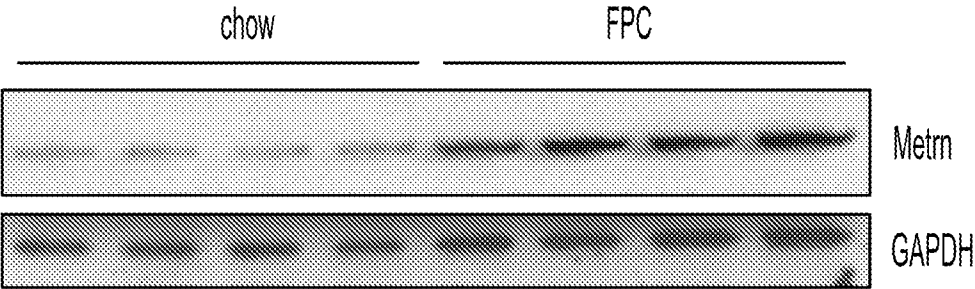


FIG. 20C

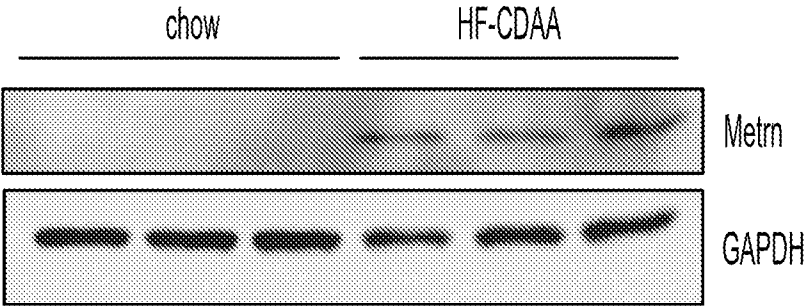


FIG. 20D

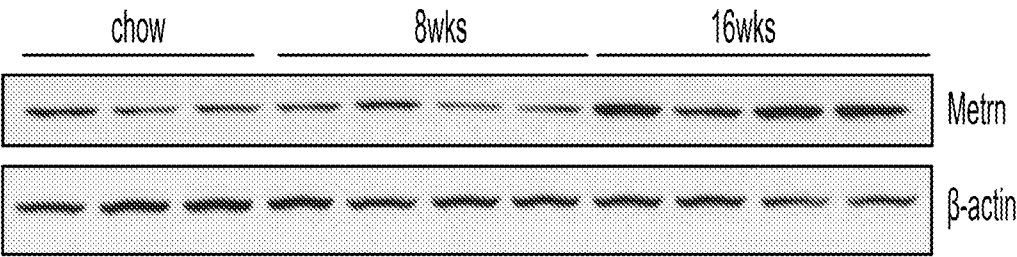


FIG. 20E

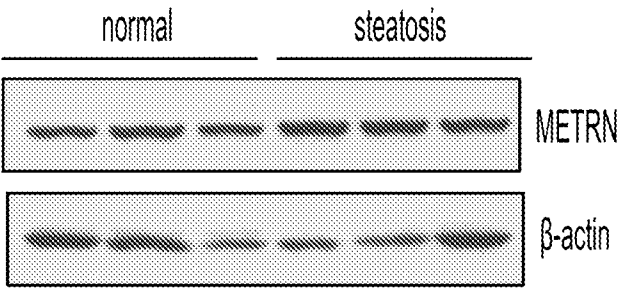


FIG. 20F



FIG. 20G

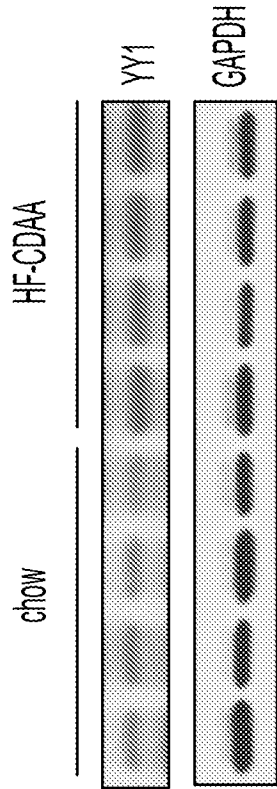


FIG. 20H

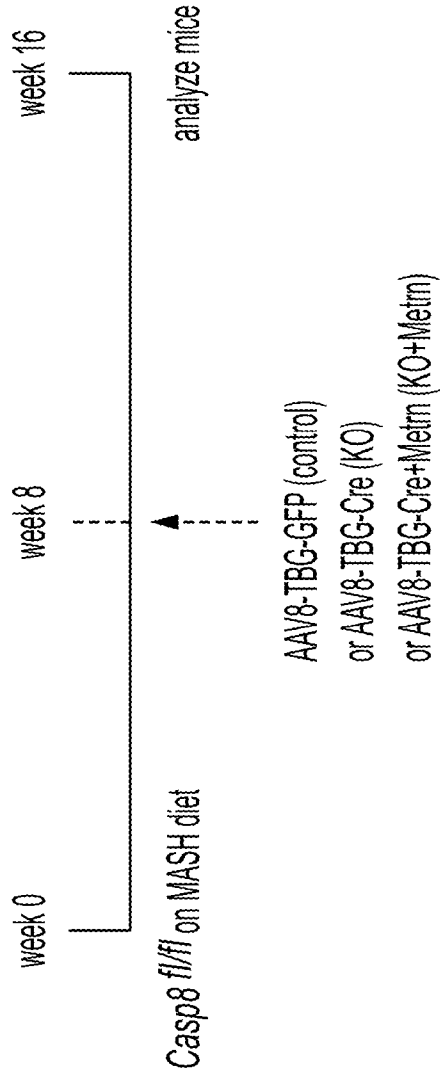


FIG. 21A

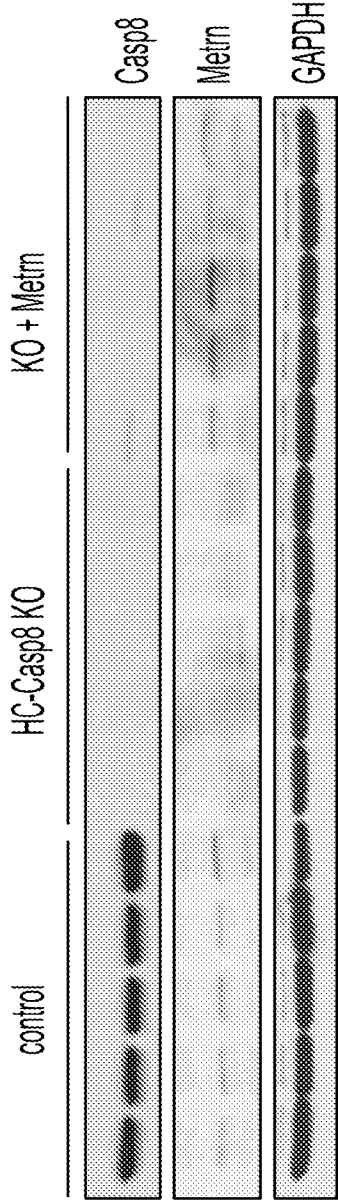


FIG. 21B

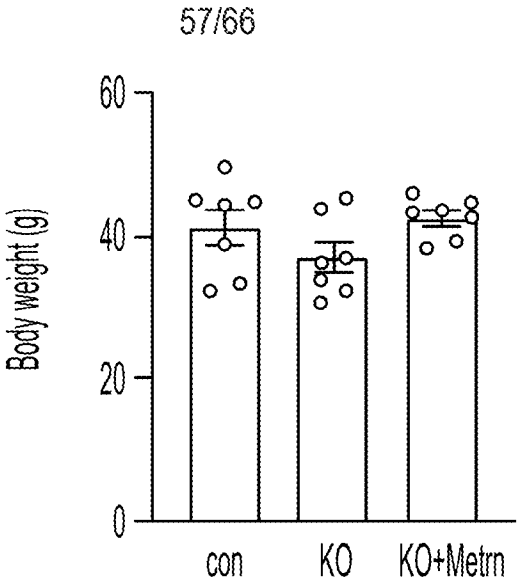


FIG. 21C

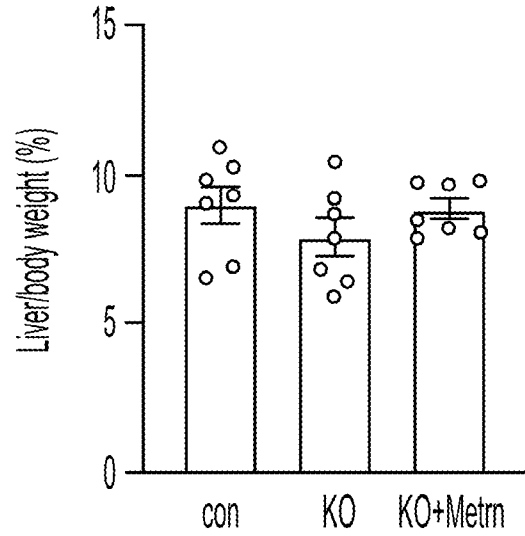


FIG. 21D

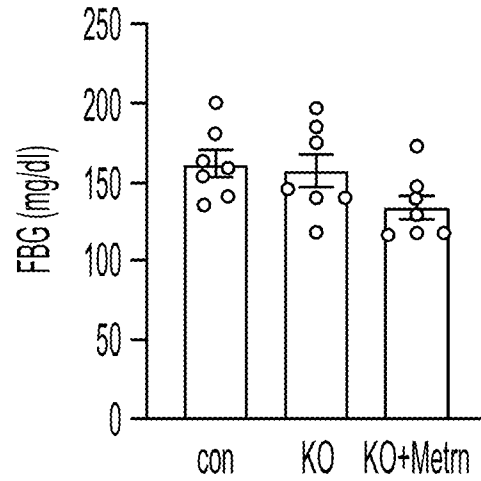


FIG. 21E

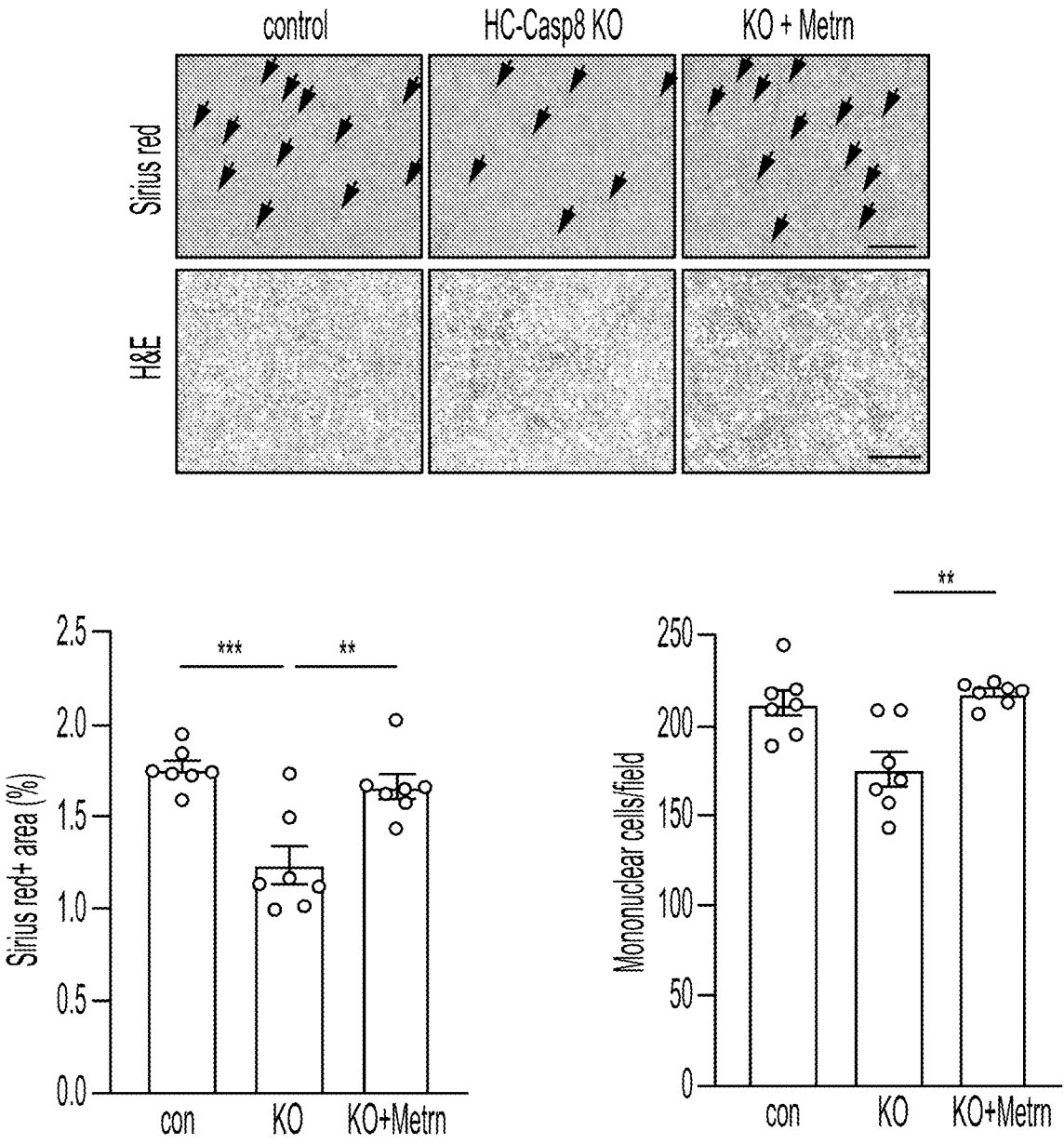


FIG. 21F

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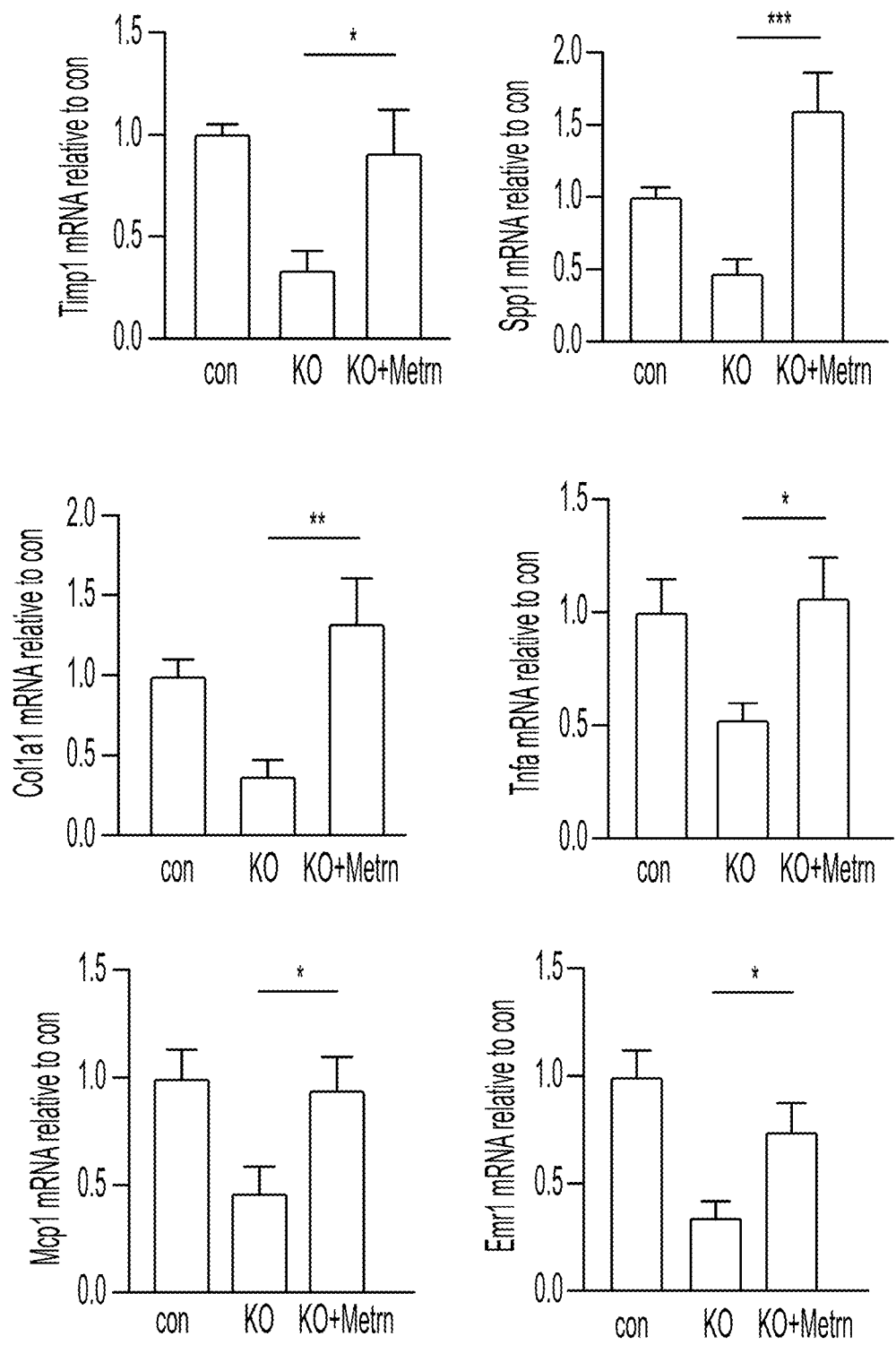


FIG. 21G

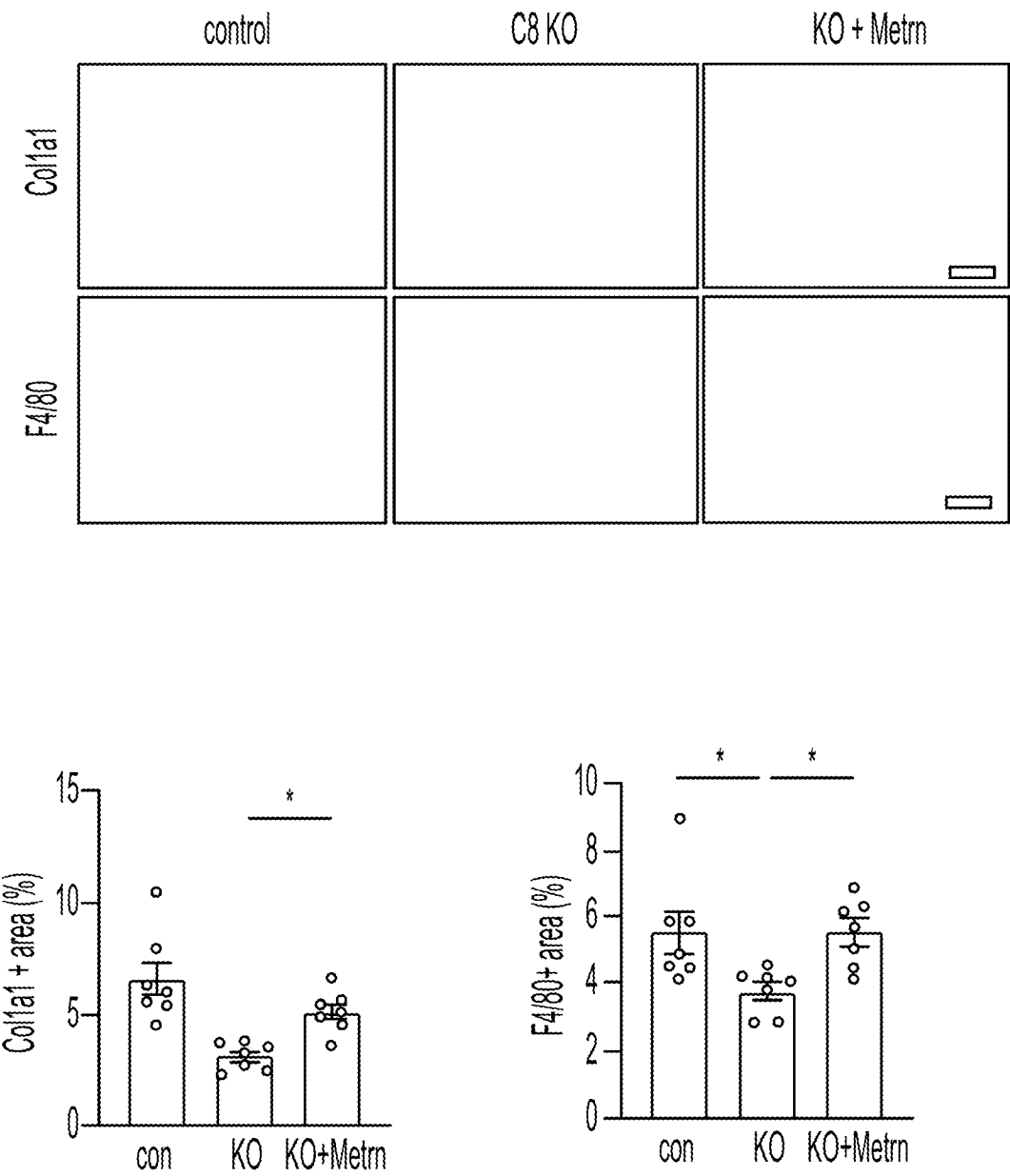


FIG. 21H

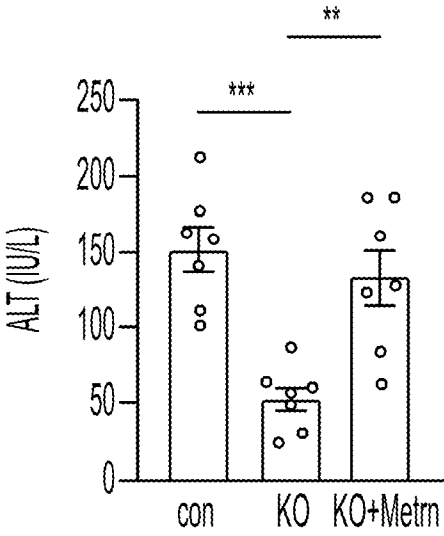


FIG. 21I

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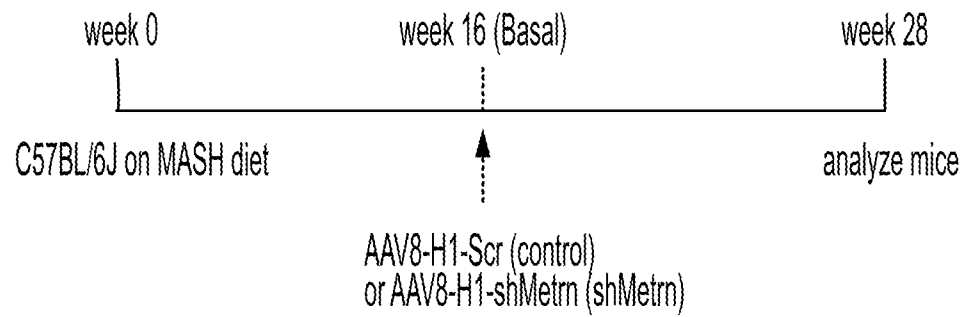


FIG. 22A

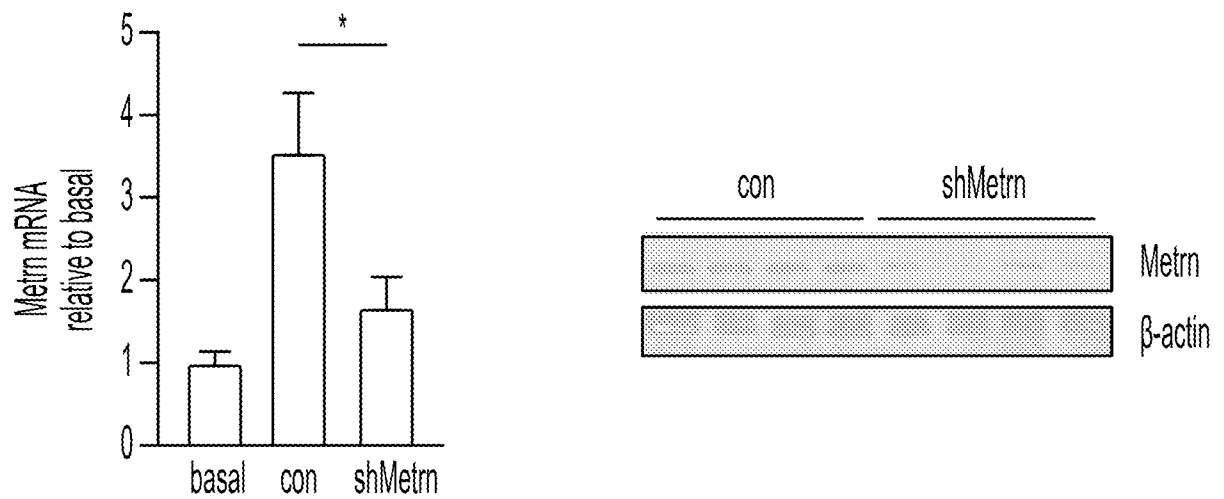


FIG. 22B

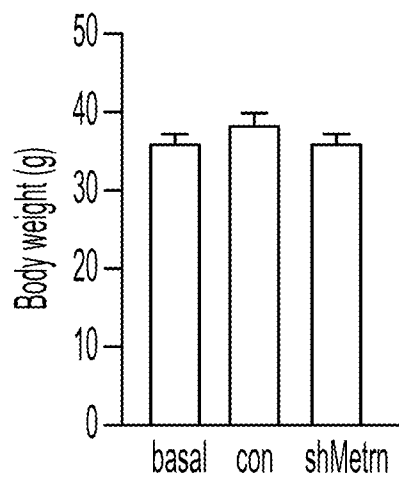


FIG. 22C

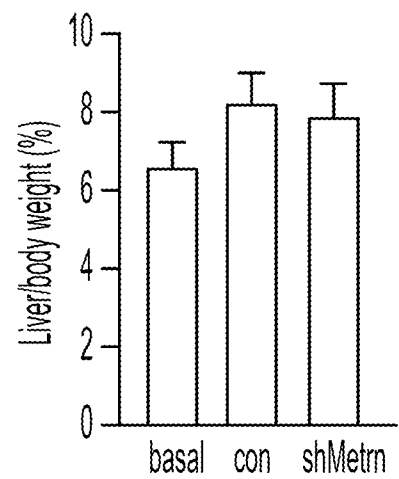


FIG. 22D

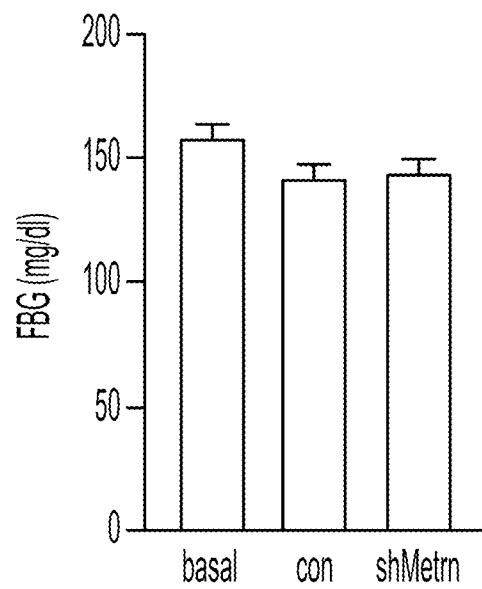


FIG. 22E

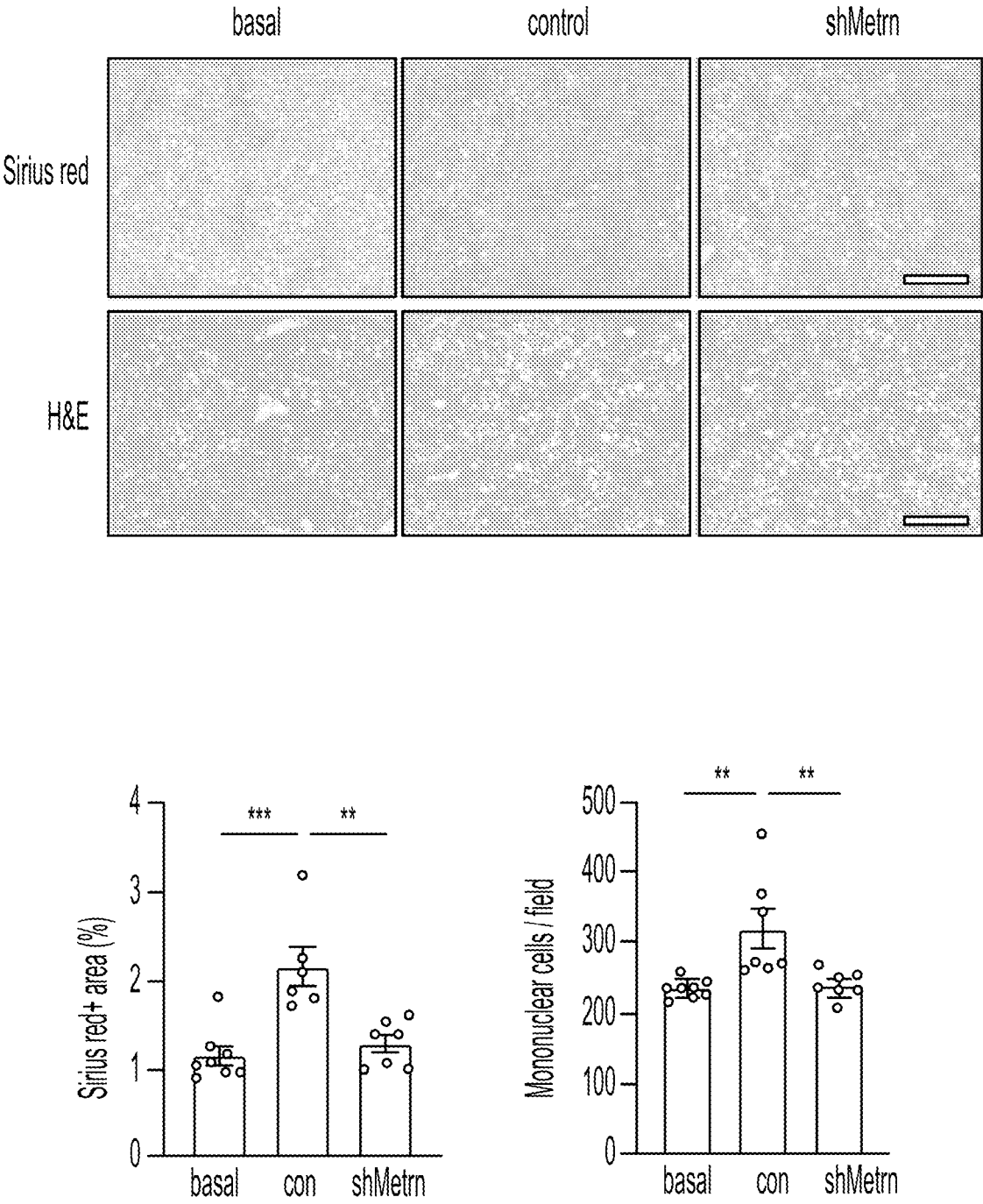


FIG. 22F

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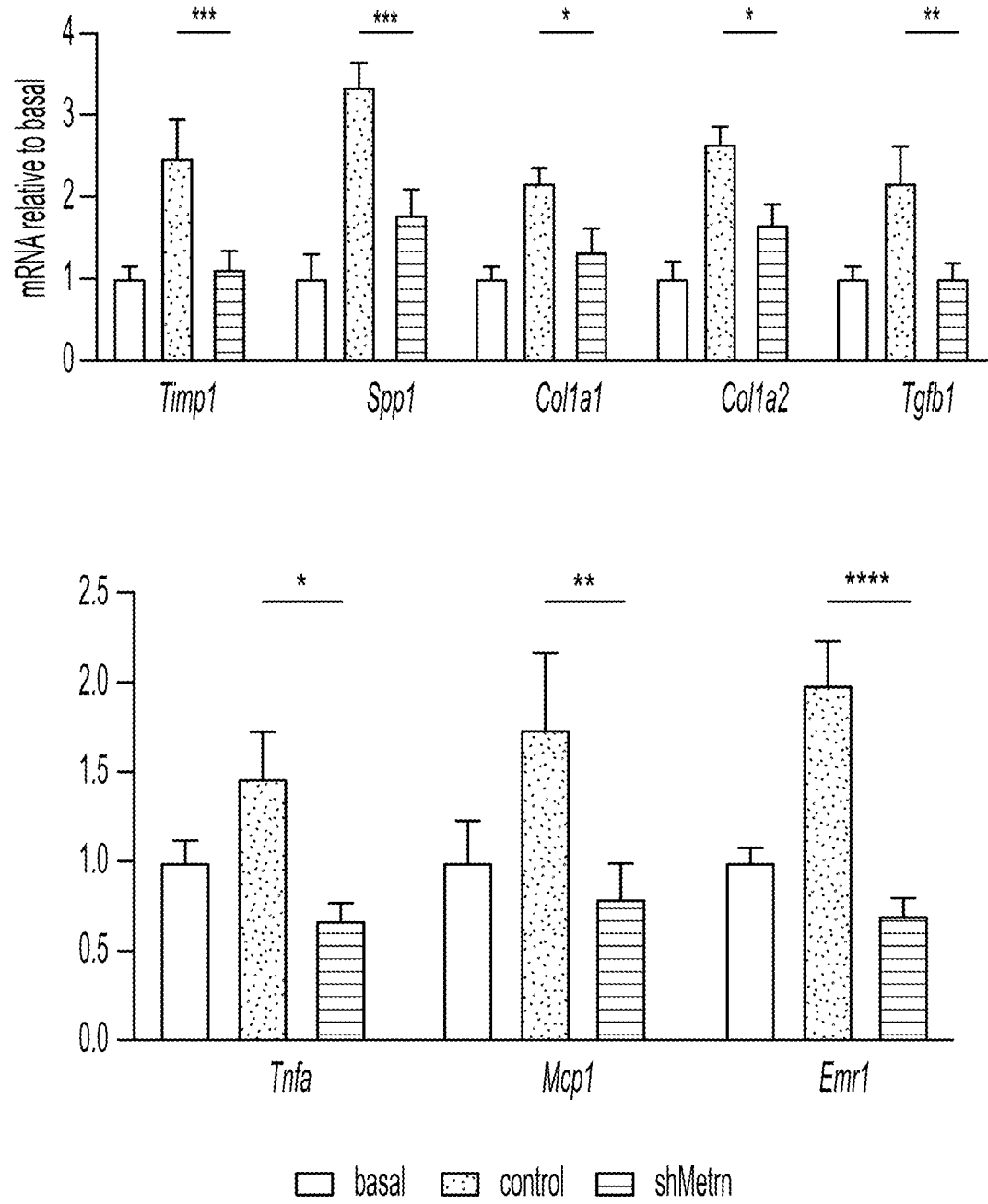


FIG. 22G

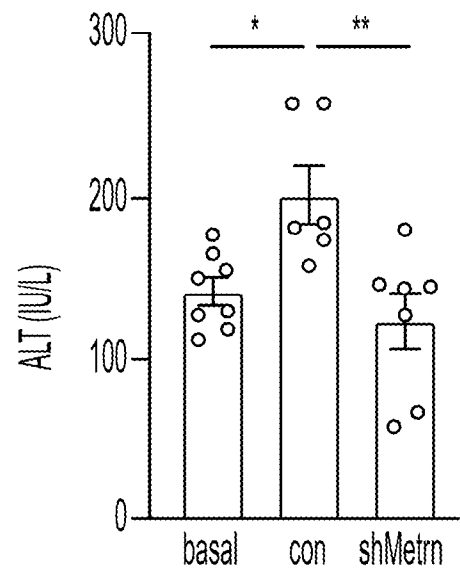


FIG. 22H

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2024/019467

A. CLASSIFICATION OF SUBJECT MATTERIPC: **A61K 48/00** (2024.01); **A61P 1/16** (2024.01); **A61P 3/00** (2024.01); **C07H 21/02** (2024.01); **C07K 14/47** (2024.01); **C12N 15/113** (2024.01); **C12Q 1/68** (2024.01)CPC: **C12N 15/113**; **A61K 48/00**; **A61P 3/00**; **C07H 21/02**; **C07K 14/47**; **C12Q 1/68**; **A61P 1/16**; **C12N 2310/14**

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History Document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History Document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HATTING et al. "Hepatocyte caspase-8 is an essential modulator of steatohepatitis in rodents," Hepatology, 06 May 2013 (06.05.2013), Vol. 57, No. 6, Pgs. 2189-2201. entire document	1, 2
Y	ZENER et al. "Caspase 8 small interfering RNA prevents acute liver failure in mice," Proceedings of the National Academy of Sciences, 24 June 2003 (24.06.2003), Vol. 100, No. 13, Pgs. 7797-7802. entire document	1, 2
Y	US 2015/0322460 A1 (DANA-FARBER CANCER INSTITUTE INC.) 12 November 2015 (12.11.2015) entire document	2
A	XU et al. "Over expression of METRN predicts poor clinical prognosis in colorectal cancer," Molecular Genetics and Genomic Medicine, 20 December 2019 (20.12.2019), Vol. 8, No. 3, Pgs. 1-7. entire document	1-3



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"D" document cited by the applicant in the international application

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

09 May 2024 (09.05.2024)

Date of mailing of the international search report

03 July 2024 (03.07.2024)

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US
Commissioner for Patents
P.O. Box 1450, Alexandria, VA 22313-1450

Facsimile No. **571-273-8300**

Authorized officer

MATOS
TAINA

Telephone No. **571-272-4300**

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2012/0144504 A1 (WALLACH et al.) 07 June 2012 (07.06.2012) entire document	1-3
A	US 2016/0151459 A1 (EXERKINE CORPORATION) 02 June 2016 (02.06.2016) entire document	1-3

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2024/019467

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
 - a. ☐ forming part of the international application as filed.
 - b. ☒ furnished subsequent to the international filing date for the purposes of international search (Rule 13ter.1(a)),
☒ accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.
2. ☐ With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.
3. Additional comments:

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: **4-48, 52, 53, 61, 62, 75, 76**
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees need to be paid.

Group I: claims 1-3 are drawn to methods for treating or preventing metabolic dysfunction-associated steatohepatitis (MASH) in a subject in need thereof, and kits.

Group II+: claims 49-51, and 54-60, and 63-71 are drawn to compositions for treating or preventing MASH, nucleic acids comprising siRNAs thereof, and nucleic acids comprising shRNAs thereof.

Group III: claims 72-74 are drawn to methods of diagnosing metabolic dysfunction-associated steatohepatitis (MASH) in a subject.

The first invention of Group II+ is restricted to a nucleic acid sequence targeting CASP8 gene comprising rGrGrArArCrCrUrGrGrUrArUrArUrUrCrArGrUrCrArCrUTT, compositions for treating or preventing MASH thereof, nucleic acids comprising siRNAs thereof, and nucleic acids comprising shRNAs thereof, and kits. The first named invention has been selected based on the guidance set forth in section 10.54 of the PCT International Search and Preliminary Examination Guidelines. Specifically, the first named invention was selected based on nucleic acid sequences listed in Table 1 found in instant claim 59. It is believed claims 49-51, and 54-60, and 63-71 read on this first named invention and thus these claims will be searched without fee to the extent that they read on a nucleic acid sequence targeting CASP8 gene comprising rGrGrArArCrCrUrGrGrUrArUrArUrUrCrArGrUrCrArCrUTT, compositions for treating or preventing MASH thereof, nucleic acids comprising siRNAs thereof, and nucleic acids comprising shRNAs thereof.

Applicant is invited to elect additional nucleic acid sequences to be searched by paying additional fee for each set of election. An exemplary election would be a nucleic acid sequence targeting METRN comprising rGrArGrCrUrGrCrArArGrArArUrCrArGrUrCrArUrCrArCTG, compositions for treating or preventing MASH thereof, nucleic acids comprising siRNAs thereof, and nucleic acids comprising shRNAs thereof. Additional nucleic acids will be searched upon the payment of additional fees. Applicants must specify the claims that read on any additional elected inventions. Applicants must further indicate, if applicable, the claims which read on the first named invention if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined.

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

The inventions listed in Groups I, II+ and III do not relate to a single general inventive concept under PCT Rule 13.1, because under PCT Rule 13.2 they lack the same or corresponding special technical features for the following reasons:

The Groups II+ formulas do not share a significant structural element, requiring the selection of alternative nucleic acids, where “the composition comprises at least one of the small interfering ribonucleic acid (siRNA) sequences from Table 1,” where “the hepatocyte-targeted nucleic acid comprises any one of the small interfering ribonucleic acid (siRNA) sequences from Table 1,” where “a sequence of any one of the small interfering ribonucleic acid (siRNA) designs from Table 1,” and where “the shRNA comprises a nucleic acid sequence provided in Table 1.”

The special technical features of Group I, methods for treating or preventing metabolic dysfunction-associated steatohepatitis (MASH) in a subject in need thereof, are not present in Groups II+ and III; the special technical features of Group II+, compositions for treating or preventing MASH thereof, nucleic acids comprising siRNAs thereof, and nucleic acids comprising shRNAs thereof, are not present in Groups I and III; and the special technical features of Group III, methods of diagnosing metabolic dysfunction-associated steatohepatitis (MASH) in a subject, are not present in Groups I and II+.

Additionally, even if Groups I, II+, and III were considered to share the technical features of a composition targeting a caspase 8 (CASP8) pathway in hepatocytes; a composition for treating or preventing MASH comprising a hepatocyte-targeted nucleic acid capable of targeting the caspase 8 (CASP8) pathway; a nucleic acid comprising a sequence of any one of the small interfering ribonucleic acid (siRNA); and a nucleic acid comprising a sequence encoding a short-hairpin RNA (shRNA), these shared technical features do not represent a contribution over the prior art as disclosed by the publication entitled “Caspase 8 small interfering RNA prevents acute liver failure in mice,” by Zender et al. (hereinafter “Zender”), the publication entitled “Hepatocyte caspase-8 is an essential modulator of steatohepatitis in rodents,” by Hatting et al. (hereinafter, “Hatting”), and US 2012/0144504 to Wallach et al. (hereinafter, “Wallach”).

Zender teaches a composition targeting a caspase 8 (CASP8) pathway in hepatocytes (Pg. 7800, left column, first full paragraph, Inhibition of Caspase 8 by siRNA RNAi targeted against death receptor-mediated apoptosis would be an important intervention to protect hepatocytes and maintain liver function. Caspase 8 is presumed to be the apex of the death receptor-mediated apoptosis pathways), a composition comprising a hepatocyte-targeted nucleic acid capable of targeting the caspase 8 (CASP8) pathway (Pg. 7800, left column, first full paragraph, Inhibition of Caspase 8 by siRNA RNAi targeted against death receptor-mediated apoptosis would be an important intervention to protect hepatocytes and maintain liver function. Caspase 8 is presumed to be the apex of the death receptor-mediated apoptosis pathways); a composition comprising a hepatocyte-targeted nucleic acid (Pg. 7800, left column, second partial paragraph, siRNA Is Delivered Effectively into Hepatocytes by Systemic Large-Volume Injection and Inhibits Liver Gene Expression); and a nucleic acid comprising a sequence of any one of the small interfering ribonucleic acid (siRNA) (Pg. 7800, left column, first full paragraph, Inhibition of Caspase 8 by siRNA RNAi targeted against death receptor-mediated apoptosis would be an important intervention to protect hepatocytes and maintain liver function. Caspase 8 is presumed to be the apex of the death receptor-mediated apoptosis pathways; Pg. 7797, right column, second full paragraph, The AA-N19 mRNA targets caspase 8 target sequence 1 (5'-AAC CUC GGG GAU ACU GUC UGA-3'), caspase 8 target sequence 2 (5'-AAG AAG CUC UUC UUC CCU CCC U-3')).

Hatting teaches a composition for treating or preventing MASH (Abstract, nonalcoholic steatohepatitis (NASH) ... Conclusion: Selective ablation of Casp8 in hepatocytes ameliorates development of NASH by modulating liver injury. Casp8-directed therapy might be a plausible treatment for patients with steatohepatitis; Instant Application, Pg. 1, MASH was previously referred to as nonalcoholic liver disease, specifically nonalcoholic steatohepatitis (NASH). References to MASH herein are intended to refer to MASH as well as the prior nomenclature of NASH) comprising a hepatocyte-targeted nucleic acid capable of targeting the caspase 8 (CASP8) pathway (Pg. 2190, left column, first partial paragraph, Generation and Housing of Casp8^{hep} Mice. The Casp8^{hep} strain was generated by crossing recently described Casp8^{f/f} mice¹³ with transgenic animals expressing the Cre transgene solely in hepatocytes under control of a postnatal activated albumin promoter).

Wallach teaches a nucleic acid comprising a sequence encoding a short-hairpin RNA (shRNA) (Para. [0089], an inhibitor of expression is a specific short hairpin RNA (shRNA). Designing and cloning strategies for

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

constructing shRNA expression vectors are known in the art; Para. [0024], inhibitor of a natural inhibitor of caspase-8 activation may be an inhibitor of cFLIP short, cFLIP long, and caspase-8- and caspase-10-associated RING proteins, such as siRNA or shRNA specific to, cFLIP short, cFLIP long, or caspase-8- and caspase-10-associated RING proteins),

The inventions listed in Groups I, II+ and III therefore lack unity under Rule 13 because they do not share a same or corresponding special technical features.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: **1-3**

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.