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(54) **Title:** THERAPEUTIC TARGETS FOR NASH-INDUCED HEPATOCELLULAR CARCINOMA

(57) **Abstract:** The present invention relates to methods and compositions for specifically modulating the Hippo pathway transcription factor TAZ (WWTR1), as a therapeutic target for inhibiting or preventing hepatocellular carcinoma (HCC) including the progression of NASH to HCC.

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THERAPEUTIC TARGETS FOR NASH-INDUCED HEPATOCELLULAR CARCINOMA

Cross Reference to Related Application

This present application claims priority to U.S. Provisional Patent Application No. 62/608,298 filed on December 20, 2017, which is incorporated herein by reference in its entirety.

Sequence Listing

The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on December 20, 2018, is named 01001_006430-WO0_ST25.txt and is 18799 bytes in size.

Statement of Government Support

This invention was made with government support under HL087123 and DK116620 awarded by the National Institutes of Health. The government has certain rights in the invention.

Field of the Invention

The present invention relates to methods and compositions for specifically modulating the Hippo pathway transcription factor TAZ (WWTR1), as a therapeutic target for inhibiting or preventing hepatocellular carcinoma (HCC), including the progression of NASH to HCC.

Background

The obesity epidemic has led to a surge in patients developing nonalcoholic steatohepatitis (NASH), which is marked by liver inflammation, dysfunctional fibrosis, and hepatocyte death. NASH has emerged as the leading cause of hepatocellular carcinoma (HCC), a fatal cancer whose only treatment option is surgical removal with or without liver transplantation if caught at an early stage. There are no effective non-surgical treatment options for HCC.

NASH has become the leading cause of chronic liver disease worldwide. However, there is a dearth of treatment options and no FDA-approved drugs for NASH, which is due in

large part to a poor understanding of NASH pathophysiology, particularly in the conversion of the relatively benign steatosis to NASH. NASH is a common and serious complication of obesity and type 2 diabetes, but many gaps remain in our understanding of its pathophysiology, leading to a lack of treatment options (White et al., 2012). NASH most likely develops as a result of multiple hits (Day and James, 1998), including steatosis, driven by hyperinsulinemia and elevated free fatty acid delivery to the liver, in combination with insults that promote inflammation, fibrosis, and hepatocyte death (Singh et al., 2015). However, the molecular mechanisms corresponding to these pathogenic processes and their integration are poorly understood. The incomplete understanding of NASH can be explained in part by the paucity of animal models that combine steatosis, obesity/insulin resistance, and key features of NASH, such as inflammation and fibrosis, as well as by insufficient integration of experimental results with human NASH findings.

Our previous work has identified a role for the Hippo pathway transcription factor Taz in the development of NASH; however, an incomplete understanding of the pathophysiology of NASH-induced HCC has impaired the development of mechanism-based therapeutic targets for this deadly disease.

There is a great need for improved models that mimic the relevant human disease conditions, as well as for new therapeutic targets for treating or preventing HCC and related conditions.

Summary

The present disclosure provides for a method for treating or preventing hepatocellular carcinoma (HCC) in a subject.

The present disclosure provides for a method for preventing or delaying progression of non-alcoholic steatohepatitis (NASH) to hepatocellular carcinoma (HCC) in a subject.

The method may comprise administering to the subject an effective amount of an inhibitor of TAZ (and/or an inhibitor of YAP).

The method may comprise administering to the subject an effective amount of a polynucleotide encoding an inhibitor of TAZ (and/or an inhibitor of YAP).

The inhibitor of TAZ (and/or the inhibitor of YAP), or the polynucleotide encoding an inhibitor of TAZ (and/or an inhibitor of YAP), may be administered to at least one hepatocyte of the subject.

The HCC may be derived from a progression of non-alcoholic steatohepatitis (NASH) in the subject.

The method may further comprise administering to the subject an effective amount of an inhibitor of Indian hedgehog (Ihh), an inhibitor of YAP, an inhibitor of TEAD1, an inhibitor of TEAD2, an inhibitor of TEAD3, an inhibitor of TEAD4, or any combination thereof.

The method may further comprise administering to the subject at least one additional therapeutic agent for treatment of steatosis hepatis, steatohepatitis, non-alcoholic fatty liver disease, non-alcoholic steatohepatitis, adiposity and combinations thereof.

The additional therapeutic agent may be antidiabetic drugs and insulin sensitizers selected from the group consisting of: Rosiglitazone; Pioglitazone; Losartan; Simtuzumab (anti-LOXL2); GR-MD-02; Obeticholic acid (OCA) and combinations thereof.

The method may further comprise administering to the subject a cytotoxic agent. The cytotoxic agent may be an alkylating agent, an anti-metabolite, an anti-microtubule agent, a topoisomerase inhibitor, a cytotoxic antibiotic, or an endoplasmic reticulum stress inducing agent.

The present disclosure provides for a method for inhibiting growth, or increasing cell death, of a hepatocellular carcinoma (HCC) cell. The method may comprise contacting the hepatocellular carcinoma cell with an effective amount of an inhibitor of TAZ (and/or an inhibitor of YAP).

Also encompassed by the present disclosure is a method for preventing or delaying progression of a hepatocyte to a hepatocellular carcinoma (HCC) cell. The method may comprise contacting the hepatocyte with an effective amount of an inhibitor of TAZ (and/or an inhibitor of YAP).

The contacting may be *in vitro* or *in vivo*.

The method may further comprise contacting the hepatocellular carcinoma cell or hepatocyte with an effective amount of an inhibitor of Indian hedgehog (Ihh), an inhibitor of YAP, an inhibitor of TEAD1, an inhibitor of TEAD2, an inhibitor of TEAD3, an inhibitor of TEAD4, or any combination thereof.

The method may further comprise contacting the hepatocellular carcinoma cell or hepatocyte with at least one additional therapeutic agent for treatment of steatosis hepatis, steatohepatitis, non-alcoholic fatty liver disease, non-alcoholic steatohepatitis, adiposity and combinations thereof. The additional therapeutic agent may be antidiabetic drugs and insulin sensitizers selected from the group consisting of: Rosiglitazone; Pioglitazone; Losartan; Simtuzumab (anti-LOXL2); GR-MD-02; Obeticholic acid (OCA) and combinations thereof.

The method may further comprise contacting the hepatocellular carcinoma cell or hepatocyte with a cytotoxic agent. The cytotoxic agent may be an alkylating agent, an anti-metabolite, an anti-microtubule agent, a topoisomerase inhibitor, a cytotoxic antibiotic, or an endoplasmic reticulum stress inducing agent.

The inhibitor of TAZ (and/or the inhibitor of YAP) may be a small molecule, a nucleic acid, a protein or polypeptide, an antibody or antigen-binding portion thereof, or combinations thereof.

The nucleic acid may be a small interfering RNA (siRNA), a short hairpin RNA (shRNA), an antisense oligonucleotide, and combinations thereof.

The nucleic acid may be SEQ ID NO:1 or SEQ ID NO:2, or any nucleic acid selected from the group consisting of SEQ ID NO:55-SEQ ID NO:72 and SEQ ID NO:81.

The nucleic acid may be formulated in a nanoparticle. In one embodiment, the nanoparticle comprises: (a) a hydrophobic inner core, (b) a hydrophilic outer shell and (c) a hepatocyte targeting ligand.

The inhibitor may comprise a CRISPR/Cas9 system.

Brief Description of the Drawings

Figure 1. siTAZ-1 and -2 silence TAZ in AML-12 cells. AML-12 cells were treated with the indicated concentrations of siRNA and then analyzed for TAZ immunoblot at 24 and 48 hours. siTAZ: siRNA that binds specifically to (against) TAZ.

Figures 2A-2B. siTAZ-1 and -2 silence TAZ *in vivo*. **A.** Male C57BL/6J mice were fed a fructose-palmitate-cholesterol diet (Wang et al., Cell Metab. 2016, 24:848-862) 9 weeks, which induces very early NASH. The mice were then injected once subcutaneously with PBS, control RNA, or siTAZ-1 or -2, as indicated in the scheme. **B.** After 3 days, the mice were euthanized, and the livers were immunoblotted for YAP and TAZ.

Figures 3. Experimental design for steatosis-to-NASH progression experiment. Male C57BL/6J mice were fed a fructose-palmitate-cholesterol diet (Wang et al. Cell Metab. 2016) for 8 weeks, which induces steatosis, not NASH, and is the time when hepatocyte TAZ levels begin to rise. The mice were then injected once per week subcutaneously with PBS, control RNA, or siTAZ-1 or -2, as indicated in the scheme. At week 16, the mice were euthanized and analyzed.

Figures 4A-4B. siTAZ-1 and -2 silence hepatic TAZ in NASH mice, with unexpected moderate suppression of YAP.

Figures 5A-5F. siTAZ-1 and -2 do not affect metabolic endpoints in NASH mice. Blood glucose was assayed 5 hours after food withdrawal. TG, triglyceride. None of the differences are statistically different. **Figure 5G.** siTAZ-1 and -2 lower plasma ALT in NASH mice. n=8 mice/group; mean ± SEM; groups with different symbols are statistically different from each other at $P < 0.05$.

Figures 6A-6B. siTAZ-1 and -2 lower liver fibrosis in NASH mice. A. Representative images of Sirius red-stained sections of liver. B. Quantification. n=8 mice/group; mean ± SEM; groups with different symbols are statistically different from each other at $P < 0.05$.

Figure 7. siTAZ-1 and -2 lower hepatic expression of mRNAs encoding fibrosis-related proteins in NASH mice. n=8 mice/group; mean ± SEM; *$P < 0.05$ vs. all other groups.

Figure 8. siTAZ-1 and -2 lower hepatic expression of mRNAs encoding inflammation-related proteins in NASH mice. n=8 mice/group; mean ± SEM; *$P < 0.05$ vs. all other groups.

Figure 9. siTAZ-1 and -2 lower cell death in the livers of NASH mice. Cell death was assayed using the TUNEL method. n=8 mice/group; mean ± SEM; *$P < 0.01$ vs. all other groups.

Figures 10A-10F. HCC in long-term NASH diet-fed mice and prevention by HC TAZ silencing. **A.** H&E staining of a typical liver nodule (Nod) from a C57 mouse fed the NASH diet for 15 months, showing abnormal cells and nuclei within the nodule. **B.** HCC nodules show characteristic loss of reticulin fibers and positive glypican-3 immunostaining (Bars, 100 μm). **C-E.** TAZ and YAP were assayed in the livers of control mice (Con) and in surrounding tissue (ST) or tumors (Tu) in 3 HCC models. High TAZ/YAP was found in all tumors. High TAZ in surrounding tissue was found only in the NASH diet model, with weak or absent TAZ in the other 2 models. **C**, Mice fed NASH diet for 15 months; **D**, C3H mice fed a high-fat, low-cholesterol diet for 12 months; **E**, chow-fed mice treated with DEN + CCl4. (**F**) Mice fed the NASH diet for 8 months were then treated with AAV8-H1-shTAZ of AAV8-H1-control RNA for 5 additional months. The mice were then analyzed for the presence and size of HCC nodules (*P<0.05, **P<0.01, n=6/group). shTAZ: shRNA that binds specifically to (against) TAZ.

Figures 11A-11E. Silencing hepatocyte TAZ reverses HCC tumors in L-NICD NASH-HCC model. **A.** 12-week old male NICD fl/fl mice were injected with AAV-TBG-Cre vector to induce NICD expression in hepatocytes and fed with NASH diet for 3 months to induce NASH-HCC development, then were introduced AAV8-H1-shTAZ or control vector to knockdown TAZ expression in hepatocytes. After another 2 months NASH diet feeding, the mouse livers were harvested for analysis. **B.** Immunoblot of TAZ in surrounding tissue of tumors. **C.** Liver images from shTAZ or shControl-treated L-NICD mice. **D.** Quantification of nodule numbers. **E.** Quantification of average tumor size. (*p<0.05, mean ± SEM; n=8 or 7 mice/group).

Detailed Description

The present disclosure provides for methods and compositions for specifically modulating the Hippo pathway transcription factor TAZ (WWTR1), as a therapeutic target for inhibiting or preventing hepatocellular carcinoma (HCC), including the progression of NASH to HCC.

The present methods and compositions may be used to treat or prevent liver cancer, e.g., hepatocellular carcinoma (HCC), hepatoblastoma, cholangiocarcinoma, angiosarcoma, and/or hemangiosarcoma.

The present methods and compositions may be used to treat or prevent HCC associated with, or progressed from, chronic liver disease, cirrhosis of the liver, chronic liver inflammation, chronic viral hepatitis (hepatitis B or C), exposure to toxins (e.g., alcohol or aflatoxin), hemochromatosis, metabolic syndrome (e.g., (NASH, Type 2 diabetes), metabolic congenital disorders (e.g., alpha 1-antitrypsin deficiency, Wilson's disease, hemophilia), certain benign liver tumors (e.g., hepatocellular adenoma), congenital liver disorders (e.g., biliary atresia, infantile cholestasis, glycogen-storage diseases, and other cirrhotic diseases of the liver). In one embodiment, the present methods and compositions may be used to treat or prevent HCC associated with, or progressed from, NASH.

The present disclosure provides for a method for treating or preventing hepatocellular carcinoma (HCC) in a subject. The method may comprise administering to the subject an effective amount of an inhibitor of TAZ (and/or an inhibitor of YAP).

The inhibitor of TAZ (and/or the inhibitor of YAP)may be administered to at least one hepatocyte of the subject.

In one embodiment, the HCC derives from a progression of non-alcoholic steatohepatitis (NASH) in the subject.

The present method may further comprise administering to the subject an effective amount of an inhibitor of Indian hedgehog (Ihh), an inhibitor of YAP, an inhibitor of TEAD1, an inhibitor of TEAD2, an inhibitor of TEAD3, an inhibitor of TEAD4, or any combination thereof.

The present method may further comprise administering to the subject at least one additional therapeutic agent for treatment of steatosis hepatis, steatohepatitis, non-alcoholic fatty liver disease, non-alcoholic steatohepatitis, adiposity and combinations thereof. The additional therapeutic agent may be antidiabetic drugs and/or insulin sensitizers, such as

Rosiglitazone; Pioglitazone; Losartan; Simtuzumab (anti-LOXL2); GR-MD-02; Obeticholic acid (OCA) and combinations thereof.

The present method may further comprise administering to the subject a cytotoxic agent. For example, the cytotoxic agent may be an alkylating agent, an anti-metabolite, an anti-microtubule agent, a topoisomerase inhibitor, a cytotoxic antibiotic, and/or an endoplasmic reticulum stress inducing agent.

Also encompassed by the present disclosure is a method for inhibiting the growth, or increasing the cell death, of a hepatocellular carcinoma (HCC) cell. The method may comprise contacting the hepatocellular carcinoma cell with an effective amount of an inhibitor of TAZ (and/or an inhibitor of YAP).

The present disclosure provides for method for preventing or delaying progression of a hepatocyte to a hepatocellular carcinoma (HCC) cell. The method may comprise contacting the hepatocyte with an effective amount of an inhibitor of TAZ (and/or an inhibitor of YAP).

The contacting may be *in vitro* or *in vivo*.

The present method may further comprise contacting the hepatocellular carcinoma cell or hepatocyte with an effective amount of an inhibitor of Indian hedgehog (Ihh), an inhibitor of YAP, an inhibitor of TEAD1, an inhibitor of TEAD2, an inhibitor of TEAD3, an inhibitor of TEAD4, or any combination thereof.

The present method may further comprise contacting the hepatocellular carcinoma cell or hepatocyte with at least one additional therapeutic agent for treatment of steatosis hepatis, steatohepatitis, non-alcoholic fatty liver disease, non-alcoholic steatohepatitis, adiposity and combinations thereof. The additional therapeutic agent may be antidiabetic drugs and/or insulin sensitizers, such as Rosiglitazone; Pioglitazone; Losartan; Simtuzumab (anti-LOXL2); GR-MD-02; Obeticholic acid (OCA) and combinations thereof.

The present method may further comprise contacting the hepatocellular carcinoma cell or hepatocyte with a cytotoxic agent.

The inhibitor of TAZ may be a small molecule, a nucleic acid, a protein or polypeptide, an antibody or antigen-binding portion thereof, or combinations thereof.

The the nucleic acid may be a small interfering RNA (siRNA), a short hairpin RNA (shRNA), an antisense oligonucleotide, or combinations thereof. In certain embodiments, the nucleic acid is SEQ ID NO:1 or SEQ ID NO:2, or any nucleic acid selected from the group consisting of SEQ ID NO:55-SEQ ID NO:72 and SEQ ID NO:81.

The inhibitor may comprise a CRISPR/Cas9 system.

In one embodiment, the nucleic acid is formulated in a nanoparticle. For example, the nanoparticle may comprise: (a) a hydrophobic inner core, (b) a hydrophilic outer shell and (c) a hepatocyte targeting ligand.

In various embodiments, the present methods reduce cancer cell growth, proliferation, and/or metastasis, as measured according to routine techniques in the diagnostic art. Specific examples of relevant responses include reduced size, mass, or volume of a tumor, or reduction in cancer cell number.

The present compositions and methods can have one or more of the following effects on cancer cells or the subject: cell death; decreased cell proliferation; decreased numbers of cells; inhibition of cell growth; apoptosis; necrosis; mitotic catastrophe; cell cycle arrest; decreased cell size; decreased cell division; decreased cell survival; decreased cell metabolism; markers of cell damage or cytotoxicity; indirect indicators of cell damage or cytotoxicity such as tumor shrinkage; improved survival of a subject; preventing, inhibiting or ameliorating the cancer in the subject, such as slowing progression of the cancer, reducing or ameliorating a sign or symptom of the cancer; reducing the rate of tumor growth in a patient; preventing the continued growth of a tumor, reducing the size of a tumor; and/or disappearance of markers associated with undesirable, unwanted, or aberrant cell proliferation. In certain embodiments, the present compositions and methods reduce the number and/or size of tumor nodules (e.g., HCC nodules).

Methods and compositions of the present invention can be used for prophylaxis as well as amelioration of signs and/or symptoms of liver cancer.

The present composition may be administered alone or in combination with other treatment methods including radiation, standard chemotherapy, surgery (e.g., surgical resection), ablation (e.g., radiofrequency ablation (RFA), cryoablation, and percutaneous ethanol injection), arterial catheter-based treatment (e.g., transcatheter arterial chemoembolization (TACE), and selective internal radiation therapy (SIRT)), portal vein embolization (PVE), high intensity focused ultrasound (HIFU), Yttrium-90 radioembolization, and/or liver transplantation.

Nucleic Acids

The present disclosure provides for certain constructs and nucleic acids encoding the complete or portions of the TAZ protein described herein. Certain constructs and sequences, including selected TAZ inhibitory sequences SEQ ID NO:1, SEQ ID NO:2, and any of SEQ ID NO:55-SEQ ID NO:72, or SEQ ID NO:81 may be useful in certain embodiments.

Preferably, the nucleic acids hybridize under low, moderate or high stringency conditions. A first nucleic acid molecule is "hybridizable" to a second nucleic acid molecule when a single stranded form of the first nucleic acid molecule can anneal to the second nucleic acid molecule under the appropriate conditions of temperature and solution ionic strength (see Sambrook, *et al.*, supra). The conditions of temperature and ionic strength determine the "stringency" of the hybridization. Typical low stringency hybridization conditions include 55°C, 5X SSC, 0.1% SDS and no formamide; or 30% formamide, 5X SSC, 0.5% SDS at 42°C. Typical moderate stringency hybridization conditions are 40% formamide, with 5X or 6X SSC and 0.1% SDS at 42°C. High stringency hybridization conditions are 50% formamide, 5X or 6X SSC at 42°C or, optionally, at a higher temperature (e.g., 57°C, 59°C, 60°C, 62°C, 63°C, 65°C or 68°C). In general, SSC is 0.15M NaC1 and 0.015M Na-citrate. Hybridization requires that the two nucleic acids contain complementary sequences, although, depending on the stringency of the hybridization, mismatches between bases are possible. The appropriate stringency for hybridizing nucleic acids depends on the length of the nucleic acids and the degree of complementation, variables well known in the art. The greater the degree of similarity or homology between two nucleotide sequences, the higher the stringency under which the nucleic acids may hybridize. For hybrids of greater than 100 nucleotides in length, equations for calculating the melting temperature have been derived (see Sambrook, *et al.*, supra, 9.50-9.51). For hybridization with shorter nucleic acids, *e.g.*, oligonucleotides, the position of mismatches becomes more important, and the length of the oligonucleotide determines its specificity (see Sambrook, *et al.*, supra, 11.7-11.8).

Inhibitory Nucleic Acids that Hybridize to TAZ or YAP

It is noted that in addition to TAZ, YAP induced liver fibrosis is another process which could potentially be blocked by inhibitory compounds in a similar manner as described herein for TAZ. Any number of means for inhibiting TAZ and/or YAP activity or gene expression can be used in the present methods. For example, a nucleic acid molecule complementary to at least a portion of a human TAZ and/or YAP encoding nucleic acid can be used to inhibit TAZ and/or YAP gene expression. Means for inhibiting gene expression using short RNA molecules, for example, are known. Among these are short interfering RNA (siRNA), small temporal RNAs (stRNAs), and micro-RNAs (miRNAs). Short interfering RNAs silence genes through an mRNA degradation pathway, while stRNAs and miRNAs are approximately 21 or 22 nt RNAs that are processed from endogenously encoded hairpin-structured precursors, and function to silence genes via translational repression. See, e.g., McManus et al., RNA,

8(6):842-50 (2002); Morris et al., Science, 305(5688):1289-92 (2004); He and Hannon, Nat Rev Genet. 5(7):522-31 (2004).

"RNA interference, or RNAi" a form of post-transcriptional gene silencing ("PTGS"), describes effects that result from the introduction of double-stranded RNA into cells (reviewed in Fire, A. Trends Genet 15:358-363 (1999); Sharp, P. Genes Dev 13:139-141 (1999); Hunter, C. Curr Biol 9:R440-R442 (1999); Baulcombe. D. Curr Biol 9:R599-R601 (1999); Vaucheret et al. Plant J 16: 651-659 (1998)). RNA interference, commonly referred to as RNAi, offers a way of specifically inactivating a cloned gene, and is a powerful tool for investigating gene function.

The active agent in RNAi is a long double-stranded (antiparallel duplex) RNA, with one of the strands corresponding or complementary to the RNA which is to be inhibited. The inhibited RNA is the target RNA. The long double stranded RNA is chopped into smaller duplexes of approximately 20 to 25 nucleotide pairs, after which the mechanism by which the smaller RNAs inhibit expression of the target is largely unknown at this time. While RNAi was shown initially to work well in lower eukaryotes, for mammalian cells, it was thought that RNAi might be suitable only for studies on the oocyte and the preimplantation embryo.

More recently, it was shown that RNAi would work in human cells if the RNA strands were provided as pre-sized duplexes of about 19 nucleotide pairs, and RNAi worked particularly well with small unpaired 3' extensions on the end of each strand (Elbashir et al. Nature 411: 494-498 (2001)). In this report, "short interfering RNA" (siRNA, also referred to as small interfering RNA) were applied to cultured cells by transfection in oligofectamine micelles. These RNA duplexes were too short to elicit sequence-nonspecific responses like apoptosis, yet they efficiently initiated RNAi. Many laboratories then tested the use of siRNA to knock out target genes in mammalian cells. The results demonstrated that siRNA works quite well in most instances.

For purposes of reducing the activity of TAZ/YAP, siRNAs to the gene encoding the TAZ/YAP can be specifically designed using computer programs. Illustrative nucleotide sequences encoding the amino acid sequences of the various YAP isoforms are known and published, e.g., in GenBank Accession Nos. NM_001130145, NP_001123617, yorkie homolog isoform 1; NM_006106; NP_006097; yorkie homolog isoform 2; NM_001195044, NP-_001181973, yorkie homolog isoform 3; NM_001195045, NP_001181974, yorkie homolog isoform 4; NM_001282097; NM_001282098; NP_001181973; NP_001269026; NP_001269027. The NCBI RefSeq accession numbers for murine YAP protein may include,

NP_001164618 and NP_033560. The NCBI RefSeq accession numbers for murine YAP mRNA may include NM_001171147 and NM_009534.

Furthermore, exemplary nucleotide sequences encoding the amino acid sequences of the various TAZ isoforms are known and published, e.g., in GenBank Accession Nos. NM_001168278, NP_001161750; NM_001168280, NP_001161752; NM_015472, NP_056287; NM_001348362, NP_001335291. The NCBI RefSeq accession numbers for murine TAZ protein may include, NP_001161753 and NP_598545. The NCBI RefSeq accession numbers for murine TAZ mRNA may include NM_001168281 and NM_133784. see also, Kanai, et al., The EMBO Journal (2000) 19(24):6778-6791.

Software programs for predicting siRNA sequences to inhibit the expression of a target protein are commercially available and find use. One program, siDESIGN from Dharmacon, Inc. (Lafayette, Colo.), permits predicting siRNAs for any nucleic acid sequence, and is available on the internet at dharmacon.com. Programs for designing siRNAs are also available from others, including Genscript (available on the internet at genscript.com/ssl-bin/app/rnai) and, to academic and non-profit researchers, from the Whitehead Institute for Biomedical Research found on the worldwide web at "jura.wi.mit.edu/pubint/http://iona.wi.mit.edu/siRNAext/."

Any suitable viral knockdown system could be utilized for decreasing TAZ mRNA levels - including AAV, lentiviral vectors, or other suitable vectors that are capable of being targeted specifically to the liver. (See Zuckerman and Davis 2015).

Additionally, specifically targeted delivery of sh*Taz* mRNA or other TAZ blocking molecule (nucleic acid, peptide, or small molecule) could be delivered by targeted liposome, nanoparticle or other suitable means.

As described herein we provide methods as well as one or more agents/compounds that silence or inhibit TAZ for the treatment, prophylaxis or alleviation of liver cancer (e.g., HCC), or related liver conditions, or predisposition to such a condition.

An approach for therapy of such disorders is to express anti-sense constructs directed against TAZ polynucleotides as described herein, and specifically administering them to liver cells, to inhibit gene function and prevent one or more of the symptoms and processes associated with the progression of NASH to HCC. Such treatment may also be useful in treating patients who already exhibit a progression to HCC, to reverse or alleviate one or more of the disease processes. Additionally, approaches utilizing one or more additional inhibitors including an inhibitor of Indian hedgehog (Ihh), an inhibitor of YAP, an inhibitor of TEAD1, TEAD2, TEAD3, TEAD4, or any combination of these, are also expected to be useful for

treating certain conditions. In certain instances, administering at least one additional therapeutic agent for treatment of any of the following conditions including: steatosis hepatis, steatohepatitis, non-alcoholic fatty liver disease, non-alcoholic steatohepatitis, adiposity and combinations thereof may be useful. Such additional therapeutic agents include antidiabetic drugs and insulin sensitizers including: Rosiglitazone; Pioglitazone; Losartan; Simtuzumab (anti-LOXL2); GR-MD-02; Obeticholic acid (OCA) and combinations thereof.

Anti-sense constructs may be used to inhibit gene function to prevent progression of NASH to HCC. Antisense constructs, i.e., nucleic acid, such as RNA, constructs complementary to the sense nucleic acid or mRNA, are described in detail in U.S. Pat. No. 6,100,090 (Monia et al.), and Neckers et al., 1992, Crit Rev Oncog 3(1-2):175-231.

In a particular example, liver cancer (e.g., HCC) may be treated or prevented by reducing the amount, expression or activity of TAZ in whole or in part in hepatocytes, for example by siRNAs capable of binding to and destroying TAZ mRNA. Examples of such anti-TAZ agents/compounds are provided herein, which function to downregulate TAZ by RNA interference. The anti-TAZ agent/compound may comprise a small interfering RNA (siRNA) or short hairpin RNA (shRNA). A specific example of an anti-TAZ agent includes SEQ ID NO:1, SEQ ID NO:2, and any of SEQ ID NO:55-SEQ ID NO:72 and SEQ ID NO:81, which may be useful in certain embodiments as described below and in the Examples.

RNA interference (RNAi) is a method of post transcriptional gene silencing (PTGS) induced by the direct introduction of double-stranded RNA (dsRNA) and has emerged as a useful tool to knock out expression of specific genes in a variety of organisms. RNAi is described by Fire et al., Nature 391:806-811 (1998). Other methods of PTGS are known and include, for example, introduction of a transgene or virus. Generally, in PTGS, the transcript of the silenced gene is synthesized but does not accumulate because it is rapidly degraded. Methods for PTGS, including RNAi are described, for example, in the Ambion.com world wide web site, in the directory "/hottopics/", in the "rnai" file.

Suitable methods for RNAi in vitro are described herein. One such method involves the introduction of siRNA (small interfering RNA). Current models indicate that these 21-23 nucleotide dsRNAs can induce PTGS. Methods for designing effective siRNAs are described, for example, in the Ambion web site described above. RNA precursors such as short hairpin RNAs (shRNAs) can also be encoded by all or a part of the TAZ nucleic acid sequence.

Alternatively, double-stranded (ds) RNA is a powerful way of interfering with gene expression in a range of organisms that has recently been shown to be successful in mammals (Wianny and Zernicka-Goetz, 2000, Nat Cell Biol 2:70-75). Double stranded RNA

corresponding to the sequence of a TAZ polynucleotide can be introduced into or expressed in oocytes and cells of a candidate organism to interfere with TAZ activity.

Other methods of modulating TAZ gene expression are known to those skilled in the art and include dominant negative approaches. An example of this approach, which could be utilized in the context of inhibiting, preventing, or treating NASH and NASH related conditions is utilizing a TAZ mutant such as TAZ S51A to block TAZ/TEAD interaction or a small molecule chemical or mimetic which can block TAZ/TEAD interaction. (Zhang H, et al., J Biol Chem. 2009 May 15; 284(20):13355-62). TAZ WW domain mutations also block its binding to some transcriptional factors. Other TAZ peptide inhibitors are described in WO2015063747A2. Yet another approach is to use non-functional variants of TAZ polypeptide that compete with the endogenous gene product resulting in inhibition of function. Inhibitors of TAZ co-factors TEAD1, TEAD2, TEAD3, and TEAD4 can be targeted, and these are also expected to serve as useful in the context of inhibiting, preventing, or treating NASH and NASH related conditions.

Non-limiting examples of inhibitors of TAZ include, dasatinib, statins, pazopanib, verteporfin, agents described in U.S. Patent No. 9,649,300; U.S. Patent No. 10,077,294; U.S. Patent Publication No. 20150157584; U.S. Patent Publication No. 2018297964; U.S. Patent Publication No. 2018215721; WO2017064277; WO2017053706; WO2015/031109; WO2015063747; Oku et al., Small molecules inhibiting the nuclear localization of YAP/TAZ for chemotherapeutics and chemosensitizers against breast cancers, FEBS Open Bio, Volume 5, 2015, Pages 542-549; Gibault et al., Molecular Features of the YAP Inhibitor Verteporfin: Synthesis of Hexasubstituted Dipyrrins as Potential Inhibitors of YAP/TAZ, the Downstream Effectors of the Hippo Pathway. ChemMedChem. 2017, 12(12):954-961; Crawford et al., Hippo pathway inhibition by blocking the YAP/TAZ–TEAD interface: a patent review, Expert Opinion on Therapeutic Patents, 2018, 28:12, 867-873; Stanger BZ, Quit your YAPing: a new target for cancer therapy, Genes Dev., 2012, Vol. 26, Issue 12, pp. 1263-1267.

TAZ gene expression may also be modulated by introducing peptides or small molecules which inhibit gene expression or functional activity. Thus, compounds identified by the assays described herein as binding to or modulating, such as down-regulating, the amount, activity or expression of TAZ polypeptide may be administered to liver hepatocyte cells to prevent the function of TAZ polypeptide. Such a compound may be administered along with a pharmaceutically acceptable carrier in an amount effective to down-regulate expression or activity TAZ, or by activating or down-regulating a second signal which controls TAZ expression, activity or amount, and thereby alleviating the abnormal condition.

Alternatively, gene therapy may be employed to control the endogenous production of TAZ by the relevant cells such as liver cells in the subject. For example, a polynucleotide encoding a TAZ siRNA or a portion of this may be engineered for expression in a replication defective retroviral vector, as discussed below. The retroviral expression construct may then be isolated and introduced into a packaging cell transduced with a retroviral plasmid vector containing RNA encoding an anti-TAZ siRNA such that the packaging cell now produces infectious viral particles containing the sequence of interest. These producer cells may be administered to a subject for engineering cells *in vivo* and regulating expression of the TAZ polypeptide *in vivo*. For overview of gene therapy, see Chapter 20, Gene Therapy and other Molecular Genetic-based Therapeutic Approaches, (and references cited therein) in Human Molecular Genetics, T Strachan and A P Read, BIOS Scientific Publishers Ltd (1996).

In some embodiments, the level of TAZ is decreased in a liver cell. Furthermore, in such embodiments, treatment may be targeted to, or specific to, liver cells. The expression of TAZ may be specifically decreased only in diseased liver cells (i.e., those cells which are predisposed to the liver condition, or exhibiting liver disease already), and not substantially in other non-diseased liver cells. In these methods, expression of TAZ may not be substantially reduced in other cells, i.e., cells which are not liver cells. Thus, in such embodiments, the level of TAZ remains substantially the same or similar in non- liver cells in the course of or following treatment.

Liver cell specific reduction of TAZ levels may be achieved by targeted administration, i.e., applying the treatment only to the liver cells and not other cells. However, in other embodiments, down-regulation of TAZ expression in liver cells (and not substantially in other cell or tissue types) is employed. Such methods may advantageously make use of liver specific expression vectors, for liver specific expression of for example siRNAs, as described in further detail below.

The methods and compositions described here may employ, as a means for detecting expression levels of TAZ, TAZ polynucleotides, TAZ nucleotides and TAZ nucleic acids, as well as variants, homologues, derivatives and fragments of any of these. In addition, we disclose particular TAZ fragments useful for the methods of diagnosis described here. The TAZ nucleic acids may also be used for the methods of treatment or prophylaxis described.

The terms "TAZ polynucleotide", "TAZ nucleotide" and "TAZ nucleic acid," "*Taz* nucleic acid" may be used interchangeably, and should be understood to specifically include both cDNA and genomic TAZ sequences. These terms are also intended to include a nucleic

acid sequence capable of encoding a TAZ polypeptide and/or a fragment, derivative, homologue or variant of this. SEQ ID NO:1, SEQ ID NO:2, and any of SEQ ID NO:55-SEQ ID NO:72 and SEQ ID NO:81 may be useful in certain embodiments as primers amplifying *Taz* or as sequences utilized for designing nucleic acid inhibitors (shRNA or RNAi) of TAZ, as shown in the table below. The primer sequences described herein are shown as DNA sequences; however in certain instances it would be useful to utilize the RNA equivalent, in which the sequence is identical, except the T is replaced with U.

TABLE 1: Taz-specific polynucleotides siRNA

(T can be replaced with U for any of the primers listed below, in certain instances.)

		Primers can also be useful as RNA if T is replaced with U (T/U)
SEQ ID NO:55	TCATTGCGAGATTCGGCTG	T/U
SEQ ID NO:56	GATGAATCCGTCCTCGGTG	T/U
SEQ ID NO:57	GAGGCAAGTTGAAAGGTCAGAGGCA	T/U
SEQ ID NO:58	GCTGCACCACGTTCTGCCTTTGTAC	T/U
SEQ ID NO:59	GGCAATGACGTCCTTAGCTGTTTAG	T/U
SEQ ID NO:60	AGGCAGCTTGGTCCAGGAAGTGATT	T/U
SEQ ID NO:61	ACCTCTTCAACTCTGTCATGAA	T/U
SEQ ID NO:62	CGCCCTTTCTAACCTGGCTGTA	T/U
SEQ ID NO:63	TGCCACCGTTCATCATTTTCCTGCT	T/U
SEQ ID NO:64	TCCCCGAGTCCCCAGAAAGATGAAT	T/U
SEQ ID NO:65	CCAGCTCATGGCGGAAAAAGATCCT	T/U
SEQ ID NO:66	ACCCCAGGAAGGTGATGAATCAGCC	T/U

SEQ ID NO:67	GGGCCTTGCGGACCAAGTGATGAGG	T/U
SEQ ID NO:68	GCCCTTGACTGTTTACTAATAGATA	T/U
SEQ ID NO:69	CCAAATCCATCAGATGAAACCATTT	T/U
SEQ ID NO:70	GCCTGCATTTCTGTGGCAGATA	T/U
SEQ ID NO:71	GCCATGAGCACAGATATGAGATCT	T/U

The present nucleic acid or polynucleotide may be double-stranded (e.g., comprising a sense strand and an antisense strand) or single-stranded. The RNAi (e.g., siRNA) agent may be double-stranded or single-stranded. The RNAi agents can have any suitable form, such as single-stranded, double-stranded, linear, circular (e.g., a plasmid), nicked circular, coiled, supercoiled, concatemerized, or charged. Additionally, nucleotides may contain 5' and 3' sense and antisense strand terminal modifications and can have blunt or overhanging terminal nucleotides (e.g., UU or TT at the 3'-terminus), or combinations thereof.

Included in the present disclosure are oligomers (e.g., PNAs, LNAs, 2'-OMe, MOE) that consist of about 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 bases, in which at least about 6, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 contiguous or non-contiguous bases are complementary to their target sequence, or variants thereof.

The present nucleic acid or polynucleotide may contain modified oligonucleotide backbones. For example, the ribose or another pentose sugar may be alternate or modified groups. Examples of modified oligonucleotide backbones include, without limitation, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity wherein the adjacent pairs of nucleoside units are linked 3'-5' to 5'-3' or 2'-5' to 5'-2'. Also contemplated are peptide nucleic acids (PNAs), locked nucleic acids (LNAs), 2'-O-Methyl oligonucleotides (2'-OMe), 2'-methoxyethoxy oligonucleotides (MOE), among other oligonucleotides known in the art.

Modifications can be made to the present nucleic acid or polynucleotide and may include conjugate groups attached to one of the termini, selected nucleobase positions, sugar positions or to one of the internucleoside linkages. Possible modifications include, but are not limited to, 2'-fluoro (2'-F), 2'-OMethyl (2'-OMe), 2'-O-(2-methoxyethyl) (2'-MOE) high affinity sugar modifications, inverted abasic caps, deoxynucleobases, and bicyclic nucleobase analogs, such as locked nucleic acids (LNA) and ethylene-bridged nucleic acids (ENA).

Modified nucleic acids, including modified DNA or RNA molecules, may be used in place of naturally occurring nucleic acids in the polynucleotides (e.g., RNAi agents) described herein. Modified nucleic acids can improve the half-life, stability, specificity, delivery, solubility, nuclease resistance, and/or potency of the polynucleotides described herein. For example, siRNA agents can be partially or completed composed of nucleotide analogs that confer the beneficial qualities described above. As described in Elmen et al. (Nucleic Acids Res. 33:439-447 (2005)), synthetic, RNA-like nucleotide analogs (e.g., locked nucleic acids (LNA)) can be used to construct siRNA molecules that exhibit silencing activity against a target gene product.

The phosphorothioate (PS) backbone modification, where a non-bridging oxygen in the phosphodiester bond is replaced by sulfur, may be used to in the present nucleic acid or polynucleotide. In certain embodiments, the present nucleic acid or polynucleotide may comprise the phosphorothioate (PS) modification which is restricted to 1, 2, 3, 4, 5, or more bases at the 3' and/or 5' ends.

The boranophosphate linker can be used in the present nucleic acid or polynucleotide (Hall et al., Nucleic Acids Res. 32:5991-6000 (2004)). Other exemplary modifications to the oligonucleotide backbone include methylphosphonates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, alkyl phosphonates (e.g., 3'-alkylene phosphonate), chiral phosphonates, phosphinates, phosphoramidates (e.g., 3'-amino phosphoramidate), aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, and a protein nucleotide (PNA) backbone having repeating N-(2-aminoethyl)-glycine units linked by peptide bonds, where representative PNA compounds include, but are not limited to, those disclosed in U.S. Pat. Nos. 5,539,082, 5,714,331, and 5,719,262, and Nielsen et al., Science 254:1497-1500 (1991). In certain embodiments, the modification is restricted to 1, 2, 3, 4, 5, or more bases at the 3' and/or 5' ends.

Other modifications to the backbone of the present nucleic acid or polynucleotide include those replacing the phosphorous atom with short chain alkyl or cycloalkyl

internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages (e.g., morpholino linkages; siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; alkene containing backbones; sulphamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH_2 component parts).

Certain modified nucleobases may be used in the present composition and method, such as 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines (e.g., 2-aminopropyladenine, 5-propynyluracil, 5-propynylcytosine, and 5-methylcytosine). Exemplary modified nucleobases include 5-methylcytosine (5-me-C or m5c); 5-hydroxymethyl cytosine, xanthine, and hypoxanthine; 2-aminoadenine, 6-methyl, and other alkyl derivatives of adenine and guanine; 2-propyl and other alkyl derivatives of adenine and guanine; 2-thiouracil; 2-thiothymine; 2-thiocytosine; 5-halouracil and cytosine; 5-propynyl uracil and cytosine; 6-azo uracil, cytosine, and thymine; 5-uracil (pseudouracil); 4-thiouracil; 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxy, and other 8-substituted adenines and guanines; 5-halo, particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines; 7-methylguanine; 7-methyladenine; 8-azaguanine; 8-azaadenine; 7-deazaguanine; 7-deazaadenine; 3-deazaguanine; and 3-deazaadenine. These modified nucleobases may be combined, in particular embodiments, with other modifications, such as any sugar modification described herein.

The purine or pyrimidine base pairing moiety may be adenine, cytosine, guanine, uracil, thymine or inosine. Also included are bases such as pyridin-4-one, pyridin-2-one, phenyl, pseudouracil, 2,4,6-trime115thoxy benzene, 3-methyl uracil, dihydrouridine, naphthyl, aminophenyl, 5-alkylcytidines (e.g., 5-methylcytidine), 5-alkyluridines (e.g., ribothymidine), 5-halouridine (e.g., 5-bromouridine) or 6-azapyrimidines or 6-alkylpyrimidines (e.g. 6-methyluridine), propyne, quesosine, 2-thiouridine, 4-thiouridine, wybutosine, wybutoxosine, 4-acetyltidine, 5-(carboxyhydroxymethyl)uridine, 5'-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluridine, .beta.-D-galactosylqueosine, 1-methyladenosine, 1-methylinosine, 2,2-dimethylguanosine, 3-methylcytidine, 2-methyladenosine, 2-methylguanosine, N6-methyladenosine, 7-methylguanosine, 5-methoxyaminomethyl-2-thiouridine, 5-methylaminomethyluridine, 5-methylcarbonyhnethyluridine, 5-methyloxyuridine, 5-methyl-2-thiouridine, 2-methylthio-N6-isopentenyladenosine, .beta.-D-mannosylqueosine, uridine-5-oxyacetic acid, 2-thiocytidine,

threonine derivatives and others (Burgin et al., 1996, Biochemistry, 35, 14090; Uhlman & Peyman, supra). By "modified bases" in this aspect is meant nucleotide bases other than adenine (A), guanine (G), cytosine (C), thymine (T), and uracil (U), as illustrated above; such bases can be used at any position in the present nucleic acid or polynucleotide. Persons skilled in the art will appreciate that depending on the uses of the oligomers, T and U may be interchangeable.

Modified oligonucleotides may also contain one or more substituted sugar moieties, where modifications can be made at any reactive site of the ribose ring (e.g., the 2'-OH of the ribose ring), or one or more universal bases. Exemplary modifications include 2'-halo, such as F, Br, or Cl; 2'-O-alkyl, 2'-S-alkyl, or 2'-N-alkyl, such as 2'-OMe; 2'-O-(alkyl-O)n-alkyl, such as 2'-O-methoxyethyl (2'-O-MOE), 2'-O[(CH_2)nO]mCH_3, 2'-O(CH_2)nOCH_3, 2'-O$(CH_2)_2$ON$(CH_3)_2$O(CH_2)nNH_2, O(CH_2)nCH_3, 2'-O(CH_2)nONH_2, and 2'-O(CH_2)nON[(CH_2)nCH_3)]$_2$, where n and m are from 1 to about 10; 2'-O-alkenyl, 2'-S-alkenyl, or 2'-N-alkenyl; 2'-O-alkynyl, 2'-S-alkynyl, or 2'-N-alkynyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C_{1-10} alkyl or C_{2-10} alkenyl and alkynyl, as well as a bridging modification between the 2' and 4' positions of ribose to form a locked nucleic acid (LNA). Exemplary universal bases include a heterocyclic moiety located at the 1' position of a nucleotide sugar moiety in a modified nucleotide, or the equivalent position in a nucleotide sugar moiety substitution, such as 1-beta-D-ribofuranosyl-5-nitroindole and 1-beta-D-ribofuranosyl-3-nitropyrrole.

In certain embodiments, nucleic acids possessing described forms of modification and/or patterns of modification can be employed. Additional detail regarding exemplary modifications and modification patterns of nucleic acids can be found, e.g., in at least the following references: US 2010/0240734; WO 2010/080129; WO 2010/033225; US 2011/0021604; WO 2011/075188; WO2011/072292; WO 2010/141724; WO 2010/141726; WO 2010/141933; WO 2010/115202; WO 2008/136902; WO/2011/109294; WO/2011/075188; PCT/US11/42810; PCT/US11/42820; U.S. Ser. No. 61/435,304; U.S. Ser. No. 61/478,093; U.S. Ser. No. 61/497,387; U.S. Ser. No. 61/529,422; U.S. Pat. No. 7,893,245; WO 2007/051303; and US 2010/0184209. Each of the preceding documents is hereby incorporated by reference in its entirety.

The present nucleic acid or polynucleotide may be nuclease-resistant. A "nuclease-resistant" oligomeric molecule (oligomer) refers to one whose backbone is substantially resistant to nuclease cleavage, in non-hybridized or hybridized form; by common extracellular and intracellular nucleases in the body; that is, the oligomer shows little or no

nuclease cleavage under normal nuclease conditions in the body or in vitro to which the oligomer is exposed.

Contacting a cell in vitro may be done, for example, by incubating the cell with the RNAi agent. Contacting a cell in vivo may be done, for example, by injecting the RNAi agent into or near the tissue where the cell is located, or by injecting the RNAi agent into another area, the bloodstream or the subcutaneous space, such that the agent will subsequently reach the tissue where the cell to be contacted is located. For example, the RNAi agent may contain and/or be coupled to a ligand, e.g., a GalNAc3 ligand, that directs the RNAi agent to a site of interest, e.g., the liver. Combinations of in vitro and in vivo methods of contacting are also possible. In connection with the methods of the invention, a cell might also be contacted in vitro with an RNAi agent and subsequently transplanted into a subject.

The present nucleic acid or polynucleotide may be conjugated to at least one ligand which is one or more GalNAc derivatives attached through a bivalent or trivalent branched linker. One strand (e.g., the sense strand or the antisense strand) or both strands (e.g., the sense strand or the antisense strand) of the present nucleic acid or polynucleotide may be conjugated to at least one ligand (e.g., 1, 2, 3, 4, 5, 6, 7, 8, or more GalNAc derivatives, or GalNAc clusters, or trivalent or bivalent GalNAc clusters) attached through a bivalent or trivalent branched linker at the 3' and/or 5' ends of the nucleic acid or polynucleotide.
The targeting ligand can be any ligand that is capable of targeting a specific receptor. Non-limiting examples of the ligands include, folate, GalNAc, galactose, mannose, mannose-6P, clusters of sugars such as GalNAc cluster, mannose cluster, galactose cluster, or an aptamer. A cluster is a combination of two or more sugar units. The targeting ligands also include integrin receptor ligands, Chemokine receptor ligands, transferrin, biotin, serotonin receptor ligands, PSMA, endothelin, GCPII, somatostatin, LDL and HDL ligands. The ligands can also be based on nucleic acid, e.g., an aptamer. The aptamer can be unmodified or have any combination of modifications disclosed herein.

The ligand can be attached to the sense strand, antisense strand or both strands, at the 3'-end, 5'-end or both ends. For instance, the ligand may be conjugated to the sense strand. In some embodiments, the ligand is conjugated to the 3'-end of the sense strand. In one embodiment, the ligand is a GalNAc ligand. In one embodiment, the ligand is GalNAc3. Contacting a cell may be accomplished via a targeting ligand, including any ligand described herein or known in the art. In some embodiments, the targeting ligand is a carbohydrate moiety, e.g., a GalNAc3 ligand, or any other ligand that directs the RNAi agent to a site of interest, e.g., the liver of a subject. Any suitable ligand in the field of RNA interference may

be used, such as a carbohydrate, e.g., monosaccharide (such as GalNAc), disaccharide, trisaccharide, tetrasaccharide, and polysaccharide.

Linkers that conjugate the ligand to the nucleic acid include those discussed above. For example, the ligand can be one or more GalNAc (N-acetylglucosamine) derivatives attached through a bivalent or trivalent branched linker.

The present nucleic acid or polynucleotide (e.g., one strand, the sense strand, antisense strand or both strands) may comprise at least one (e.g., 1, 2, 3, 4, 5, or more) phosphorothioate or methylphosphonate internucleotide linkage at the 3'-end, 5'-end or both ends. The present nucleic acid or polynucleotide (e.g., one strand, the sense strand, antisense strand or both strands) may comprise 1, 2, 3, 4, 5, 6, 7, 8 or more phosphorothioate internucleotide linkages between the 3'-end 1, 2, 3, 4, 5, 6, 7, 8 or more terminal nucleotides (and/or between the 5' end 1, 2, 3, 4, 5, 6, 7, 8 or more terminal nucleotides). The present nucleic acid or polynucleotide (e.g., one strand, the sense strand, antisense strand or both strands) may comprise at least one phosphorothioate internucleotide linkages between the 3'-end terminal nucleotide and a ligand (and/or between the 5' end terminal nucleotide and a ligand).

The present nucleic acid or polynucleotide (e.g., one strand, the sense strand, antisense strand or both strands) may comprise 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 or more modified nucleotides each independently selected from the group consisting of 2'-O-methyl (2'-OMe), 2'-fluoro (2'-F), and 2'-deoxythymidine (dT).

Where reference is made to a TAZ nucleic acid, this should be taken as a reference to any member of the TAZ family of nucleic acids. Of particular interest are TAZ nucleic acids selected from the group consisting of: NM_015472, NM_133784, NM_001037696, XM_001193047 and NM_001024869, as well as NM_001168280; NM_001168278; XM_011512661; NM_001168281. For example, the TAZ nucleic acid may comprise a human TAZ sequence having GenBank Accession Number NM_015472.

In certain instances, the following RNA sequences are useful:

the target sequence of mouse Ihh siRNA: UGC GGA CAA UCA UAC AGA ACC AGC A (SEQ ID NO:82);

target sequence of mouse Ihh siRNA: ACC ACC UUC AGU GAU GUG CUU A (SEQ ID NO:83);

a target sequence of human Taz siRNA: GGA UAC UAG UUG UGA AAU GGA AAG A (SEQ ID NO:84).

TAZ nucleic acids may be used for a variety of means, for example, administration to an individual suffering from, or suspected to be suffering from, liver cancer (e.g., HCC), or related liver conditions, or predisposition to such a condition, for the treatment thereof. The expression of elevated levels of TAZ nucleic acids may be detected for diagnosis or detection of liver cancer (e.g., HCC), or related liver conditions, or predisposition to such a condition. Such a "TAZ diagnostic NASH" test would utilize a liver biopsy in order to obtain a suitable patient test sample. Preliminary data indicates that in humans with NASH, TAZ protein levels are elevated in the range of 5-20 fold above normal. A bank of human non-NASH liver specimens would provide an average baseline immunoblot signal using densitometry quantification based on β-actin load. Values ≥2-fold greater than the averaged baseline would be indicative of a NASH diseased condition, or likelihood of progression to NASH. Methods including IHC would be useful in detecting elevated TAZ levels. The data described herein indicate that *Taz* nucleic acid levels or TAZ protein liver levels would be a good marker of risk for progression of benign steatosis to clinically significant NASH. RNAseq is another method that may be useful for such testing. Additionally, monitoring samples for elevated levels of *Taz* nucleic acids in patients undergoing treatments as described herein, may provide an indication of treatment efficacy and/or effectiveness. *Taz* nucleic acids may also be used for the expression or production of TAZ polypeptides. Additionally, methods for diagnosing liver cancer (e.g., HCC) or susceptibility to liver cancer (e.g., HCC) in a human subject can also utilize detecting an elevated level of TAZ, Indian hedgehog (Ihh), YAP, TEAD1, TEAD2, TEAD3, TEAD4, or any combination of these proteins, wherein an elevated level above baseline, of any one or more of these proteins: TAZ, Indian hedgehog (Ihh), YAP, TEAD1, TEAD2, TEAD3, TEAD4 indicates susceptibility to NASH or ongoing NASH in the human subject.

SEQ ID NO:1, SEQ ID NO:2, and any of SEQ ID NO:55-SEQ ID NO:72 and SEQ ID NO:81 may be useful in certain embodiments as primers amplifying *Taz* or as sequences utilized for designing nucleic acid inhibitors (*Taz* shRNA or RNAi), as shown in Table 1. Such sequences are expected to be useful in diagnostic and methods for detecting NASH or susceptibility to NASH, and for monitoring treatment by any of the methods as described herein.

By "down-regulation" included is any negative effect on the condition being studied; this may be total or partial. Thus, where binding is being detected, candidate antagonists are

capable of reducing, ameliorating, or abolishing the binding between two entities. The down-regulation of binding (or any other activity) achieved by the candidate molecule may be at least 10%, such as at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90% or more compared to binding (or which-ever activity) in the absence of the candidate molecule. Thus, a candidate molecule suitable for use as an antagonist is one which is capable of reducing by at least 10% the binding or other activity.

The term "compound" refers to a chemical compound (naturally occurring or synthesized), such as a biological macromolecule (e.g., nucleic acid, protein, non-peptide, or organic molecule), or an extract made from biological materials such as bacteria, plants, fungi, or animal (particularly mammalian) cells or tissues, or even an inorganic element or molecule. The compound may be an antibody.

Examples of potential antagonists of TAZ include antibodies, small molecules, nucleotides and their analogues, including purines and purine analogues, oligonucleotides or proteins which are closely related to a binding partner of TAZ, e.g., a fragment of the binding partner, or small molecules which bind to the TAZ polypeptide but do not elicit a response, so that the activity of the polypeptide is prevented, etc.

In some embodiments, the anti-TAZ agent is provided as an injectable or intravenenous composition and administered accordingly. The dosage of the anti-TAZ agent inhibitor may be between about 5 mg/kg/2 weeks to about 10 mg/kg/2 weeks. The anti-TAZ agent inhibitor may be provided in a dosage of between 10-300 mg/day, such as at least 30 mg/day, less than 200 mg/day or between 30 mg/day to 200 mg/day.

The anti-TAZ agent may downregulate TAZ by RNA interference, such as by comprising a small interfering RNA (siRNA) or short hairpin RNA (shRNA).

Additionally, TAZ polypeptide fragments could be utilized as inhibitors, for example See, WO2015063747A2, which describes peptides that block TAZ/TEAD interaction.

TAZ polypeptides or polypeptide fragments comprising amino acid sequences that are at least about 70% identical, preferably at least about 80% identical, more preferably at least about 90% identical and most preferably at least about 95% identical (e.g., 95%, 96%, 97%, 98%, 99%, 100%) to the mouse TAZ or human TAZ amino acid sequences with reference to sequences described above, are contemplated with respect to inhibiting TAZ expression and or function, when the comparison is performed by a BLAST algorithm wherein the parameters of the algorithm are selected to give the largest match between the respective sequences over the entire length of the respective reference sequences. Polypeptides comprising amino acid sequences that are at least about 70% similar, preferably at least about 80% similar, more

preferably at least about 90% similar and most preferably at least about 95% similar (e.g., 95%, 96%, 97%, 98%, 99%, 100%) to any of the reference TAZ amino acid sequences when the comparison is performed with a BLAST algorithm wherein the parameters of the algorithm are selected to give the largest match between the respective sequences over the entire length of the respective reference sequences, are also included in constructs and methods of the present invention.

Sequence identity refers to the degree to which the amino acids of two polypeptides are the same at equivalent positions when the two sequences are optimally aligned. Sequence similarity includes identical residues and nonidentical, biochemically related amino acids. Biochemically related amino acids that share similar properties and may be interchangeable are discussed above.

"Homology" refers to sequence similarity between two polynucleotide sequences or between two polypeptide sequences when they are optimally aligned. When a position in both of the two compared sequences is occupied by the same base or amino acid monomer subunit, e.g., if a position in each of two DNA molecules is occupied by adenine, then the molecules are homologous at that position. The percent of homology is the number of homologous positions shared by the two sequences divided by the total number of positions compared ×100. For example, if 6 of 10 of the positions in two sequences are matched or homologous when the sequences are optimally aligned then the two sequences are 60% homologous. Generally, the comparison is made when two sequences are aligned to give maximum percent homology.

The following references relate to BLAST algorithms often used for sequence analysis: BLAST ALGORITHMS: Altschul, S.F., *et al.*, (1990) J. Mol. Biol. 215:403-410; Gish, W., *et al.*, (1993) Nature Genet. 3:266-272; Madden, T.L., *et al.*, (1996) Meth. Enzymol. 266:131-141; Altschul, S.F., *et al.*, (1997) Nucleic Acids Res. 25:3389-3402; Zhang, J., *et al.*, (1997) Genome Res. 7:649-656; Wootton, J.C., *et al.*, (1993) Comput. Chem. 17:149-163; Hancock, J.M. *et al.*, (1994) Comput. Appl. Biosci. 10:67-70; ALIGNMENT SCORING SYSTEMS: Dayhoff, M.O., *et al.*, "A model of evolutionary change in proteins." in Atlas of Protein Sequence and Structure, (1978) vol. 5, suppl. 3. M.O. Dayhoff (ed.), pp. 345-352, Natl. Biomed. Res. Found., Washington, DC; Schwartz, R.M., *et al.*, "Matrices for detecting distant relationships." in Atlas of Protein Sequence and Structure, (1978) vol. 5, suppl. 3." M.O. Dayhoff (ed.), pp. 353-358, Natl. Biomed. Res. Found., Washington, DC; Altschul, S.F., (1991) J. Mol. Biol. 219:555-565; States, D.J., *et al.*, (1991) Methods 3:66-70; Henikoff, S., *et al.*, (1992) Proc. Natl. Acad. Sci. USA 89:10915-10919; Altschul, S.F., *et al.*, (1993) J. Mol. Evol. 36:290-300; ALIGNMENT STATISTICS: Karlin, S., *et al.*, (1990) Proc. Natl. Acad.

Sci. USA 87:2264-2268; Karlin, S., *et al.*, (1993) Proc. Natl. Acad. Sci. USA 90:5873-5877; Dembo, A., *et al.*, (1994) Ann. Prob. 22:2022-2039; and Altschul, S.F. "Evaluating the statistical significance of multiple distinct local alignments." in Theoretical and Computational Methods in Genome Research (S. Suhai, ed.), (1997) pp. 1-14, Plenum, New York.

In certain aspects, the present invention also provides expression vectors comprising various nucleic acids, wherein the nucleic acid is operably linked to control sequences that are recognized by a host cell when the host cell is transfected with the vector.

Combination Therapy

An inhibitor of TAZ (and/or an inhibitor of YAP) may be administered alone, or in combination with a second treatment (e.g., radiation, surgery, chemotherapeutic agents, other agents, etc.). An inhibitor of TAZ (and/or an inhibitor of YAP) may also be co-administered with antiviral agents, anti-inflammatory agents or antibiotics. The agents may be administered concurrently or sequentially. An inhibitor of TAZ (and/or an inhibitor of YAP) can be administered before, during or after radiation, surgery, or the administration of the other active agent(s).

The second treatments may include, but are not limited to, sorafenib, thalidomide, protein kinase inhibitors (e.g., tyrosine kinase inhibitors including VEGF inhibitors such as axitinib), inhibitors of ribonucleotide reductase subunit 2 (R2), a taxane and/or an albumin and/or another agent (such as an agent that inhibits microtubule disassembly), immunotherapy (e.g., anti-PD-1 antibodies), IL-17A inhibitors (e.g., IL-17A blocking antibodies), etc.

The inhibitor of TAZ (and/or the inhibitor of YAP) may be used in combination with radiation therapy. In one embodiment, the present disclosure provides for a method of treating cancer/tumor cells (e.g., liver cancer, or liver cancer cells) with radiation, where the subject/cells are treated with an effective amount of an inhibitor of TAZ (and/or an inhibitor of YAP), and then exposed to radiation. The inhibitor of TAZ (and/or the inhibitor of YAP) treatment may be before, during and/or after radiation.

The present disclosure provides for a method of treating cancer/tumor cells with chemotherapy or cytotoxic agent treatment, where the subject/cells are treated with an effective amount of an inhibitor of TAZ (and/or an inhibitor of YAP), and then exposed to chemotherapy. The inhibitor of TAZ (and/or the inhibitor of YAP) treatment may be before, during and/or after chemotherapy or cytotoxic agent treatment.

The present method for treating liver cancer (e.g., HCC) may comprise the step of administering to a subject an inhibitor of TAZ (and/or an inhibitor of YAP).

The present method for treating liver cancer (e.g., HCC) may comprise the step of administering to a subject an inhibitor of TAZ (and/or an inhibitor of YAP) and a cytotoxic agent (e.g., a chemotherapeutic agent).

This may be achieved by administering a pharmaceutical composition that includes both agents (an inhibitor of TAZ (and/or an inhibitor of YAP) and a cytotoxic agent (e.g., a chemotherapeutic agent)), or by administering two pharmaceutical compositions, at the same time or within a short time period, wherein one composition comprises an inhibitor of TAZ (and/or an inhibitor of YAP), and the other composition includes a cytotoxic agent (e.g., a chemotherapeutic agent).

The combination of the inhibitor of TAZ (and/or the inhibitor of YAP) and a cytotoxic agent (e.g., a chemotherapeutic agent) may produce an additive or synergistic effect (i.e., greater than additive effect) in treating the cancer compared to the effect of the inhibitor of TAZ (or YAP) alone or the cytotoxic agent (e.g., a chemotherapeutic agent) alone. For example, the combination may result in a synergistic increase in apoptosis of cancer cells, and/or a synergistic reduction in tumor volume, and a synergistic decrease in the number and/or size of tumor nodules (e.g., HCC nodules). In certain embodiments, depending on the combination and the effective amounts used, the combination of agents can inhibit tumor growth, achieve tumor stasis, or achieve substantial or complete tumor regression. In some embodiments, the combination therapy results in a synergistic effect, for example, the inhibitor of TAZ (and/or the inhibitor of YAP) and a cytotoxic agent (e.g., a chemotherapeutic agent) act synergistically, for example, in the apoptosis of cancer cells, inhibition of proliferation/survival of cancer cells, in the production of tumor stasis.

In various embodiments, the present invention provides methods to reduce cancer cell growth, proliferation, and/or metastasis, as measured according to routine techniques in the diagnostic art. Specific examples of relevant responses include reduced size, mass, or volume of a tumor, or reduction in cancer cell number.

Methods and compositions of the present invention can be used for prophylaxis as well as amelioration of signs and/or symptoms of liver cancer.

As used herein, the term “synergy” (or "synergistic") means that the effect achieved with the methods and combinations of this disclosure is greater than the sum of the effects that result from using the individual agents alone, e.g., using the inhibitor of TAZ (or YAP) alone and the cytotoxic agent (e.g., a chemotherapeutic agent) alone. For example, the effect

(e.g., apoptosis of cells, a decrease in cell viability, cytotoxicity, a decrease in cell proliferation, a decrease in cell survival, inhibition of tumor growth, a reduction in tumor volume, and/or tumor stasis, etc. as described herein) achieved with the combination of an inhibitor of TAZ (and/or an inhibitor of YAP) and a cytotoxic agent (e.g., a chemotherapeutic agent) is about 1.1 fold, about 1.2 fold, about 1.3 fold, about 1.4 fold, about 1.5 fold, about 1.6 fold, about 1.7 fold, about 1.8 fold, about 1.9 fold, about 2 fold, about 2.5 fold, about 3 fold, about 3.5 fold, about 4 fold, about 4.5 fold, about 5 fold, about 5.5 fold, about 6 fold, about 6.5 fold, about 7 fold, about 8 fold, about 9 fold, about 10 fold, about 12 fold, about 15 fold, about 20 fold, about 25 fold, about 30 fold, about 50 fold, about 100 fold, at least about 1.2 fold, at least about 1.5 fold, at least about 2 fold, at least about 2.5 fold, at least about 3 fold, at least about 3.5 fold, at least about 4 fold, at least about 4.5 fold, at least about 5 fold, at least about 5.5 fold, at least about 6 fold, at least about 6.5 fold, at least about 7 fold, at least about 8 fold, at least about 9 fold, at least about 10 fold, of the sum of the effects that result from using the inhibitor of TAZ (or YAP) alone and the inhibitor of the cytotoxic agent (e.g., a chemotherapeutic agent) alone.

Synergistic effects of the combination may also be evidenced by additional, novel effects that do not occur when either agent is administered alone, or by reduction of adverse side effects when either agent is administered alone.

In vitro efficacy of the present composition/agent can be determined using methods well known in the art. For example, the cytoxicity of the present composition/agent and/or the therapeutic agents may be studied by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] cytotoxicity assay. MTT assay is based on the principle of uptake of MTT, a tetrazolium salt, by metabolically active cells where it is metabolized into a blue colored formazon product, which can be read spectrometrically. J. of Immunological Methods 65: 55 63, 1983. The cytoxicity of the present composition/agent and/or the therapeutic agents may be studied by colony formation assay. Functional assays for inhibition of VEGF secretion and IL-8 secretion may be performed via ELISA. Cell cycle block by the present composition/agent and/or the therapeutic agents may be studied by standard propidium iodide (PI) staining and flow cytometry. Invasion inhibition may be studied by Boyden chambers. In this assay a layer of reconstituted basement membrane, Matrigel, is coated onto chemotaxis filters and acts as a barrier to the migration of cells in the Boyden chambers. Only cells with invasive capacity can cross the Matrigel barrier. Other assays include, but are not limited to cell viability assays, apoptosis assays, and morphological assays.

Cytotoxicity effects can be determined by any suitable assay *in vitro*, including, but not limited to, assessing cell membrane integrity (using, e.g., dyes such as trypan blue or propidium iodide, or using lactate dehydrogenase (LDH) assay), measuring enzyme activity, measuring cell adherence, measuring ATP production, measuring co-enzyme production, measuring nucleotide uptake activity, crystal violet method, Tritium-labeled Thymidine uptake method, measuring lactate dehydrogenase (LDH) activity, 3-(4, 5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) or MTS assay, sulforhodamine B (SRB) assay, WST assay, clonogenic assay, cell number count, monitoring cell growth, etc.

Apoptosis of cells may be assayed by any suitable method, including, but not limited to, TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) assay, assaying levels of cytochrome C release, assaying levels of cleaved/activated caspases, assaying 5-bromo-2'-deoxyuridine labeled fragmented DNA, assaying levels of survivin etc.

Other methods that can be used to show the synergistic effects of the present methods, pharmaceutical compositions and combinations include, but are not limited to, clonogenic assay (colony formation assay) to show decrease in cell survival and/or proliferation, studying tumor volume reduction in animal models (such as in mice, etc.)

In one embodiment, advantageously, such synergy provides greater efficacy at the same doses, lower side effects, and/or prevents or delays the build-up of multi-drug resistance.

The cytotoxic agent (e.g., a chemotherapeutic agent) and the inhibitor of TAZ may be administered simultaneously, separately or sequentially. They may exert an advantageously combined effect (e.g., additive or synergistic effects).

For sequential administration, either an inhibitor of TAZ (and/or an inhibitor of YAP) is administered first and then a cytotoxic agent (e.g., a chemotherapeutic agent), or a cytotoxic agent (e.g., a chemotherapeutic agent) is administered first and then an inhibitor of TAZ (and/or an inhibitor of YAP). In embodiments where an inhibitor of TAZ (and/or an inhibitor of YAP) and a cytotoxic agent (e.g., a chemotherapeutic agent) are administered separately, administration of a first agent can precede administration of a second agent by seconds, minutes, hours, days, or weeks. The time difference in non-simultaneous administrations may be greater than 1 minute, and can be, for example, precisely, at least, up to, or less than 5 minutes, 10 minutes, 15 minutes, 30 minutes, 45 minutes, 60 minutes, 2 hours, 3 hours, 6 hours, 9 hours, 12 hours, 24 hours, 36 hours, or 48 hours, or more than 48 hours. The two or more agents can be administered within minutes of each other or within about 0.5, about 1, about 2, about 3, about 4, about 6, about 9, about 12, about 15, about 18,

about 24, or about 36 hours of each other or within about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14 days of each other or within about 2, 3, 4, 5, 6, 7, 8, 9, or 10 weeks of each other. In some cases, longer intervals are possible.

The present invention also provides for a pharmaceutical composition comprising (i) the inhibitor of TAZ (and/or an inhibitor of YAP); (ii) a cytotoxic agent (e.g., a chemotherapeutic agent); and (iii) at least one pharmaceutically acceptable excipient.

Combinations may be administered either concomitantly, e.g., as an admixture, separately but simultaneously or concurrently; or sequentially. This includes presentations in which the combined agents are administered together as a therapeutic mixture, and also procedures in which the combined agents are administered separately but simultaneously, e.g., as through separate intravenous lines into the same individual. Administration in combination further includes the separate administration of one of the compounds or agents given first, followed by the second.

This may be achieved by administering a pharmaceutical composition that includes both agents, or by administering two pharmaceutical compositions, at the same time or within a short time period.

In certain embodiments, the combination of the present inhibitor and the second treatment produces an additive or synergistic effect (i.e., greater than additive effect) in treating a disorder as discussed herein, compared to the effect of the inhibitor alone or the second treatment alone.

As used herein, the term “synergy” (or "synergistic") means that the effect achieved with the methods and combinations of the combination therapy is greater than the sum of the effects that result from using the individual agents alone, e.g., using the inhibitor of TAZ (or YAP) alone and the second treatment alone. For example, the effect achieved with the combination of the inhibitor of TAZ (and/or an inhibitor of YAP) and the second treatment is about 1.1 fold, about 1.2 fold, about 1.3 fold, about 1.4 fold, about 1.5 fold, about 1.6 fold, about 1.7 fold, about 1.8 fold, about 1.9 fold, about 2 fold, about 2.5 fold, about 3 fold, about 3.5 fold, about 4 fold, about 4.5 fold, about 5 fold, about 5.5 fold, about 6 fold, about 6.5 fold, about 7 fold, about 8 fold, about 9 fold, about 10 fold, about 12 fold, about 15 fold, about 20 fold, about 25 fold, about 30 fold, about 50 fold, about 100 fold, at least about 1.2 fold, at least about 1.5 fold, at least about 2 fold, at least about 2.5 fold, at least about 3 fold, at least about 3.5 fold, at least about 4 fold, at least about 4.5 fold, at least about 5 fold, at least about 5.5 fold, at least about 6 fold, at least about 6.5 fold, at least about 7 fold, at least about 8

fold, at least about 9 fold, at least about 10 fold, of the sum of the effects that result from using the inhibitor of TAZ (or YAP) alone or the second treatment alone.

In one embodiment, advantageously, such synergy provides greater efficacy at the same doses, and/or lower side effects.

For sequential administration, either an inhibitor of TAZ (and/or an inhibitor of YAP) is administered first and then a second treatment, or the second treatment is administered first and then an inhibitor of TAZ (and/or an inhibitor of YAP). In embodiments where the inhibitor of TAZ (and/or an inhibitor of YAP) and the second treatment are administered separately, administration of a first agent can precede administration of a second agent by seconds, minutes, hours, days, or weeks. The time difference in non-simultaneous administrations may be greater than 1 minute, and can be, for example, precisely, at least, up to, or less than 5 minutes, 10 minutes, 15 minutes, 30 minutes, 45 minutes, 60 minutes, 2 hours, 3 hours, 6 hours, 9 hours, 12 hours, 24 hours, 36 hours, or 48 hours, or more than 48 hours. The two or more agents can be administered within minutes of each other or within about 0.5, about 1, about 2, about 3, about 4, about 6, about 9, about 12, about 15, about 18, about 24, or about 36 hours of each other or within about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14 days of each other or within about 2, 3, 4, 5, 6, 7, 8, 9, or 10 weeks of each other. In some cases, longer intervals are possible.

The present disclosure may provide for a pharmaceutical composition comprising a first amount of an inhibitor of TAZ (and/or an inhibitor of YAP) and a second amount of a second agent. The combination of the first amount of an inhibitor of TAZ (and/or an inhibitor of YAP) and the second amount of the second agent may produce a synergistic effect on a cancer cell compared to the effect of the first amount of the inhibitor of TAZ (or YAP) alone or the effect of the second amount of the second agent alone.

The amount of the inhibitor of TAZ (and/or an inhibitor of YAP) or the amount of the second agent that may be used in the combination therapy may be a therapeutically effective amount, a sub-therapeutically effective amount or a synergistically effective amount.

The inhibitor of TAZ (and/or an inhibitor of YAP), and/or the second agent may be present in the pharmaceutical composition in an amount ranging from about 0.005% (w/w) to about 100% (w/w), from about 0.01% (w/w) to about 90% (w/w), from about 0.1% (w/w) to about 80% (w/w), from about 1% (w/w) to about 70% (w/w), from about 10% (w/w) to about 60% (w/w), from about 0.01% (w/w) to about 15% (w/w), or from about 0.1% (w/w) to about 20% (w/w).

The inhibitor of TAZ (and/or an inhibitor of YAP) and the second agent may be present in two separate pharmaceutical compositions to be used in a combination therapy.

The effective amount of the inhibitor of TAZ (and/or an inhibitor of YAP) or the second agent for the combination therapy may be less than, equal to, or greater than when the agent is used alone.

Pharmaceutical Compositions and Administration

To prepare pharmaceutical or sterile compositions of the compositions of the present invention, the viral vectors, RNAi, shRNA or other TAZ inhibitors, or similar compositions may be admixed with a pharmaceutically acceptable carrier or excipient. See, *e.g.*, *Remington's Pharmaceutical Sciences* and *U.S. Pharmacopeia: National Formulary*, Mack Publishing Company, Easton, PA (1984).

Formulations of therapeutic and diagnostic agents may be prepared by mixing with acceptable carriers, excipients, or stabilizers in the form of, *e.g.*, lyophilized powders, slurries, aqueous solutions or suspensions (see, *e.g.*, Hardman, *et al.* (2001) *Goodman and Gilman's The Pharmacological Basis of Therapeutics*, McGraw-Hill, New York, NY; Gennaro (2000) *Remington: The Science and Practice of Pharmacy*, Lippincott, Williams, and Wilkins, New York, NY; Avis, *et al.* (eds.) (1993) *Pharmaceutical Dosage Forms: Parenteral Medications*, Marcel Dekker, NY; Lieberman, *et al.* (eds.) (1990) *Pharmaceutical Dosage Forms: Tablets*, Marcel Dekker, NY; Lieberman, *et al.* (eds.) (1990) *Pharmaceutical Dosage Forms: Disperse Systems*, Marcel Dekker, NY; Weiner and Kotkoskie (2000) *Excipient Toxicity and Safety*, Marcel Dekker, Inc., New York, NY).

Toxicity and therapeutic efficacy of the therapeutic compositions, administered alone or in combination with another agent, can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.,* for determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index (LD_{50}/ ED_{50}). In particular aspects, therapeutic compositions exhibiting high therapeutic indices are desirable. The data obtained from these cell culture assays and animal studies can be used in formulating a range of dosage for use in human. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration.

In an embodiment of the invention, a composition of the invention is administered to a subject in accordance with the Physicians' Desk Reference 2003 (Thomson Healthcare; 57th edition (November 1, 2002)).

The mode of administration can vary. Suitable routes of administration include oral, rectal, transmucosal, intestinal, parenteral; intramuscular, subcutaneous, intradermal, intramedullary, intrathecal, direct intraventricular, intravenous, intraperitoneal, intranasal, intraocular, inhalation, insufflation, topical, cutaneous, transdermal, or intra-arterial.

In particular embodiments, the composition or therapeutic can be administered by an invasive route such as by injection (see above). In further embodiments of the invention, the composition, therapeutic, or pharmaceutical composition thereof, is administered intravenously, subcutaneously, intramuscularly, intraarterially, intra-articularly (e.g. in arthritis joints), intratumorally, or by inhalation, aerosol delivery. Administration by non-invasive routes (*e.g.*, orally; for example, in a pill, capsule or tablet) is also within the scope of the present invention.

Compositions can be administered with medical devices known in the art. For example, a pharmaceutical composition of the invention can be administered by injection with a hypodermic needle, including, e.g., a prefilled syringe or autoinjector.

The pharmaceutical compositions of the invention may also be administered with a needleless hypodermic injection device; such as the devices disclosed in U.S. Patent Nos. 6,620,135; 6,096,002; 5,399,163; 5,383,851; 5,312,335; 5,064,413; 4,941,880; 4,790,824 or 4,596,556.

Alternately, one may administer the viral vectors, RNAi, shRNA or other TAZ 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, RNAi, shRNA or other TAZ inhibitors, or TAZ-based compound, alone or in combination with an *Ihh* RNAi construct or similar inhibitors. 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.

Any of the therapeutics described herein including: an inhibitor of TAZ, inhibitor of Indian hedgehog (Ihh), an inhibitor of YAP, an inhibitor of TEAD1, TEAD2, TEAD3, TEAD4, or any combination thereof, can also comprise a delivery vehicle, including liposomes, for administration to a subject, carriers and diluents and their salts, and/or can be present in pharmaceutically acceptable formulations. For example, methods for the delivery of nucleic acid molecules are described in Akhtar et al., 1992, *Trends Cell Bio.*, 2, 139; DELIVERY STRATEGIES FOR ANTISENSE OLIGONUCLEOTIDE THERAPEUTICS, ed. Akbtar, 1995, Maurer et al., 1999, *Mol. Membr. Biol.*, 16, 129-140; Hofland and Huang, 1999, *Handb. Exp. Pharmacol.*, 137, 165-192; and Lee et al., 2000, ACS Symp. Ser., 752, 184-192. U.S. Pat. No. 6,395,713 and PCT Publication No. WO 94/02595 further describe the general methods for delivery of nucleic acid molecules. These protocols can be utilized for the delivery of virtually any nucleic acid molecule.

Any of the therapeutics described herein including: an inhibitor of TAZ, inhibitor of Indian hedgehog (Ihh), an inhibitor of YAP, an inhibitor of TEAD1, TEAD2, TEAD3, TEAD4, or any combination thereof can also be administered to a desired target by a variety of methods known to those of skill in the art, including, but not restricted to, encapsulation in liposomes, by iontophoresis, or by incorporation into other vehicles, such as hydrogels, cyclodextrins, biodegradable nanocapsules, and bioadhesive microspheres, or by proteinaceous vectors (see PCT Publication No. WO 00/53722). Alternatively, the therapeutic/vehicle combination is locally delivered by direct injection or by use of an infusion pump. Direct injection of the composition, whether subcutaneous, intramuscular, or intradermal, can take place using standard needle and syringe methodologies, or by needle-free technologies such as those described in Conry et al., 1999, *Clin. Cancer Res.*, 5, 2330-2337 and PCT Publication No. WO 99/3 1262.

Therapeutic compositions comprising surface-modified liposomes containing poly(ethylene glycol) lipids (PEG-modified, or long-circulating liposomes or stealth liposomes) may also be suitably employed in the methods of the invention. These formulations offer a method for increasing the accumulation of drugs in target tissues. This class of drug carriers resists opsonization and elimination by the mononuclear phagocytic system (MPS or RES), thereby enabling longer blood circulation times and enhanced tissue exposure for the encapsulated drug (Lasic et al. Chem. Rev. 1995, 95, 2601-2627; Ishiwata et al., Chem. Pharm. Bull. 1995, 43, 1005-1011). The long-circulating liposomes enhance the pharmacokinetics and pharmacodynamics of DNA and RNA, particularly compared to conventional cationic liposomes which are known to accumulate in tissues of the MPS (Liu et al., J. Biol. Chem.

1995, 42, 24864-24870; PCT Publication No. WO 96/10391; PCT Publication No. WO 96/10390; and PCT Publication No. WO 96/10392). Long-circulating liposomes are also likely to protect drugs from nuclease degradation to a greater extent compared to cationic liposomes, based on their ability to avoid accumulation in metabolically aggressive MPS tissues such as the liver and spleen.

The administration regimen depends on several factors, including the serum or tissue turnover rate of the therapeutic composition, the level of symptoms, and the accessibility of the target cells in the biological matrix. Preferably, the administration regimen delivers sufficient therapeutic composition to effect improvement in the target disease state, while simultaneously minimizing undesired side effects. Accordingly, the amount of biologic delivered depends in part on the particular therapeutic composition and the severity of the condition being treated.

Determination of the appropriate dose is made by the clinician, *e.g.*, using parameters or factors known or suspected in the art to affect treatment. Generally, the dose begins with an amount somewhat less than the optimum dose and it is increased by small increments thereafter until the desired or optimum effect is achieved relative to any negative side effects. Important diagnostic measures include those of symptoms of, *e.g.*, the inflammation or level of inflammatory cytokines produced. In general, it is desirable that a biologic that will be used is derived from the same species as the animal targeted for treatment, thereby minimizing any immune response to the reagent.

As used herein, "inhibit" or "treat" or "treatment" includes a postponement of development of the symptoms associated with a disorder and/or a reduction in the severity of the symptoms of such disorder. The terms further include ameliorating existing uncontrolled or unwanted symptoms, preventing additional symptoms, and ameliorating or preventing the underlying causes of such symptoms. Thus, the terms denote that a beneficial result has been conferred on a vertebrate subject with a disorder, disease or symptom, or with the potential to develop such a disorder, disease or symptom.

As used herein, the terms "therapeutically effective amount", "therapeutically effective dose" and "effective amount" refer to an amount of a viral vector, RNAi, shRNA or other TAZ inhibitors or inhibitor compound of the invention that, when administered alone or in combination with an additional therapeutic agent to a cell, tissue, or subject, is effective to cause a measurable improvement in one or more symptoms of a disease or condition or the progression of such disease or condition. A therapeutically effective dose further refers to that amount of the compound sufficient to result in at least partial amelioration of symptoms, *e.g.*, treatment, healing, prevention or amelioration of the relevant medical condition, or an increase

in rate of treatment, healing, prevention or amelioration of such conditions. When applied to an individual active ingredient administered alone, a therapeutically effective dose refers to that ingredient alone. When applied to a combination, a therapeutically effective dose refers to combined amounts of the active ingredients that result in the therapeutic effect, whether administered in combination, serially or simultaneously. An effective amount of a therapeutic will result in an improvement of a diagnostic measure or parameter by at least 10%; usually by at least 20%; preferably at least about 30%; more preferably at least 40%, and most preferably by at least 50%. An effective amount can also result in an improvement in a subjective measure in cases where subjective measures are used to assess disease severity.

Kits

The present invention also provides kits comprising the components of the combinations of the invention in kit form. A kit of the present invention includes one or more components including, but not limited to, the viral vectors, RNAi, shRNA or other TAZ inhibitors, or TAZ/YAP/IHH-based inhibitor compounds, as discussed herein, in association with one or more additional components including, but not limited to a pharmaceutically acceptable carrier and/or a chemotherapeutic agent, as discussed herein. The viral vectors, RNAi, shRNA or other TAZ inhibitors, or TAZ/YAP/IHH-based inhibitor compounds, composition and/or the therapeutic agent can be formulated as a pure composition or in combination with a pharmaceutically acceptable carrier, in a pharmaceutical composition.

Kits may also include primers, buffers, and probes along with instructions for determining elevated levels of nucleic acid, proteins, or protein fragments of TAZ, Indian hedgehog (Ihh), YAP, TEAD1, TEAD2, TEAD3, TEAD4, or any combination thereof.

In one embodiment, a kit includes the viral vectors, RNAi, shRNA, or other TAZ inhibitors, or TAZ/YAP/IHH-based inhibitor compounds/composition of the invention or a pharmaceutical composition thereof in one container (*e.g.*, in a sterile glass or plastic vial) and a pharmaceutical composition thereof and/or a chemotherapeutic agent in another container (*e.g.*, in a sterile glass or plastic vial).

In another embodiment of the invention, the kit comprises a combination of the invention, including the viral vectors, RNAi, shRNA or other TAZ inhibitors, or TAZ/YAP/IHH-based inhibitor compounds, along with a pharmaceutically acceptable carrier, optionally in combination with one or more therapeutic agent components formulated together, optionally, in a pharmaceutical composition, in a single, common container.

If the kit includes a pharmaceutical composition for parenteral administration to a subject, the kit can include a device for performing such administration. For example, the kit can include one or more hypodermic needles or other injection devices as discussed above.

The kit can include a package insert including information concerning the pharmaceutical compositions and dosage forms in the kit. Generally, such information aids patients and physicians in using the enclosed pharmaceutical compositions and dosage forms effectively and safely. For example, the following information regarding a combination of the invention may be supplied in the insert: pharmacokinetics, pharmacodynamics, clinical studies, efficacy parameters, indications and usage, contraindications, warnings, precautions, adverse reactions, overdosage, proper dosage and administration, how supplied, proper storage conditions, references, manufacturer/distributor information and patent information.

GENERAL METHODS

Standard methods in molecular biology are described Sambrook, Fritsch and Maniatis (1982 & 1989 2nd Edition, 2001 3rd Edition) *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY; Sambrook and Russell (2001) *Molecular Cloning, 3rd ed.,* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY; Wu (1993) *Recombinant DNA*, Vol. 217, Academic Press, San Diego, CA). Standard methods also appear in Ausbel, *et al.* (2001) *Current Protocols in Molecular Biology, Vols.1-4*, John Wiley and Sons, Inc. New York, NY, which describes cloning in bacterial cells and DNA mutagenesis (Vol. 1), cloning in mammalian cells and yeast (Vol. 2), glycoconjugates and protein expression (Vol. 3), and bioinformatics (Vol. 4).

Methods for protein purification including immunoprecipitation, chromatography, electrophoresis, centrifugation, and crystallization are described (Coligan, *et al.* (2000) *Current Protocols in Protein Science, Vol. 1*, John Wiley and Sons, Inc., New York). Chemical analysis, chemical modification, post-translational modification, production of fusion proteins, glycosylation of proteins are described (see, *e.g.*, Coligan, *et al.* (2000) *Current Protocols in Protein Science, Vol. 2*, John Wiley and Sons, Inc., New York; Ausubel, *et al.* (2001) *Current Protocols in Molecular Biology, Vol. 3*, John Wiley and Sons, Inc., NY, NY, pp. 16.0.5-16.22.17; Sigma-Aldrich, Co. (2001) *Products for Life Science Research*, St. Louis, MO; pp. 45-89; Amersham Pharmacia Biotech (2001) *BioDirectory*, Piscataway, N.J., pp. 384-391). Production, purification, and fragmentation of polyclonal and monoclonal antibodies are described (Coligan, *et al.* (2001) *Current Protocols in Immunology, Vol. 1*, John Wiley and Sons, Inc., New York; Harlow and Lane (1999) *Using Antibodies*, Cold Spring Harbor

Laboratory Press, Cold Spring Harbor, NY; Harlow and Lane, *supra*). Standard techniques for characterizing ligand/receptor interactions are available (see, *e.g.*, Coligan, *et al.* (2001) *Current Protocols in Immunology, Vol. 4*, John Wiley, Inc., New York).

Abbreviations

HSCs: hepatic stellate cells;

HCC: hepatocellular carcinoma;

IHC: immunohistochemistry;

NASH: Nonalcoholic steatohepatitis;

NAFLD: Non-alcoholic fatty liver disease;

Hprt: hypoxanthine guanine phosphoribosyl transferase;

Taz (*Wwtr1*): WW domain containing transcription regulator 1, encoding the TAZ protein (Reference human nucleotide sequence: NM_015472; Reference human protein sequence: NP_056287);

Tgfβ1: transforming growth factor, beta 1;

Acta2, α-smooth muscle actin;

Vim: vimentin;

Des: desmin;

Col1a1: collagen type I alpha 1;

Col1a2: collagen type I alpha 2;

Col3a1: collagen, type III, alpha 1;

F4/80 (*Adgre1*): adhesion G protein-coupled receptor E1;

Tnfα: tumor necrosis factor alpha;

Mcp1: monocyte chemoattractant protein-1;

Ihh: Indian hedgehog;

Gli2: *GLI* family zinc finger 2;

Gli3: *GLI* family zinc finger 3;

Opn: osteopontin;

Timp1: tissue inhibitor of metalloproteinase 1;

Cpt1b: carnitine palmitoyltransferase 1B;

Pparg: peroxisome proliferator-activated receptor-γ;

Scd1: stearoyl-CoA desaturase;

Fasn: fatty acid synthase;

Acaca: acetyl-CoA carboxylase-α;

Cd3: *CD3* antigen;

Cd20: B-lymphocyte antigen;

Ihh intron: specific TAZ/TEAD binding area in 1st intron of *Ihh* gene;

Ihh non-specific: non-specific TAZ/TEAD binding site in mouse *Ihh* gene distal promoter.

TEA; transcriptional enhancer factor.

TEAD1: TEA Domain Family Member 1 (also known as SV40 Transcriptional Enhancer Factor or TEF-1), (Reference human nucleotide sequence: NM_021961; Reference human protein sequence: NP_068780).

TEAD2: Transcriptional enhancer factor TEF-4 also known as TEA domain family member 2. (Reference human nucleotide sequence: NM_001256658; Reference human protein sequence: NP_001243587).

TEAD3: TEA domain transcription factor 3 encodes transcriptional enhancer factor TEF-5. (Reference human nucleotide sequence: NM_003214; Reference human protein sequence: NP_003205).

TEAD4: TEA Domain Family Member 4; (Reference human nucleotide sequence: NM_003213; Reference human protein sequence: NP_003204).

YAP: Yes-associated protein 1, the gene encoding this protein is known as YAP1 or YAP65 (Reference human nucleotide sequence:_ NM_001282101; Reference human protein sequences: UniProtKB/Swiss-Prot P46937.2 or NCB1 NP_001 123617.1).

So that the invention may be more readily understood, certain technical and scientific terms are specifically defined below. Unless specifically defined elsewhere in this document, all other technical and scientific terms used herein have the meaning commonly understood by one of ordinary skill in the art to which this invention belongs.

As used herein, including the appended claims, the singular forms of words such as "a," "an," and "the," include their corresponding plural references unless the context clearly dictates otherwise.

"Activation," "stimulation," and "treatment," as it applies to cells or to receptors, may have the same meaning, e.g., activation, stimulation, or treatment of a cell or receptor with a ligand, unless indicated otherwise by the context or explicitly. "Ligand" encompasses natural and synthetic ligands, e.g., cytokines, cytokine variants, analogues, muteins, and binding compounds derived from antibodies. "Ligand" also encompasses small molecules, e.g., peptide mimetics of cytokines and peptide mimetics of antibodies. "Activation" can refer to cell activation as regulated by internal mechanisms as well as by external or environmental factors. "Response," e.g., of a cell, tissue, organ, or organism, encompasses a change in biochemical or physiological behavior, e.g., concentration, density, adhesion, or migration within a biological compartment, rate of gene expression, or state of differentiation, where the change is correlated with activation, stimulation, or treatment, or with internal mechanisms such as genetic programming.

"Activity" of a molecule may describe or refer to the binding of the molecule to a ligand or to a receptor, to catalytic activity; to the ability to stimulate gene expression or cell signaling, differentiation, or maturation; to antigenic activity, to the modulation of activities of other molecules, and the like. "Activity" of a molecule may also refer to activity in modulating or maintaining cell-to-cell interactions, e.g., adhesion, or activity in maintaining a structure of a cell, e.g., cell membranes or cytoskeleton. "Activity" can also mean specific activity, e.g., [catalytic activity]/[mg protein], or [immunological activity]/[mg protein], concentration in a biological compartment, or the like. "Activity" may refer to modulation of components of the innate or the adaptive immune systems.

"Administration" and "treatment," as it applies to an animal, human, experimental subject, cell, tissue, organ, or biological fluid, refers to contact of an exogenous pharmaceutical, therapeutic, diagnostic agent, or composition to the animal, human, subject, cell, tissue, organ, or biological fluid. "Administration" and "treatment" can refer, e.g., to therapeutic, pharmacokinetic, diagnostic, research, and experimental methods. Treatment of a cell encompasses contact of a reagent to the cell, as well as contact of a reagent to a fluid, where

the fluid is in contact with the cell. "Administration" and "treatment" also means *in vitro* and *ex vivo* treatments, e.g., of a cell, by a reagent, diagnostic, binding compound, or by another cell. The term "subject" includes any organism, preferably an animal, more preferably a mammal (*e.g.*, rat, mouse, dog, cat, rabbit) and most preferably a human, including a human patient.

"Treat" or "treating" refers to administering a therapeutic agent, such as a composition containing any of the liver targeted viral vectors, RNAi, shRNA or other TAZ inhibitors, or similar compositions described herein, internally or externally to a subject or patient having one or more disease symptoms, or being suspected of having a disease or being at elevated at risk of acquiring a disease, for which the agent has therapeutic activity. Gene editing technology such as CRISPR/cas9 methods may also be utilized to carry out liver specific reduction of TAZ and/or related TAZ co-factors such as one or more inhibitors of IHH or TEAD1-TEAD-4. Typically, the agent is administered in an amount effective to alleviate one or more disease symptoms in the treated subject or population, whether by inducing the regression of or inhibiting the progression of such symptom(s) by any clinically measurable degree. The amount of a therapeutic agent that is effective to alleviate any particular disease symptom (also referred to as the "therapeutically effective amount") may vary according to factors such as the disease state, age, and weight of the patient, and the ability of the drug to elicit a desired response in the subject Whether a disease symptom has been alleviated can be assessed by any clinical measurement typically used by physicians or other skilled healthcare providers to assess the severity or progression status of that symptom. While an embodiment of the present invention (e.g., a treatment method or article of manufacture) may not be effective in alleviating the target disease symptom(s) in every subject, it should alleviate the target disease symptom(s) in a statistically significant number of subjects as determined by any statistical test known in the art such as the Student's t-test, the chi^2-test, the U-test according to Mann and Whitney, the Kruskal-Wallis test (H-test), Jonckheere-Terpstra-test and the Wilcoxon-test.

"Treatment," as it applies to a human, veterinary, or research subject, refers to therapeutic treatment, prophylactic or preventative measures, to research and diagnostic applications. "Treatment" as it applies to a human, veterinary, or research subject, or cell, tissue, or organ, encompasses transfection of any of the liver-targeted viral vectors, delivery of RNAi, shRNA or other TAZ inhibitors, or similar compositions, including gene editing technology such as CRISPR/cas9 methods, which may be utilized to carry out liver specific reduction of TAZ and/or related TAZ co-factors such as one or more inhibitors of IHH, or

TEAD1-TEAD-4, or related methods described herein as applied to a human or animal subject, a cell, tissue, physiological compartment, or physiological fluid.

"Isolated nucleic acid molecule" means a DNA or RNA of genomic, mRNA, cDNA, or synthetic origin or some combination thereof which is not associated with all or a portion of a polynucleotide in which the isolated polynucleotide is found in nature, or is linked to a polynucleotide to which it is not linked in nature. For purposes of this disclosure, it should be understood that "a nucleic acid molecule comprising" a particular nucleotide sequence does not encompass intact chromosomes. Isolated nucleic acid molecules "comprising" specified nucleic acid sequences may include, in addition to the specified sequences, coding sequences for up to ten or even up to twenty or more other proteins or portions or fragments thereof, or may include operably linked regulatory sequences that control expression of the coding region of the recited nucleic acid sequences, and/or may include vector sequences.

The phrase "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to use promoters, polyadenylation signals, and enhancers.

A nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

As used herein, the expressions "cell," "cell line," and "cell culture" are used interchangeably and all such designations include progeny. Thus, the words "transformants" and "transformed cells" include the primary subject cell and cultures derived therefrom without regard for the number of transfers. It is also understood that not all progeny will have precisely identical DNA content, due to deliberate or inadvertent mutations. Mutant progeny that have

the same function or biological activity as screened for in the originally transformed cell are included. Where distinct designations are intended, it will be clear from the context.

With respect to cells, the term "isolated" refers to a cell that has been isolated from its natural environment (e.g., from a tissue or subject). The term "cell line" refers to a population of cells capable of continuous or prolonged growth and division in vitro. Often, cell lines are clonal populations derived from a single progenitor cell. It is further known in the art that spontaneous or induced changes can occur in karyotype during storage or transfer of such clonal populations. Therefore, cells derived from the cell line referred to may not be precisely identical to the ancestral cells or cultures, and the cell line referred to includes such variants. As used herein, the terms "recombinant cell" refers to a cell into which an exogenous DNA segment, such as DNA segment that leads to the transcription of a biologically-active polypeptide or production of a biologically active nucleic acid such as anRNA, has been introduced.

The term "vector" includes any genetic element, such as a plasmid, phage, transposon, cosmid, chromosome, artificial chromosome, virus, virion, etc., which is capable of replication when associated with the proper control elements and which can transfer gene sequences between cells. Thus, the term includes cloning and expression vehicles, as well as viral vectors. In some embodiments, useful vectors are contemplated to be those vectors in which the nucleic acid segment to be transcribed is positioned under the transcriptional control of a promoter. A "promoter" refers to a DNA sequence recognized by the synthetic machinery of the cell, or introduced synthetic machinery, required to initiate the specific transcription of a gene. The phrases "operatively positioned," “operatively linked,” "under control," or "under transcriptional control" means that the promoter is in the correct location and orientation in relation to the nucleic acid to control RNA polymerase initiation and expression of the gene. The term "expression vector or construct" means any type of genetic construct containing a nucleic acid in which part or all of the nucleic acid encoding sequence is capable of being transcribed. In some embodiments, expression includes transcription of the nucleic acid, for example, to generate a biologically-active polypeptide product or inhibitory RNA (e.g., shRNA, miRNA) from a transcribed gene.

As used herein, "polymerase chain reaction" or "PCR" refers to a procedure or technique in which specific nucleic acid sequences, RNA and/or DNA, are amplified as described in, *e.g.*, U.S. Pat. No. 4,683,195. Generally, sequence information from the ends of the region of interest or beyond is used to design oligonucleotide primers. These primers will be identical or similar in sequence to opposite strands of the template to be amplified. The 5'

terminal nucleotides of the two primers can coincide with the ends of the amplified material. PCR can be used to amplify specific RNA sequences, specific DNA sequences from total genomic DNA, and cDNA transcribed from total cellular RNA, bacteriophage or plasmid sequences, etc. *See generally* Mullis *et al.* (1987) *Cold Spring Harbor Symp. Quant. Biol.* 51:263; Erlich, ed., (1989) PCR TECHNOLOGY (Stockton Press, N.Y.) As used herein, PCR is considered to be one, but not the only, example of a nucleic acid polymerase reaction method for amplifying a nucleic acid test sample comprising the use of a known nucleic acid as a primer and a nucleic acid polymerase to amplify or generate a specific piece of nucleic acid.

Example 1

Our studies show that the transcription regulator TAZ (WWTR1) is markedly increased in hepatocytes in both human and murine NASH liver compared with normal or steatotic liver. In several murine models of NASH, silencing of hepatocyte TAZ prevents or reverses hepatic inflammation, hepatocyte death, and fibrosis. Moreover, hepatocyte-targeted expression of TAZ in a mouse model of steatosis promoted NASH features, including fibrosis. Mechanistic studies using cultured cells and molecular-genetic causation studies in mice indicate that a key mechanism linking hepatocyte TAZ to NASH fibrosis is TAZ/TEA domain (TEAD)-mediated induction of Indian hedgehog (Ihh), a secretory factor that activates fibrogenic genes in hepatic stellate cells. Thus, TAZ represents a previously unrecognized factor and a novel therapeutic target in the critical process of steatosis-to-NASH progression.

This study directly links the transcription factor TAZ to the development of HCC in a diet-induced NASH mouse model. Specifically, the study utilized a mouse model of diet-induced NASH that normally develops reticulin-negative, glypican-3-positive HCC tumors after 13 months. However, silencing TAZ with an adenoviral vector in these mice starting at 8 months completely prevents the development of HCC tumors. Unlike surgical treatment options that are used after HCC diagnosis, this technology targets a molecular pathway that induces tumor formation to halt the progression of NASH to HCC. These findings attribute a role to the transcription factor TAZ in hepatocytes and propose an important therapeutic strategy for a widespread, currently untreatable disease.

Two different siTAZ constructs (#1 and #2) efficiently silenced TAZ in AML-12 hepatocytes (Fig. 1).

siTAZ-1 and -2 silence hepatic TAZ *in vivo* in NASH mice (Figs. 2A-2B). TAZ levels were not affected in spleen, kidney, lung, heart or skeletal muscle.

The following study shows that siTAZ-1 and -2 suppress steatosis-to-NASH progression in NASH mice. Experimental design is shown in Fig. 3. The levels of TAZ and YAP in the liver are shown in Figs. 4A-4B. The silencing of hepatic TAZ after 8 weeks of treatment was almost complete. YAP was also partially suppressed—and we verified this in our own AAV8-H1-shTAZ model. It is possible that this phenomenon may be relatively specific for hepatocytes and/or NASH. There is no evidence that TAZ directly controls YAP expression, so the mechanism may involve a change in the liver due to TAZ silencing, which then secondarily affects a pathway controlling YAP.

In the end, the partial silencing of YAP may be beneficial for NASH, and it does not seem to lead to adverse effects based on our multiple models of TAZ silencing. In fact, most investigators knockout both factors in mice to probe their roles in diseases (e.g., Tazfl/fl Yapfl/fl mice x Cre). Moreover, as described below, our studies show that silencing TAZ suppresses NASH-induced HCC. As YAP is reported to promote non-NASH HCC, it is possible that the decrease in YAP in siTAZ-treated mice also contributes to the HCC-protective effect.

siTAZ did not affect metabolic endpoints in NASH mice (Figs. 5A-5F).

siTAZ treatment of NASH mice lowered plasma ALT (Fig. 5G).

siTAZ treatment of NASH mice lowered liver fibrosis (Figs. 6A-6B). Fibrosis was quantified as percent Sirius red-positive area.

siTAZ treatment of NASH mice lowered expression of mRNAs encoding fibrosis-related proteins in the livers of NASH mice (Fig. 7).

siTAZ treatment of NASH mice lowered expression of mRNAs encoding inflammation-related proteins in the livers of NASH mice (Fig. 8).

siTAZ treatment of NASH mice lowered cell death in the livers of NASH mice (Fig. 9).

Fibrosis reversal. The goal of a clinically useful NASH therapy is to reverse liver fibrosis, which was achievable with AAV8-shTAZ (Wang et al. Cell Metab. 2016). Therefore, we will test whether the siRNAs can reverse fibrosis in NASH mice. The overall strategy is to start with 16-week NASH diet-fed mice and then treat them for 12 additional weeks.

Specifically, we will begin with a pilot experiment to determine efficacy and dosing interval of siTAZ in mice that have liver fibrosis. The 16-week mice will be treated with siTAZ-1 and -2 at doses of 5 and 15 mg/kg; the control for this pilot experiment will be 15 mg/kg control RNA. Some of the mice will be treated once (week 16) and some twice (weeks 16 and 17). The mice will then be euthanized at week 18 and the livers will be analyzed by immunoblot

for TAZ. We will determine if siTAZ can suppress TAZ in mice with fibrosis and if we can use biweekly dosing. We will also probe YAP expression in the liver in view of the data in Fig. 4B. For this pilot experiment, we plan to use 3 mice/group x 5 groups x 2 dosing intervals = 30 mice (3 x 5 x 2).

Based on the results above, we will conduct a full reversal experiment, which involves treatment from weeks 16 through 27, with euthanasia and analysis at week 28. The primary endpoint will be hepatic fibrosis, which is quantified by measuring Sirius red-positive area, hepatic stellate cell area, and fibrosis-related gene expression. In addition, all other relevant NASH endpoints will be included as detailed in our Cell Metab. paper, i.e., inflammation by histology and gene expression, cell death by TUNEL staining, plasma ALT, liver triglyceride content, and basic metabolic parameters (body weight, plasma glucose and insulin, plasma lipids). To help evaluate the "on-target" mechanism, we will also assay liver Ihh by immunoblot, as Ihh is the TAZ-induced factor that promotes HSC activation and fibrosis (Wang et al. Cell Metab 2016). Further, we will assay YAP protein, and *Yap* mRNA, to see if it remains suppressed as it did in the NASH-prevention experiment (Fig. 4). In view of the duration of this experiment, and the requirement for 8 mice/group, we will choose one dose of an siRNA based on the above experiment, *e.g.*, the lower dose if that dose efficiently silences TAZ, and one dosing interval, *e.g.*, the 2-wk interval if it is successful. We will include both control RNA and PBS controls in view of our prior findings. We plan to use 8 mice/group x 3 groups (siTAZ, control RNA, PBS) = 24 mice (8 x 3).

Screening and *in vivo* testing of additional siTAZs. While the fibrosis reversal experiment is underway, we will screen and test additional siTAZs. For the initial *in vivo* screening, we will inject subcutaneously into 9-week NASH diet-fed mice once the following: (1) one of three doses of two siTAZ constructs (3 x 2 = 6 groups), 2 control RNAs at the middle dose (2 groups), and PBS (1 group) (9 groups total). Three days later, the mice will be euthanized, and the livers will be analyzed by immunoblot for TAZ and YAP expression, as well as for Ihh expression as a mechanistic link to fibrosis. The goals are: (1) to compare the 2 siTAZ constructs for silencing of TAZ and suppression of YAP; (b) to find the lowest dose of siTAZ that lowers TAZ (and Ihh) by at least ~80%, which we know from our previous work is enough to suppress NASH progression, without lowering YAP; and (3) to evaluate the control RNAs for effects on TAZ (and YAP) expression. We plan to use 3 mice/group for the 9 condition = 27 mice (3 x 9).

Preclinical testing of additional siTAZs in fibrosis reversal. We will conduct a NASH reversal experiment, as we have already shown that AAV8-shTAZ (Wang et al. Cell

Metab 2016) and siTAZ can prevent steatosis-to-NASH progression. We will use the 2-phase strategy as described above, beginning with pilot dose/interval probing experiment and then moving on to a complete reversal experiment. This will require 30 mice and then 24 mice = 54 mice total.

Hepatocellular carcinoma (HCC). Our data show that (a) NASH mice develop reticulin-negative/glypican-3-positive HCC tumors after 13 months on diet (Figs. 10A-B); (b) these tumors (as well as tumors in other models of HCC) express high levels of TAZ (Fig. 10C-E); and importantly, (c) silencing TAZ using AAV8-shTAZ starting at month 8 completely eliminates these HCC tumors (Fig. 10F).

To further explore the therapeutic potential of TAZ silencing, NICD fl/fl transgenic mice were injected with AAV-TBG-Cre vector to induce NICD expression in hepatocytes. The mice were then placed on NASH diet for 3 months to allow NASH-HCC development, treated with AAV8-H1-shTAZ or AAV8-H1-shControl vector, and continued on the diet for an additional 2 months (Figure 11A). The results showed that TAZ protein was significantly decreased in AAV8-H1-shTAZ treated mouse livers (Figure 11B). As expected for 5 months of NASH diet feeding, the control mice had marked HCC development and nodule formation, whereas AAV8-H1-shTAZ treated group had fewer nodules and smaller average tumor size (Figures 11C-11E). These data suggested that shTAZ treatment reduced HCC nodules even after NASH-HCC has developed. NICD: Notch intracellular domain. See, Villanueva et al., Notch Signaling is Activated in Human Hepatocellular Carcinoma and Induces Tumor Formation in Mice, Gastroenterology. 2012, 143(6): 1660–1669.e7. Razumilava et al., Notch-driven carcinogenesis: The merging of hepatocellular cancer and cholangiocarcinoma into a common molecular liver cancer subtypes, J. Hepatol. 2013, 58(6): 10.1016/j.jhep.2013.01.017.

Given the importance of HCC as a devastating consequence of NASH, even before cirrhosis develops in NASH subjects, and the lack of any successful therapies, we will test whether siTAZ can prevent HCC development in mice with established NASH. We will use the strategy described above: mice will be fed the NASH diet for 8 months and then treated for 5 additional months with siTAZ (choosing the best construct and best dose from the fibrosis reversal experiment), control RNA, and PBS, using a dosing interval of one injection every 2 weeks. At 13 months, the mice will be analyzed for size and number of tumors, TAZ and YAP expression in tumor (although there may be no tumors in the siTAZ group based on Fig. 10F) and surrounding tissue, as well as all other NASH parameters as outlined above. Given the reported role of YAP in non-NASH HCC, and the fact that YAP is suppressed in siTAZ mice

after 8 weeks of treatment (Fig. 4), it will be interesting to see if YAP remains low in this longer term siTAZ experiment. We plan to use 8 mice in each of the 3 groups: 24 mice (8 x 3).

Additional studies:

(a) Plasma biomarkers: The goal here is to determine if silencing hepatic TAZ is associated with a mechanistically linked plasma biomarker. Ihh is a key target of TAZ-induced fibrosis, the synthesis and secretion from hepatocytes of which is blocked by silencing TAZ. We will use ELISA to test whether silencing TAZ results in a decrease of plasma Ihh. Other hepatocyte-derived secreted molecules mechanistically linked to both TAZ and fibrosis include amphiregulin, soluble osteopontin, and Gas6, which we will also assay using ELISA. For this experiment, we will use plasma drawn 4 weeks after the initiation of siTAZ in the fibrosis-reversal experiments described above. The results of the assays will then be used in correlation analyses with silencing of liver TAZ and improvement in NASH, particularly fibrosis, at the end of the experiment, i.e., 8 weeks after the plasma samples are drawn.

We will carry out an unbiased proteomic and/or metabolomic screen of plasma, using the same overall strategy as above, coupled with biostatistical analyses to determine correlations with TAZ silencing and improvement in fibrosis, with a focus on positive hits that have potential mechanistic links with the TAZ-Ihh-HSC activation pathway.

(b) RNAseq: Elucidating gene expression changes in mice treated with siTAZ can help further define the mechanism, suggest possible additional benefits of siTAZ, and importantly, provide clues to possible adverse effects of siTAZ. There may be two types of gene expression changes: those that are due to TAZ silencing; and "off-target" effects in which the siRNA affects the expression of other genes. Within each of these categories, the effects can be primary, i.e., suppression of direct TAZ target genes (or off-target genes), or secondary, i.e., gene changes secondary to alterations in physiology that result from the initial suppression of TAZ target genes (or off-target genes).

One option is to use methods that can specifically probe changes in actively translated (polysome-associated) mRNAs in hepatocytes as opposed to global RNAseq in whole liver. Moreover, while a focus on mechanism might benefit from looking at changes soon after treatment with siTAZ, a more complete picture of siTAZ-induced changes, including possible adverse effects, can be done after longer-term treatments using a clinically relevant model, e.g., the fibrosis reversal protocol.

Summary

Obesity-associated nonalcoholic steatohepatitis (NASH) has emerged as the leading cause of hepatocellular carcinoma (HCC), a fatal disease the only treatment option for which is surgical removal with or without liver transplantation if caught at an early stage. We discovered that the Hippo pathway transcription factor Taz is markedly increased in the livers of patients with NASH and in the livers of various mouse models that develop key features of human NASH. One of these models uses a newly modified diet that causes weight gain, insulin resistance, hepatic steatosis, inflammation, and fibrosis. Our data show that (a) these NASH mice develop reticulin-negative/glypican-3-positive HCC tumors after 13 months on diet (Figs. 1A-1B); (b) these tumors (as well as tumors in other models of HCC) express high levels of TAZ (Figs. 1C-1E). Importantly, silencing TAZ using AAV8-shTAZ starting at month 8 completely eliminates these HCC tumors (Fig. 1F). These findings reveal a heretofore unrecognized transcription factor in hepatocytes that contributes to the progression of NASH to HCC and suggest that suppression of TAZ can represent a novel treatment strategy for HCC.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies

The following antibodies were used for immunoblots: GAPDH (#3683), β-actin (#5125), CHOP (#5554), TAZ (#8418), p-eIF2α (#3398), eIF2α (#5324), Lamin A/C (#4777) from Cell Signaling; p-TAZ (sc-17610) and Col1a1 (sc-8784) from Santa Cruz; Ihh (ab39634) from Abcam; and Timp1 (AF980) from R & D. The following antibodies were using for immunostaining of liver: α-SMA (ACTA2) (C6198, F3777) and TAZ (HPA007415) from Sigma; F4/80 (MCA497GA) from AbD Serotec; Ly6g (#127601) from Biolegend; Ly6b (MCA771G) from Bio-Rad; OPN (AF808) from R & D; 4-hydroxynonenal (4-HNE) (AB5605) from Millipore; and HNF4α (sc-6556) from Santa Cruz. The following plasma assay kits were used in this study: insulin ELISA (#90080) from Crystal Chem; MCP1 ELISA (#88-7391-22) from eBiosciences; cholesterol (#439-17501) and triglyceride (#465-09791, #461-09891) from Wako; and ALT (#006A-CR) and AST (#004A-CR) from BQ Kits, Inc. AAV8-shRNA targeting murine *Taz* was made by annealing complementary oligonucleotides (5'-CACCAcagccgaatctcgcaatgaatCTCGAGATTCATTGCGAG ATTCGGCTG-3') (SEQ ID NO:1), which were then ligated into the pAAV-RSV-GFP-H1 vector, as described previously (Lisowski et al., 2014). The resultant constructs were amplified by the Salk Institute Gene Transfer, Targeting, and Therapeutics Core.

Animal Studies

Male wild-type mice C57BL/6J (#000664, 8-10 weeks/old) and MC4R-negative *loxTB Mc4r* mice (#006414, 6 weeks/old), referred to here as *Mc4r*$^{-/-}$ mice, were obtained from Jackson Laboratory (Bar Harbor, ME) and were allowed to adapt to housing in the Columbia University Medical Center Institute of Comparative Medicine for 1 week prior to random assignment to experimental cohorts. The mice were then fed the following diets for the times indicated in the figure legends: (a) chow diet (Picolab rodent diet 20, #5053); (b) "fast-food" (FF) diet (TestDiet 1810060): high-fat diet with drinking water containing 42 g/L glucose and fructose (55%/45%, w/w); or (c) fructose-palmitate-cholesterol ("FPC") diet (Teklad, TD.140154): similar to FF diet but with 1.25% added cholesterol and with palmitic acid, anhydrous milk fat, and Primex as the sources of fat and with a ~60% decrease in vitamin E and a ~35% decrease in choline compared with typical mouse diets. For several experiments, groups of mice were placed on a methionine-choline-deficient diet (Teklad, TD. 90262) for 8 weeks, as described (Dixon et al., 2012). Adeno-associated virus (2 X 10^{11} genome copy/mouse) was delivered by tail vein injection either 1 week prior to diet initiation or after 8 weeks of the FPC diet. Animals were housed in standard cages at 22°C in a 12-12-h light-dark cycle. All animal experiments were performed in accordance with institutional guidelines and regulations and approved by the Institutional Animal Care and Use Committee at Columbia University.

Blood and Plasma Analyses

Fasting blood glucose was measured using a glucose meter (One Touch Ultra, Life-scan) in mice that were fasted for 4-5 h, with free access to water. Complete blood counts were obtained with the FORCYTE Veterinary Hematology Analyzer (Oxford Science, Inc.). Total plasma triglyceride and cholesterol were assayed using a commercially available kit from Wako. For insulin, MCP1, AST, ALT, TC, TG are measured following kit instruction by using plasma.

Histopathological Analysis, Immunohistochemistry, and Immunofluorescence Microscopy

Formalin-fixed, paraffin-embedded liver sections were stained with hematoxylin and eosin (H&E) and evaluated for severity of NAFLD by a trained hepatopathologist blinded to the clinical diagnosis, αaccording to criteria described by Brunt et al. (Kleiner et al., 2005; Liang et al., 2014). Liver fibrosis was assessed by Picrosirius (Sirius) red (Polysciences, #24901) or by Masson's trichrome staining (Sigma, HT15), with aniline blue-positive areas quantified as a measure of collagen content in the trichrome-stained sections. TUNEL staining was conducted using a kit from Roche (#12156792910). For immunofluorescence

microscsopy, paraffin sections were rehydrated, subjected to antigen retrieval by placing in a pressure cooker for 10 mins in Target Retrieval Solution (Dako, S1699), and then blocked with serum. Sections were labeled with primary antibodies overnight, using a 1:150 dilution except for α-SMA and 4-HNE (1:200) and TAZ (1:400), followed by incubation with a fluorophore-conjugated secondary antibody for 1 h. The stained sections were mounted with DAPI-containing mounting medium (Life Technologies, P36935) and then viewed on an Olympus IX 70 fluorescence microscope. For filipin (Sigma, F9765) staining, frozen sections were fixed in 4% paraformaldehyde for 1 h at room temperature, then rinsed using glycine/PBS and stained 0.25 mg/ml filipin 2 h at room temperature. Fluorescence microscopic images were analyzed using ImageJ software. For immunohistochemistry, the deparaffinization, rehydration, and antigen retrieval processes were the same as with immunofluorescence staining. The slides were treated with 3% hydrogen peroxide for 10 min and then blocked with Serum-Free Protein Block (Dako, X0909) for 30 min. Sections were incubated with OPN, F4/80, or α-SMA primary antibody (1:100) overnight and then developed using DAB substrate kit (Cell Signaling, #8059) for OPN and F4/80, FITC-labeled anti-HRP secondary antibody for α-SMA.

Measurement and Analysis of Liver Tissue Fatty Acids and Cholesterol

Liver specimens (~20 mg) were homogenized in 600 µl of 5% ethanol, and then 6 µl was added to 100 µl KOH (1M, 9:1 methanol:H_2O). The suspension was heated at 100°C for 30 min and then clarified by centrifugation, followed by addition of 80 µl HCl to the supernate. Fatty acids in this solution were identified and quantified by gas chromatography in the Columbia Biomarker Core Laboratory. For liver cholesterol quantification, liver tissue was homogenized in H_2O. Color Reagent Solution from the Wako Total Cholesterol assay kit was added at a 1:20 ratio (v/v) to the liver lysates. The suspension was then centrifuged, and the supernates were read in a plate reader.

Immunoblotting

Liver protein was extracted using RIPA buffer (Thermo, #89900), and the protein concentration was measured by a BCA assay (Thermo, #23227). Proteins were separated by electrophoresis on 4-20% Tris gels (Life technologies, EC60285) and transferred to a nitrocellulose membranes (Bio-Rad, #1620115). The membranes were blocked for 30 min at room temperature in Tris-buffered saline and 0.1% Tween 20 (TBST) containing 5% (wt/vol) nonfat milk and then incubated with primary antibody in the same buffer at 4°C overnight, using 1:1000 dilution except for CHOP and Ihh (1:3000). The protein bands were detected with horse radish peroxidase-conjugated secondary antibodies (Cell Signaling) and Supersignal

West Pico enhanced chemiluminescent solution (Thermo, #34080). Cultured cells were lysed in Laemmli sample buffer (Bio-Rad, #161-0737) containing 5% 2-mercaptoethanol, heated at 100°C for 5 min, and then electrophoresed and immunoblotted as above. Preparation of nuclear and cytoplasmic fractions of liver was carried out using Nuclear Extract Kit (Active Motif, #40010) according to the manufacturer's protocol.

Cell Culture

AML12 mouse hepatocytes were purchased from ATCC (CRL-2254) and cultured in DMEM/F12 medium (Lifetechnologies, #11320) with 10% FBS (Gibco, #16140-071). Hepatic stellate cells (HSCs) were isolated from 5-6 mo/o BALB/C mice as described previously (Mederacke et al., 2015). Briefly, after cannulation of the inferior vena cava, the portal vein was cut, allowing retrograde step-wise perfusion with solutions containing protease (Sigma Aldrich, P5147) and collagenase D (Roche, #11088866001). The perfusates were subjected to 9.7% Nycodenz (Accurate Chemical, #1002424) gradient centrifugation to isolate the HSCs, which were then plated in tissue culture dishes and used the next day. For conditioned medium transfer experiments, AML12 cells were cultured in DMEM containing 0.2% BSA and incubated for 24h. The media were then transferred to HSCs that had previously been incubated in DMEM, 0.2% BSA for 24 h. After 72 h, the HSCs were assayed for gene expression. For quantification of Ihh, hepatocyte conditioned medium was concentrated 10-fold by centrifugal filters (Millipore, Ultracel) and analyzed by an ELISA kit (LifeSpan Biosciences, F7953).

Quantitative RT-qPCR

Total RNA was extracted from liver tissue or primary cultured hepatocytes using the RNeasy kit (Qiagen, 74106). cDNA was synthesized from 1 μg total RNA using oligo (dT) and Superscript II (Invitrogen). qPCR was performed in an 7500 Real time PCR system (Applied Biosystems) using SYBR green chemistry (Life Technologies, #4367659).

siRNA-Mediated Gene Silencing and Transfection

siRNA sequences against mouse *Taz* and scrambled RNA were purchased from IDT; the target sequence of *Taz* siRNA was ACA UGG ACG AGA UGG AUA CAG GUG A (SEQ ID NO:2). The scrambled RNA and siRNA were transfected into AML12 cells (ATCC) using RNAiMAX (Life Technologies, #13778150) according to the manufacturer's instruction. A plasmid encoding GFP was purchased from Lonza (pmaxGFP), and a plasmid encoding murine *Ihh* was purchased from Origene (MR227435). The plasmids were transfected into AML12 cells using Lipofectamine® LTX Reagent with PLUS™ Reagent (Life Technologies, #15338100).

Mouse Liver Nuclei Preparation and ChIP Assays

Mouse liver tissues were homogenized using a Dounce homogenizer (Wheaton, #357544) with a loose pestle in 1:10 (w:v) of ice-cold NP-40 lysis buffer supplemented with a protease inhibitor cocktail. The release of nuclei from the homogenate was monitored by DAPI staining and fluorescence microscopy. To purify intact nuclei, lysates were layered over a step gradient consisting of 1 M and 0.68 M sucrose and then centrifuged at 4000 rpm for 30 min at 4°C. Following a washing step, nuclear pellets were cross-linked with 1% fresh formaldehyde in PBS for 10 min at room temperature. Cross-linking was terminated by addition of 200 mM Tris-HCl (pH 9.4) and 1 mM DTT, and after 10 mins the suspension was centrifuged at 2500 rpm for 15 min at 4°C. Nuclear pellets were suspended in SDS lysis buffer containing protease inhibitors, incubated for 10 min on ice. DNA was sheared in a cold water bath using a focused-ultrasonicator (Covaris, S2) to obtain DNA fragments with an average size of 500 bps. Fragmented chromatin was pre-cleaned by incubating with normal rabbit IgG (Santa Cruz, sc-2027) for 1 h at 4 °C, followed by 1 h of incubation with 50 μL protein G magnetic beads (Pierce, #88847) at 4 °C with rotation. Immunoprecipitation was conducted using a rabbit anti-TAZ antibody (Cell Signaling, #4883), and a control rabbit anti-HA antibody (Santa Cruz, sc-805) was used as a negative control. Immunoprecipitated chromatin fragments were reverse cross-linked, digested by proteinase K, and purified using QIAquick PCR Purification Kit (Qiagen, #28106). The presence of TAZ in *Ihh* intronic region was quantified by qPCR and expressed relative to the input genomic DNA.

Measurement of Hydroxyproline Content of Liver Tissue

Hydroxyproline liver content was measured as previously described (Bataller et al., 2003; Seki et al., 2009). Briefly, liver tissue was homogenized, and proteins were precipitated using trichloroacetic acid. Samples were hydrolyzed by incubation with 6N hydrochloric acid at 110°C for 16 h followed by neutralization with sodium hydroxide. Liver hydrolysates were oxidized using chloramine-T, followed by incubation with Ehrlich's perchloric acid reagent for color development. Absorbance was measured at 560 nm, and hydroxyproline quantities were calculated by reference to standards processed in parallel. Results are expressed as ng per mg liver weight.

Statistical Analysis

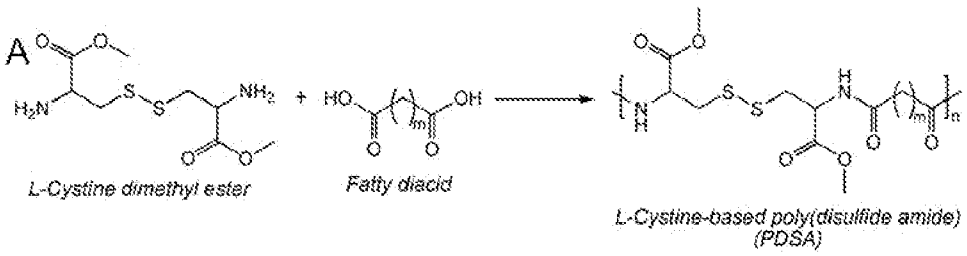
All results are presented as mean ± SEM. P values were calculated using the Student's t-test for normally distributed data and the Mann-Whitney rank sum test for non-normally distributed data. One-way ANOVA with post-hoc Tukey test was used to evaluate differences among groups when 3 or more groups were analyzed.

Redox-responsive liver-targeting nanoparticle platform for siRNA delivery

Synthesis of the L-cystine-based poly(disulfide) (PDSA) polymers

PDSA polymers were prepared by one-step polycondensation of L-cystine dimethyl ester dihydrochloride ((H-Cys-OMe)2.2HCl) and dichlorides or Bis-nitrophenol esters of different fatty diacids. A standard synthesis procedure was carried out as follows: (H-Cys-OMe)2.2HCl (10 mmol) and triethylamine (15mmol) were dissolved in 20.0 mL DMSO, then the dichloride of fatty acid (10 mmol) DMSO solution (10 mL) was added into the cystine mixture solution dropwise. The solution was stirred for 15 min to obtain a uniform mixture, precipitated twice in 250 mL of cold ethyl ether, and dried under reduced atmosphere. The final product was a yellow or brown yellow powder.

The synthesis scheme is shown below.



Redox-responsive behavior of the PDSA polymers

GPC analysis was used to study the redox-responsive behavior of the PDSA polymers. The polymer (1 mg) was dissolved in 2 mL of DMF/H_2O (9:1, V/V) and then GSH (6.2 mg, 0.02 mmol) was added to obtain a solution with GSH concentration of 10 mM. At predetermined intervals, 100 μL of the solution was taken for GPC analysis.

Preparation and characterization of nanoparticles (NPs)

The PDSA polymers were dissolved in DMF or DMSO to form a homogenous solution with a concentration of 20 mg/mL. Subsequently, 200 μL of this solution was taken and mixed with 140 μL of DSPE-PEG3000 (20 mg/mL in DMF), 50 μL of G0-C14 (5 mg/mL in DMF) and 1 nmol siRNA (0.1 nmol/μL aqueous solution). Under vigorously stirring (1000 rpm), the mixture was added dropwise to 5 mL of deionized water. The NP dispersion formed was transferred to an ultrafiltration device (EMD Millipore, MWCO 100 K) and centrifuged to remove the organic solvent and free compounds. After washing with deionized water (3 × 5 mL), the siRNA loaded NPs were dispersed in 1 mL of phosphate buffered saline (PBS, pH 7.4) solution. Size and zeta potential were determined by DLS. The morphology of NPs was visualized on TEM. To determine the siRNA encapsulation efficiency, DY547-labelled GL3 siRNA (DY547-siRNA) loaded NPs were prepared according to the method described above.

A small volume (50 μL) of the NP solution was withdrawn and mixed with 20-fold DMSO. The fluorescence intensity of DY547-siRNA was measured using a Synergy HT multi-mode microplate reader (BioTek Instruments) and compared to the free DY-547 labelled GL3 siRNA solution (1 nmol/mL PBS solution).

Redox-responsive behavior of the NPs

The siRNA loaded NPs were prepared as described above and dispersed in PBS containing 10 mM GSH. At pre-determined time point, the particle size was examined by DLS and the particle morphology was observed on TEM. To evaluate the intracellular redox-responsive behavior, the NPs with Nile red and coumarin 6 encapsulated in their hydrophobic cores were prepared and then incubated with HeLa cells for different time. The fluorescence of Nile red and coumarin 6 was observed a FV1000 confocal laser scanning microscope (CLSM, Olympus). If the NPs respond to redox stimulus, the Nile red and coumarin 6 will release and only green fluorescence of coumarin 6 can be observed under CLSM. If the NPs are intact, the fluorescence of coumarin 6 will be quenched by Nile red and only red fluorescence can be observed under CLSM.

Evaluation of endosomal escape

Luc-HeLa cells (20,000 cells) were seeded in discs and incubated in 1 mL of RPMI 1640 medium containing 10% FBS for 24 h. Subsequently, the DY547-siRNA-loaded NPs were added, and the cells were allowed to incubate for 1 or 2 h. After removing the medium and subsequently washing with PBS (pH 7.4) solution thrice, the endosomes and nuclei were stained with lysotracker green and Hoechst 33342, respectively. The cells were then viewed under CLSM.

In vitro siRNA release

DY547-labelled siRNA (DY547-siRNA) was loaded into the NPs according to the method described above. Subsequently, the NPs were dispersed in 1 mL of PBS (pH 7.4) and then transferred to a Float-a-lyzer G2 dialysis device (MWCO 100 kDa, Spectrum) that was immersed in PBS (pH 7.4) at 37 °C. At a predetermined interval, 5 μL of the NP solution was withdrawn and mixed with 20-fold DMSO. The fluorescence intensity of DY547-siRNA was determined by Synergy HT multi-mode microplate reader.

Luciferase silencing

Luciferase expressing HeLa (Luc-HeLa) cells were seeded in 96-well plates (5,000 cells per well) and incubated in 0.1 mL of RPMI 1640 medium with 10% FBS for 24 h. Thereafter, the Luc siRNA-loaded NPs were added. After incubating for 24 h, the cells were washed with fresh medium and allowed to incubate for another 48 h. The expression of firefly

luciferase in HeLa cells was determined using Steady-Glo luciferase assay kits. Cytotoxicity was measured using the Alamar Blue assay according to the manufacturer's protocol. The luminescence or fluorescence intensity was measured using a microplate reader, and the average value of five independent experiments was collected. As a control, the silencing effect of Lipo2K/Luc siRNA complexes was also evaluated according to the procedure described above and compared to that of Luc siRNA loaded NPs.

Preparation of Wwtr1 (TAZ) siRNA loaded NPs

The PDSA polymers were dissolved in DMF to form a homogenous solution with a concentration of 20 mg/mL. Subsequently, 200 μL of this solution was taken and mixed with 140 μL of DSPE-PEG3000 (20 mg/mL in DMF), 50 μL of G0-C14 (5 mg/mL in DMF) and 1 nmol TAZ siRNA (0.1 nmol/μL aqueous solution). Under vigorously stirring (1000 rpm), the mixture was added dropwise to 5 mL of deionized water. The NP dispersion formed was transferred to an ultrafiltration device (EMD Millipore, MWCO 100 K) and centrifuged to remove the organic solvent and free compounds. After washing with deionized water (3 × 5 mL), the TAZ siRNA loaded NPs were dispersed in 1 mL of phosphate buffered saline (PBS, pH 7.4) solution.

In vitro TAZ silencing

Hepatocytes (AML12 cells) were seeded in 6-well plates (50,000 cells per well) and incubated in 1 mL of DMEM/F12 (1:1, v:v) medium containing 10% FBS for 24 h. Subsequently, the cells were incubated with the TAZ siRNA loaded NPs for 24 h. After washing the cells with PBS thrice, the cells were further incubated in fresh medium for another 48 h. Thereafter, the cells were digested by trypsin and the proteins were extracted using modified radioimmunoprecipitation assay lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40 substitute, 0.25% sodium deoxycholate, 1mM sodium fluoride, 1mM Na3VO4, 1mM EDTA), supplemented with protease inhibitor cocktail and 1 mM phenylmethanesulfonyl fluoride (PMSF). The expression of TAZ was examined using the western blot analysis.

Western blot

Equal amounts of proteins were added to SDS-PAGE gels and separated by gel electrophoresis. After transferring the proteins from gel to polyvinylidene difluoride (PVDF) membrane, the blots were blocked with 3% BSA in TBST (50 mM Tris-HCl pH 7.4, 150 mM NaCl, and 0.1% Tween 20) and then incubated with a mixture of TAZ rabbit antibody (Cell Signaling, catalog # 8418S) and β-actin rabbit antibody (Cell Signaling, catalog # 13E5). The expression of TAZ was detected with horseradish peroxidase (HRP)-conjugated secondary antibody (anti-rabbit IgG HRP-linked antibody, Cell Signaling) and an enhanced

chemiluminescence (ECL) detection system (Pierce).

Animals

Healthy female C57BL/6 mice (4-5 weeks old) were purchased from Charles River Laboratories. All *in vivo* studies were performed in accordance with National Institutes of Health animal care guidelines and in strict pathogen-free conditions in the animal facility of Brigham and Women's Hospital. Animal protocol was approved by the Institutional Animal Care and Use Committees on animal care (Harvard Medical School).

Pharmacokinetics study

Healthy female C57BL/6 mice were randomly divided into two groups (n = 3) and given an intravenous injection of either (i) free DY647-labelled Luc siRNA (DY647-siRNA) or (ii) DY647-siRNA loaded NPs at a 1 nmol siRNA dose per mouse. At predetermined time intervals, orbital vein blood (20 μL) was withdrawn using a tube containing heparin, and the wound was pressed for several seconds to stop the bleeding. The fluorescence intensity of DY-647 labelled siRNA in the blood was determined using a microplate reader.

Biodistribution

Healthy female C57BL/6 mice were randomly divided into two groups (n = 3) and given an intravenous injection of either (i) free DY677-labelled Luc siRNA (DY677-siRNA) or (ii) DY677-siRNA loaded NPs at a 1 nmol siRNA dose per mouse. Twenty-four hours after the injection, the mice were imaged using the Maestro 2 In-Vivo Imaging System (Cri Inc). Main organs were then harvested and imaged. To quantify the accumulation of NPs in tumors and organs, the fluorescence intensity of each tissue was quantified by Image-J.

Immune response

Healthy female C57BL/6 mice were randomly divided into three groups (n = 3) and given an intravenous injection of either (i) PBS, (ii) naked TAZ siRNA or (iii) TAZ siRNA loaded NPs at a 1 nmol siRNA dose per mouse. Twenty-four hours after injection, blood was collected and serum isolated for measurements of representative cytokines (TNF-α, IL-6, IL-12, and IFN-γ) by enzyme-linked immunosorbent assay or ELISA (PBL Biomedical Laboratories and BD Biosciences) according to the manufacturer's instructions.

Histology

Healthy female C57BL/6 mice were randomly divided into three groups (n = 3) and administered daily intravenous injections of either (i) PBS or (ii) TAZ siRNA loaded NPs at a 1 nmol siRNA dose per mouse. After three consecutive injections (once every day), the main organs were collected 24 h post the final injection, fixed with 4% paraformaldehyde, and embedded in paraffin. Tissue sections were stained with hematoxylin-eosin (H&E) and viewed

under an optical microscope.

Additional methods relating to nanoparticle siRNA formulations can be found in U.S. Patent Publication No. 20160022835. Desirable features of nanoparticle delivery of the TAZ siRNA and related inhibitors described herein include increased stability and the ability to avoid immune degradation. It is noted that the TAZ siRNA NPs may have two main components: 1) a hydrophobic inner core that is made with redox-responsive polymers to encapsulate TAZ siRNA, and 2) a hydrophilic outer shell that can allow the TAZ NPs to evade recognition by immune system components and increase blood circulation half-life. The NPs may also include a third component: 3) a targeting ligand that can specifically bind to its receptor on hepatocytes.

A number of additional techniques will be suitable for liver-specific targeting of the present compositions including pharmaceutical compositions described herein. Such methods can be found in U.S. Patent Publication No. 20160017335 and Fitzgerald *et al.* (N. Engl. J. Med. 2017; 376:41-51; January 5, 2017 DOI: 10.1056/NEJMoa1609243).

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Many modifications and variations of this invention can be made without departing from its spirit and scope, as will be apparent to those skilled in the art. The invention is defined by the terms of the appended claims, along with the full scope of equivalents to which such claims are entitled. The specific embodiments described herein, including the following examples, are offered by way of example only, and do not by their details limit the scope of the invention.

All references cited herein are incorporated by reference to the same extent as if each individual publication, database entry (e.g. Genbank sequences or GeneID entries), patent application, or patent, was specifically and individually indicated to be incorporated by reference. This statement of incorporation by reference is intended by Applicants, pursuant to 37 C.F.R. §1.57(b)(1), to relate to each and every individual publication, database entry (e.g. Genbank sequences or GeneID entries), patent application, or patent, each of which is clearly identified in compliance with 37 C.F.R. §1.57(b)(2), even if such citation is not immediately adjacent to a dedicated statement of incorporation by reference. The inclusion of dedicated statements of incorporation by reference, if any, within the specification does not in any way weaken this general statement of incorporation by reference. Citation of the references herein is not intended as an admission that the reference is pertinent prior art, nor does it constitute any admission as to the contents or date of these publications or documents.

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and the accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. Various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims.

What is claimed is:

1. A method for treating or preventing hepatocellular carcinoma (HCC) in a subject, comprising administering to the subject an effective amount of an inhibitor of TAZ.

2. The method of claim 1, wherein the HCC derives from a progression of non-alcoholic steatohepatitis (NASH) in the subject.

3. A method for preventing or delaying progression of non-alcoholic steatohepatitis (NASH) to hepatocellular carcinoma (HCC) in a subject, the method comprising administering to the subject an effective amount of an inhibitor of TAZ.

4. The method of claims 1 or 3, wherein the inhibitor of TAZ is administered to at least one hepatocyte of the subject.

5. The method of claims 1 or 3, wherein the inhibitor of TAZ is a small molecule, a nucleic acid, a protein or polypeptide, an antibody or antigen-binding portion thereof, or combinations thereof.

6. The method of claim 5, wherein the nucleic acid is selected from the group consisting of a small interfering RNA (siRNA), a short hairpin RNA (shRNA), an antisense oligonucleotide, and combinations thereof.

7. The method of claim 5, wherein the nucleic acid is SEQ ID NO:1 or SEQ ID NO:2, or any nucleic acid selected from the group consisting of SEQ ID NO:55-SEQ ID NO:72 and SEQ ID NO:81.

8. The method of claim 5, wherein the nucleic acid is formulated in a nanoparticle.

9. The method of claim 8, wherein the nanoparticle comprises: (a) a hydrophobic inner core, (b) a hydrophilic outer shell and (c) a hepatocyte targeting ligand.

10. The method of claims 1 or 3, wherein the inhibitor comprises a CRISPR/Cas9 system.

11. The method of claims 1 or 3, further comprising administering to the subject an effective amount of an inhibitor of Indian hedgehog (Ihh), an inhibitor of YAP, an inhibitor of TEAD1, an inhibitor of TEAD2, an inhibitor of TEAD3, an inhibitor of TEAD4, or any combination thereof.

12. The method of claims 1 or 3, further comprising administering to the subject at least one additional therapeutic agent for treatment of steatosis hepatis, steatohepatitis, non-alcoholic fatty liver disease, non-alcoholic steatohepatitis, adiposity and combinations thereof.

13. The method of claim 12, wherein the additional therapeutic agent is selected from antidiabetic drugs and insulin sensitizers selected from the group consisting of: Rosiglitazone; Pioglitazone; Losartan; Simtuzumab (anti-LOXL2); GR-MD-02; Obeticholic acid (OCA) and combinations thereof.

14. The method of claims 1 or 3, further comprising administering to the subject a cytotoxic agent.

15. The method of claim 14, wherein the cytotoxic agent is an alkylating agent, an anti-metabolite, an anti-microtubule agent, a topoisomerase inhibitor, a cytotoxic antibiotic, or an endoplasmic reticulum stress inducing agent.

16. A method for inhibiting growth, or increasing cell death, of a hepatocellular carcinoma (HCC) cell, the method comprising contacting the hepatocellular carcinoma cell with an effective amount of an inhibitor of TAZ.

17. A method for preventing or delaying progression of a hepatocyte to a hepatocellular carcinoma (HCC) cell, the method comprising contacting the hepatocyte with an effective amount of an inhibitor of TAZ.

18. The method of claims 16 or 17, wherein the contacting is *in vitro* or *in vivo*.

19. The method of claims 16 or 17, wherein the inhibitor of TAZ is a small molecule, a nucleic acid, a protein or polypeptide, an antibody or antigen-binding portion thereof, or combinations thereof.

20. The method of claim 19, wherein the nucleic acid is selected from the group consisting of a small interfering RNA (siRNA), a short hairpin RNA (shRNA), an antisense oligonucleotide, and combinations thereof.

21. The method of claim 20, wherein the nucleic acid is SEQ ID NO:1 or SEQ ID NO:2, or any nucleic acid selected from the group consisting of SEQ ID NO:55-SEQ ID NO:72 and SEQ ID NO:81.

22. The method of claim 20, wherein the nucleic acid is formulated in a nanoparticle.

23. The method of claim 22, wherein the nanoparticle comprises: (a) a hydrophobic inner core, (b) a hydrophilic outer shell and (c) a hepatocyte targeting ligand.

24. The method of claims 16 or 17, wherein the inhibitor comprises a CRISPR/Cas9 system.

25. The method of claims 16 or 17, further comprising contacting the hepatocellular carcinoma cell or hepatocyte with an effective amount of an inhibitor of Indian hedgehog (Ihh), an inhibitor of YAP, an inhibitor of TEAD1, an inhibitor of TEAD2, an inhibitor of TEAD3, an inhibitor of TEAD4, or any combination thereof.

26. The method of claims 16 or 17, further comprising contacting the hepatocellular carcinoma cell or hepatocyte with at least one additional therapeutic agent for treatment of steatosis hepatis, steatohepatitis, non-alcoholic fatty liver disease, non-alcoholic steatohepatitis, adiposity and combinations thereof.

27. The method of claim 26, wherein the additional therapeutic agent is selected from antidiabetic drugs and insulin sensitizers selected from the group consisting of: Rosiglitazone; Pioglitazone; Losartan; Simtuzumab (anti-LOXL2); GR-MD-02; Obeticholic acid (OCA) and combinations thereof.

28. The method of claims 16 or 17, further comprising contacting the hepatocellular carcinoma cell or hepatocyte with a cytotoxic agent.

29. The method of claim 28, wherein the cytotoxic agent is an alkylating agent, an anti-metabolite, an anti-microtubule agent, a topoisomerase inhibitor, a cytotoxic antibiotic, or an endoplasmic reticulum stress inducing agent.

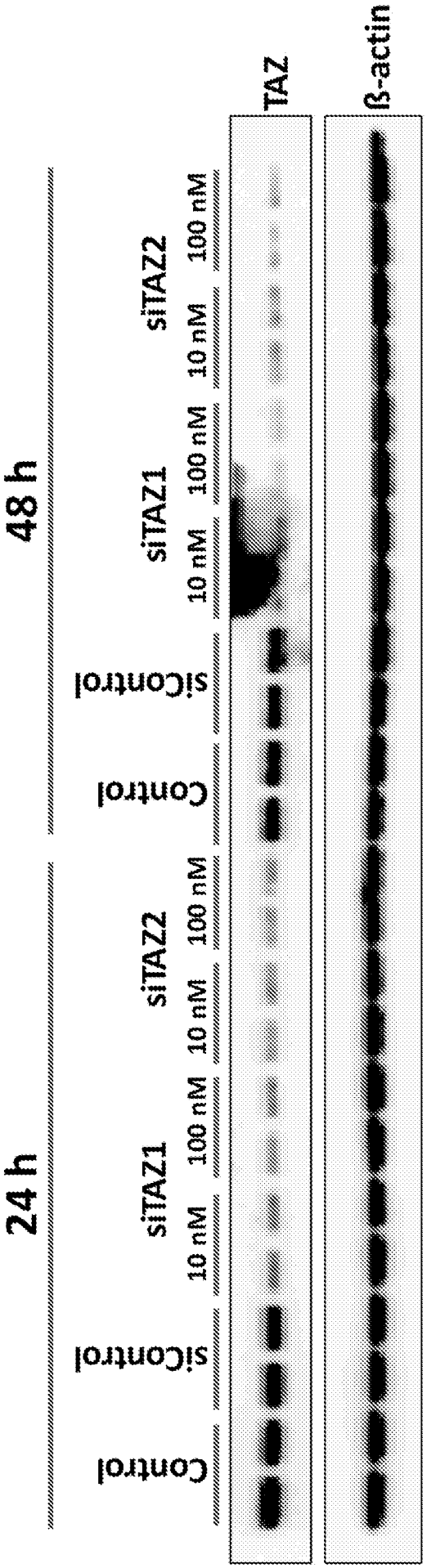

24 h
48 h
Control
siControl
siTAZ1
siTAZ2
10 nM
100 nM
TAZ
ß-actin

Fig. 1

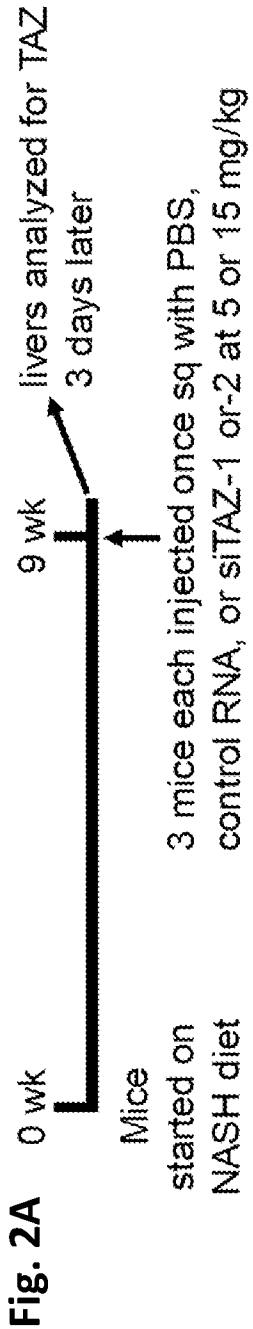

0 wk
Mice started on NASH diet
9 wk
3 mice each injected once sq with PBS, control RNA, or siTAZ-1 or-2 at 5 or 15 mg/kg
livers analyzed for TAZ 3 days later

Fig. 2A

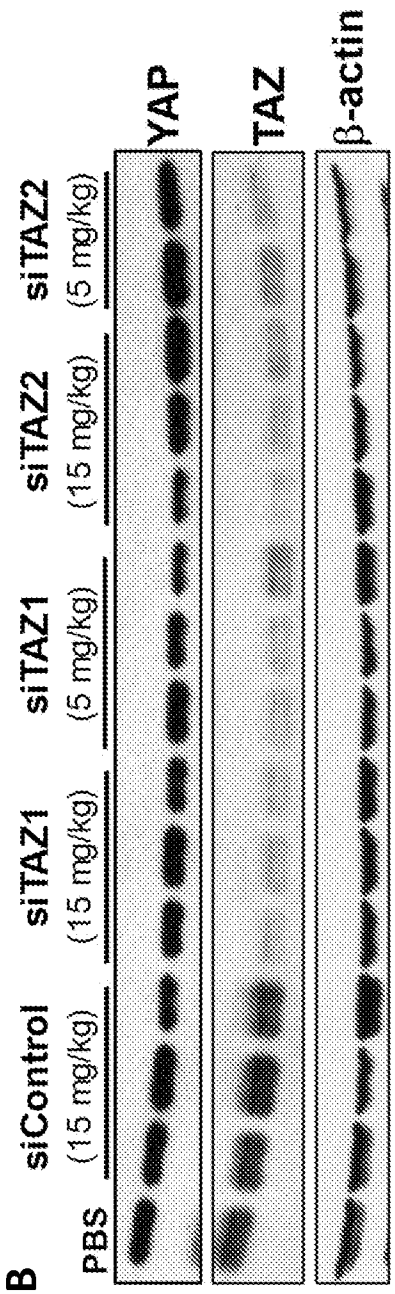

PBS
siControl (15 mg/kg)
siTAZ1 (15 mg/kg)
siTAZ1 (5 mg/kg)
siTAZ2 (15 mg/kg)
siTAZ2 (5 mg/kg)
YAP
TAZ
β-actin

Fig. 2B

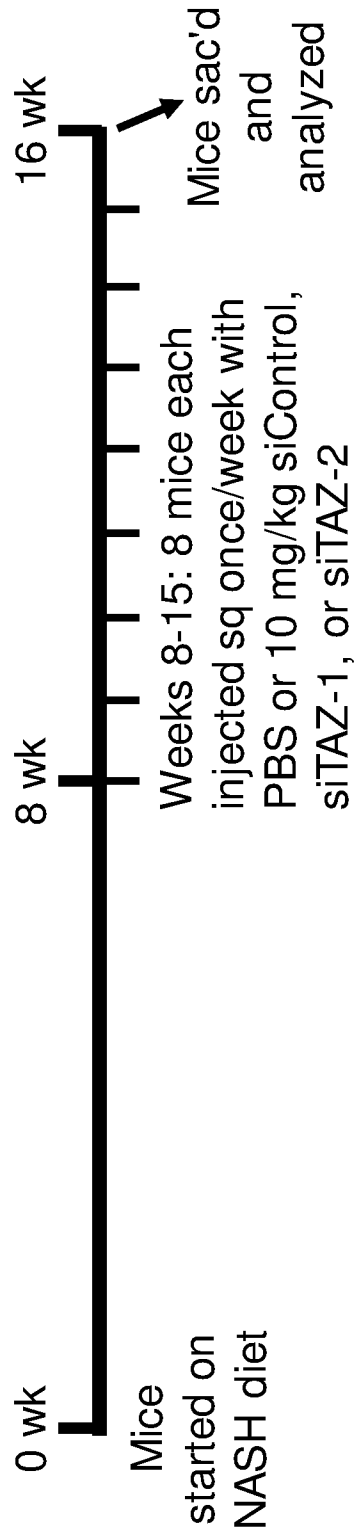

0 wk
Mice started on NASH diet
steatosis
8 wk
Weeks 8-15: 8 mice each injected sq once/week with PBS or 10 mg/kg siControl, siTAZ-1, or siTAZ-2
NASH
16 wk
Mice sac'd and analyzed

Fig. 3

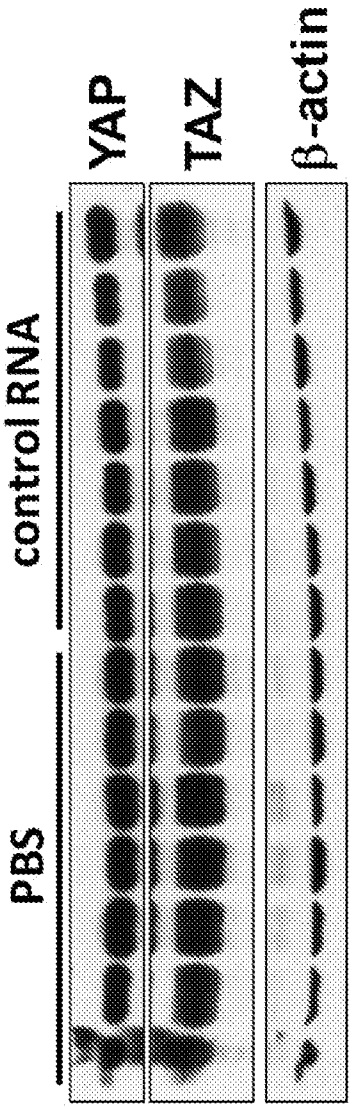
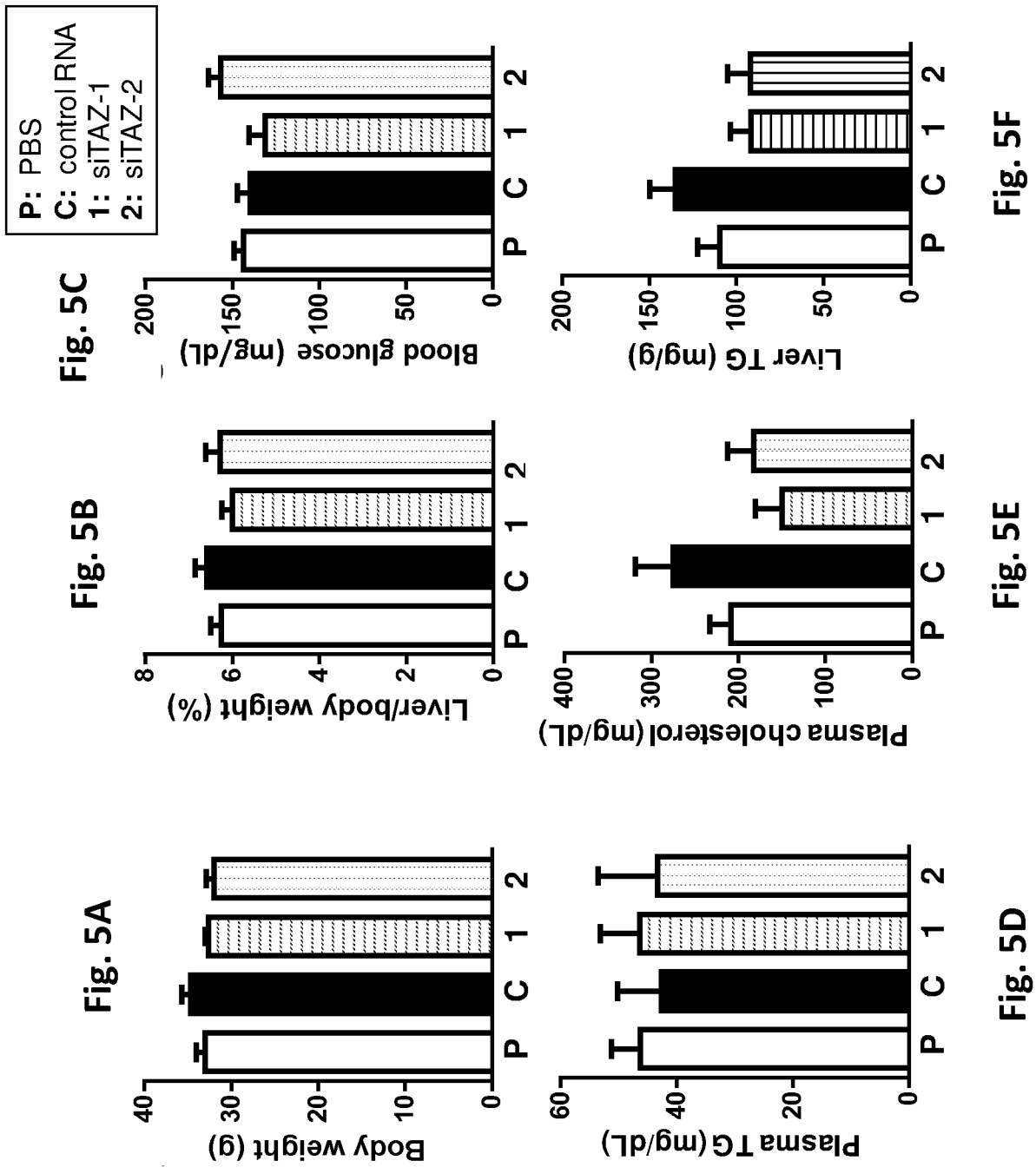

PBS
control RNA
YAP
TAZ
β-actin

Fig. 4A

PBS
siTAZ-1
siTAZ-2
YAP
TAZ
β-actin

Fig. 4B


P: PBS
C: control RNA
1: siTAZ-1
2: siTAZ-2
Fig. 5A
Body weight (g)
Fig. 5B
Liver/body weight (%)
Fig. 5C
Blood glucose (mg/dL)
Fig. 5D
Plasma TG (mg/dL)
Fig. 5E
Plasma cholesterol (mg/dL)
Fig. 5F
Liver TG (mg/g)
P C 1 2

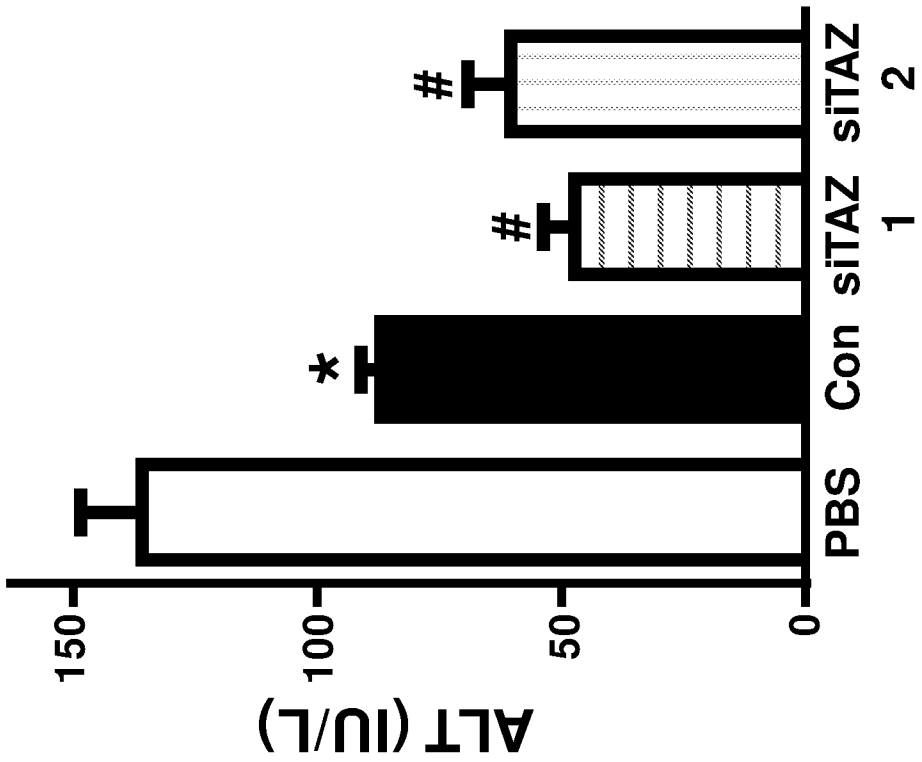


Fig. 5G

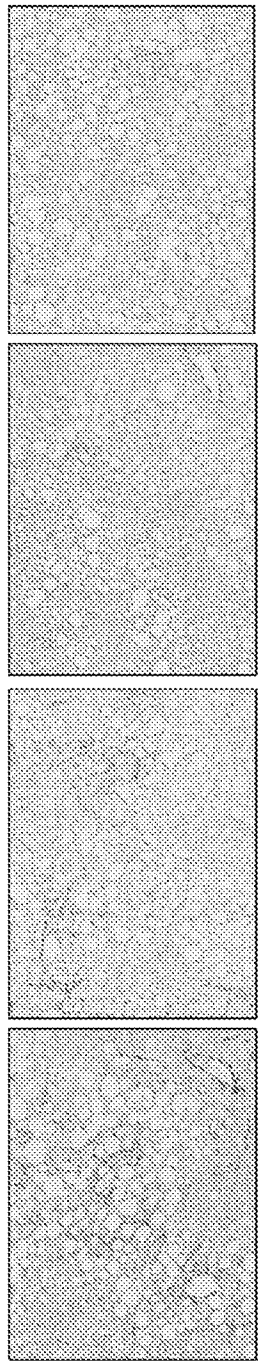

PBS
Control
siTAZ-1
siTAZ-2

Fig. 6A

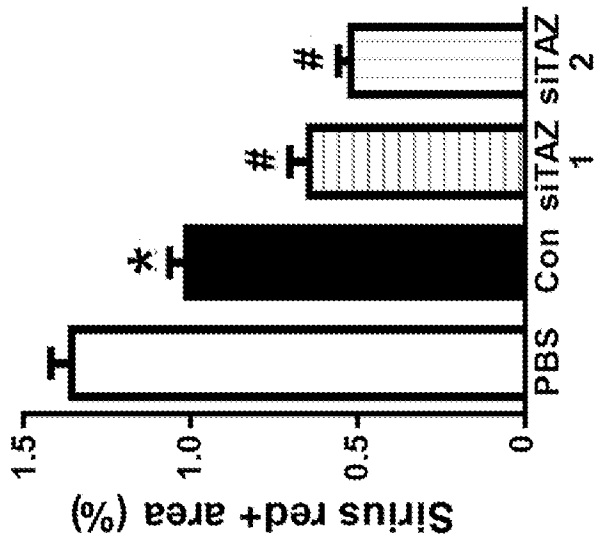

Sirius red+ area (%)
1.5
1.0
0.5
0
*
#
#
PBS
Con siTAZ 1
siTAZ 2

Fig. 6B

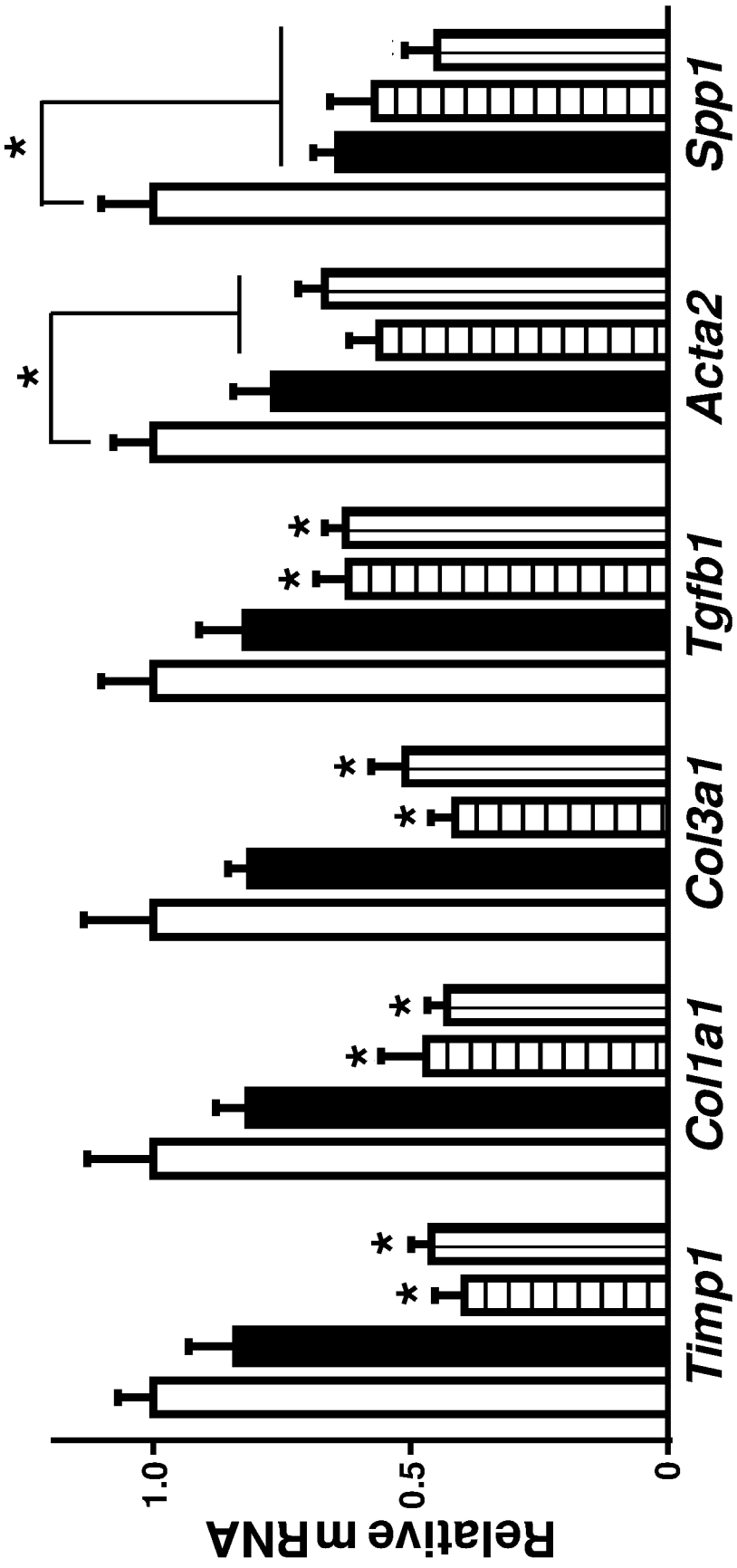


Fig. 7

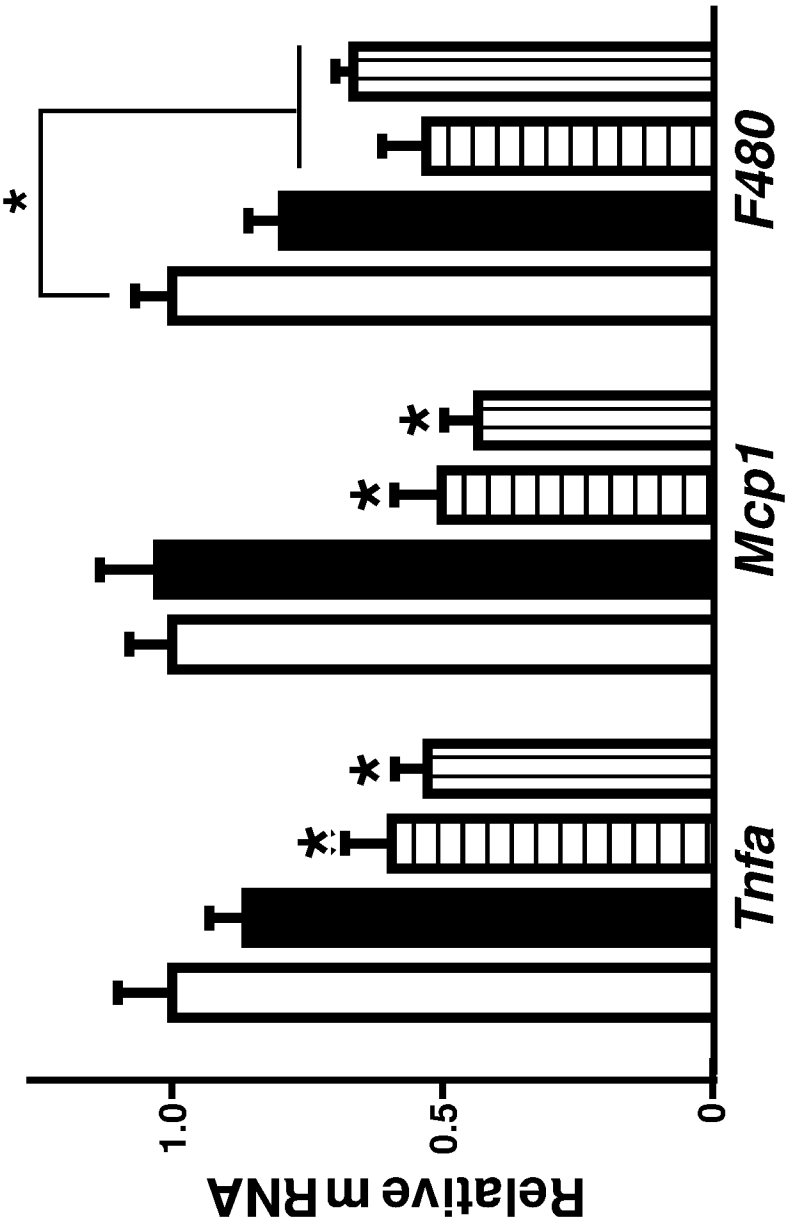


Fig. 8

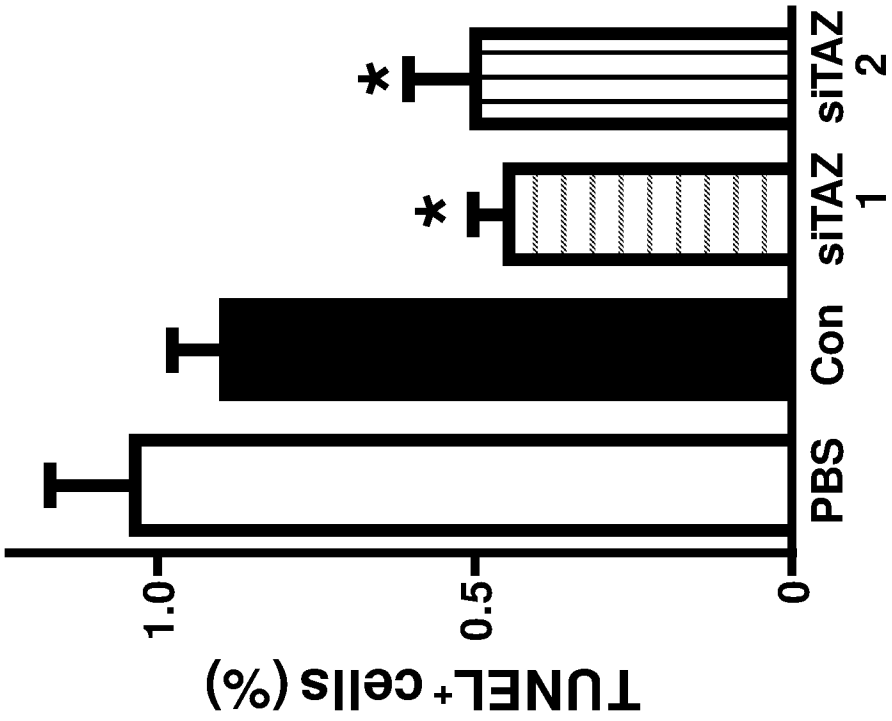


Fig. 9

Fig. 10A

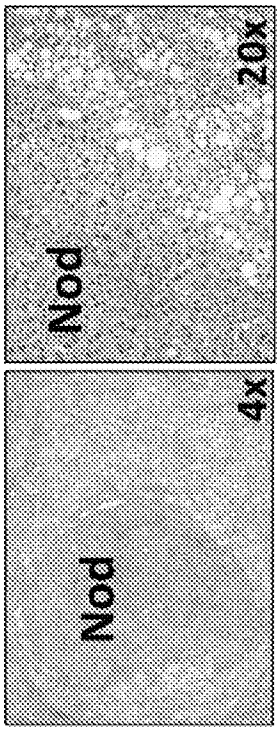


Fig. 10B

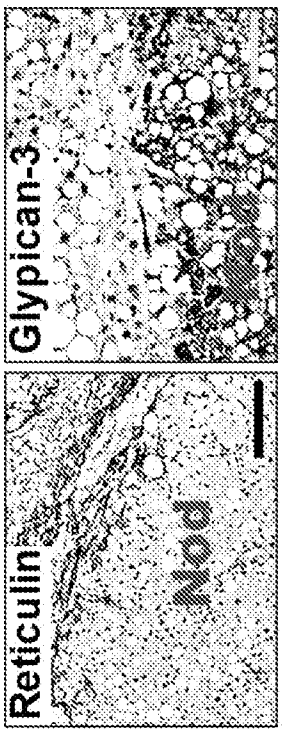


Fig. 10C

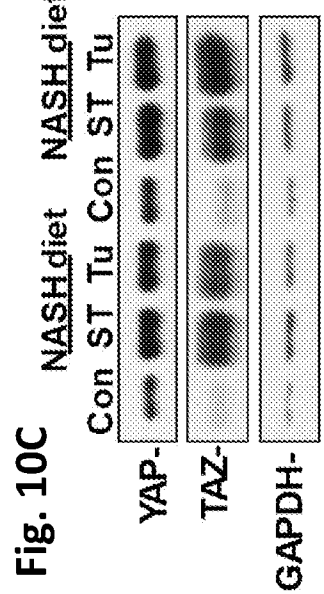


Fig. 10D

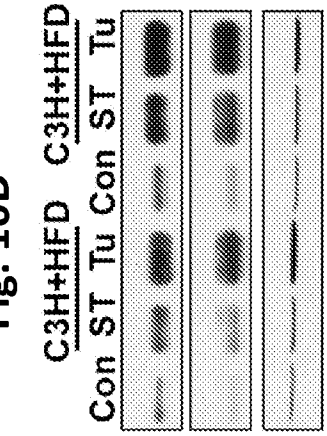


Fig. 10E

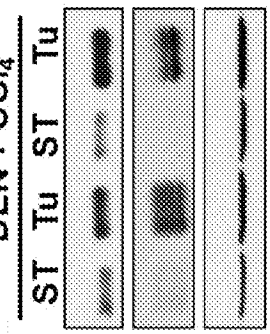
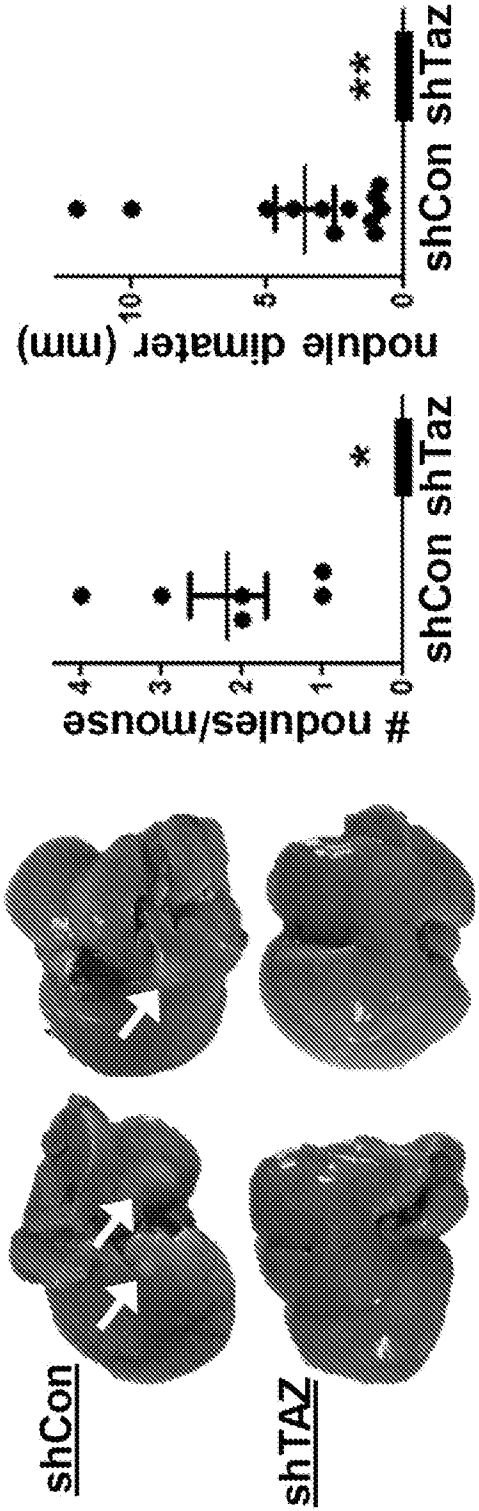


Fig. 10F



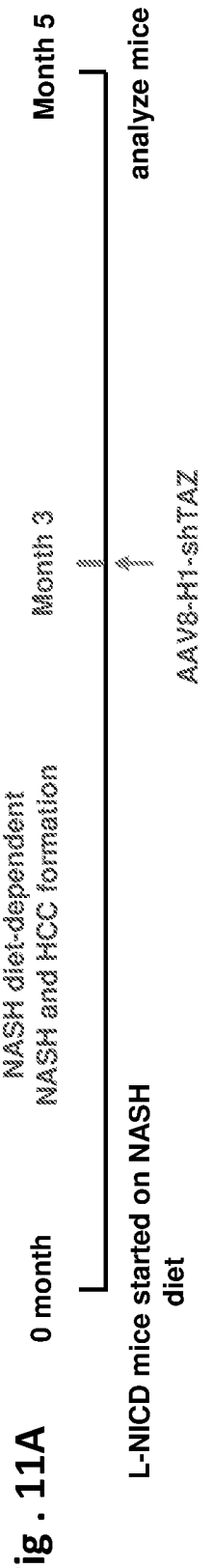


Fig . 11A
0 month
Month 3
Month 5
NASH diet-dependent
NASH and HCC formation
L-NICD mice started on NASH
diet
AAV8-H1-shTAZ
analyze mice


Fig. 11B
shControl
shTAZ
TAZ
β-actin

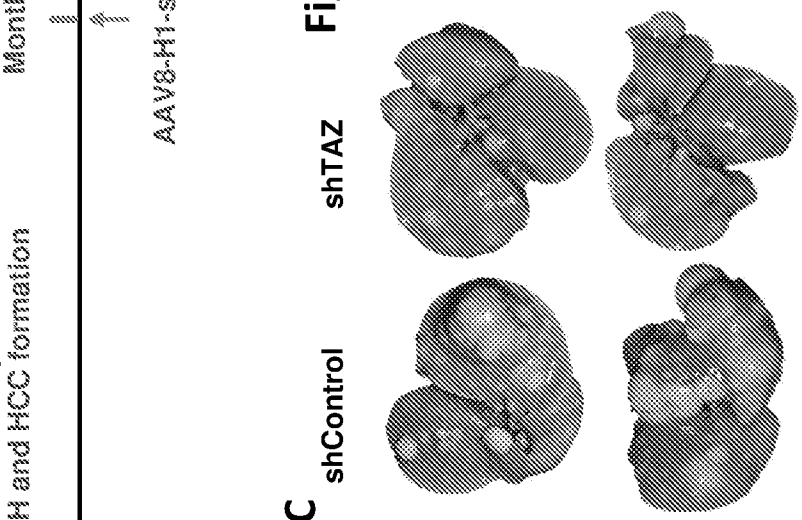


Fig. 11C
shControl
shTAZ

Fig. 11D
Nodule number/mouse
20
15
10
5
0
shControl
shTAZ
*

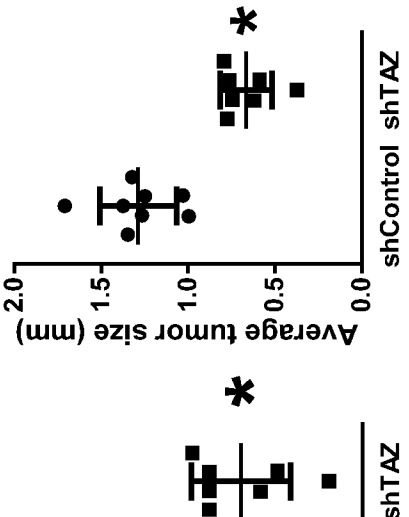


Fig. 11E
Average tumor size (mm)
2.0
1.5
1.0
0.5
0.0
shControl
shTAZ
*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 18/66837

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 38/17, C12N 15/113, C12N 5/00 (2019.01)
CPC - C12N 5/00, G01N 33/50, 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.
X --- Y	XIAO et al. TAZ regulates cell proliferation and epithelial-mesenchymal transition of human hepatocellular carcinoma. Cancer science. February 2015. Vol 106, No 2, pp 151-159. Especially Abstract; page 155, col 1, para 2	16, (18-20)/16, 25/16 --- (21-24)/16, (26-29)/16
Y	WO 2017/184586 Al (THE TRUSTEES OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK et al.) 26 October 2017 (26.10.2017) Claim 1; Claim 2; Claim 4; Claim 7; Claim 8; Claim 9; Claim 11; Claim 12; Claim 15; Claim 16; page 16, ln 8-10; page 45, ln 18-2	1-15, 17, (18-20)/17, 21-24, (25-26)/17, 27, (28-29)/17
Y	US 2016/0360735 A1 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 15 December 2016 (15.12.2016) para [0007]	1-15, 17, (18-25)/17, 26-27, (28,29)/17
Y	GENG et al. Docetaxel inhibits SMMC-7721 human hepatocellular carcinoma cells growth and induces apoptosis. World Journal of Gastroenterology. 15 April 2003. Vol 9, No 4, pp 696-700. Especially Abstract; page 696, col 1, last para; page 699, col 2, last para	14-15, 28-29

☐ 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
"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	Date of mailing of the international search report
7 March 2019	26 MAR 2019
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 18/66837

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:

      ☒ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

      ☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments: