Research Article

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RNAi-nanoparticulate manipulation of gene expression as a new functional genomics tool in the liver

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Background & Aims: The Hippo pathway controls organ size through a negative regulation of the transcription co-activator Yap1. The overexpression of hyperactive mutant Yap1 or deletion of key components in the Hippo pathway leads to increased organ size in different species. Analysis of interactions of this pathway with other cellular signals corroborating organ size control is limited in part due to the difficulties associated with development of rodent models.

Methods: Here, we develop a new model of reversible induction of the liver size in mice using siRNA-nanoparticles targeting two kinases of the Hippo pathway, namely, mammalian Ste20 family kinases 1 and 2 (Mst1 and Mst2), and an upstream regulator, neurofibromatosis type II (Nf2).

Results: The triple siRNAs nanoparticle-induced hepatomegaly in mice phenocopies one observed with *Mst1*$^{-/-}$ *Mst2*$^{-/-}$ liver-specific depletion, as shown by extensive proliferation of hepatocytes and activation of Yap1. The simultaneous co-treatment with a fourth siRNA nanoparticle against Yap1 fully blocked the liver growth. Hippo pathway-induced liver enlargement is associated with p53 activation, evidenced by its accumulation in the nuclei and upregulation of its target genes. Moreover, injections of the triple siRNAs nanoparticle in *p53*$^{LSL/LSL}$ mice shows that livers lacking p53 expression grow faster and exceed the size of livers in p53 wild-type animals, indicating a role of p53 in controlling Yap1-induced liver growth.

Conclusion: Our data show that siRNA-nanoparticulate manipulation of gene expression can provide the reversible control of organ size in adult animals, which presents a new avenue for the investigation of complex regulatory networks in liver.

Keywords: Hippo pathway; p53; Bile acids; siRNA; Nanoparticles.

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Introduction

Maintenance of proper organ size in an organism is a fundamentally important process. The proper function of organs depends on coordinated control of their size during developmental and adult stages. In contrast, loss of organ size control contributes to a number of diseases, including hypertrophy and degenerative diseases [1]. Organ size control integrates various factors, including local mechanical, autocrine/paracrine stimuli as well as soluble circulating cues and environmental factors [1–3].

One of the cues critical in controlling organ size and cellular proliferation is the Hippo signaling pathway (Reviewed in [4,5]). This pathway is composed of an evolutionarily conserved core kinase cassette and upstream modulators (reviewed in [6]). In mammals, the core kinases consist of mammalian Ste20 family kinases 1 and 2 (Mst1 and Mst2), Salvador homolog 1 (Sav1), Large tumor suppressor 1 and 2 (Lats1 and Lats2), and Mps one binder 1 (Mob1) [4,5]. The core cascade is tightly regulated by multiple upstream modulators [6]. Neurofibromatosis type II (Nf2, also known as Merlin) can activate the core kinase cassette [7], probably through direct binding and recruiting Lats1/2 to the plasma membrane [8]. The Hippo pathway negatively regulates its major downstream effector Yes-associated protein 1 (Yap1) through phosphorylation, provoking its degradation and cytoplasmic retention [9,10]. As a potent transcription co-activator, Yap1 can induce genes involved in cellular growth and apoptosis inhibition by association with the TEA domain

family and other transcription factors [9,11,12]. Overexpression of S127 mutant Yap1 in the adult liver causes massive hepatomegaly [10]. It has been reported that while one allele of either *Mst1* or *Mst2* is sufficient to maintain embryonic development, *Mst1*$^{-/-}$*Mst2*$^{-/-}$ mice are embryonically lethal [13–15]. One copy of either *Mst1* or *Mst2* can maintain quiescence of hepatocytes, but liver-specific genetic removal of both *Mst1* and *Mst2* leads to significant liver enlargement [14–16].

Hippo signaling has been shown to interact with several other pathways, including PI(3)K–mTOR [17], Wnt/beta-catenin [18], Insulin/IGF [19,20], and Sonic hedgehog (Shh) signaling pathway [21] in normal tissue and tumorigenesis. However, deciphering its complex interactions *in vivo* requires sophisticated approaches allowing to gradually and simultaneous manipulate multiple genes.

Recent advances in synthetic siRNA delivery nanoparticles make it possible to specifically suppress one or more genes simultaneously in a range of species, from rodents to primates [22–25], including humans [26]. Through manipulating the compositions of nanoparticles, siRNA delivery systems have shown potent and reversible silencing effects *in vivo* with high specificity in multiple tissues and cell types, including hepatocytes [22,23], macrophages [27] and endothelial cells [28]. Here we develop a siRNA nanoparticle-based approach to manipulate organ size through inhibition of the Hippo pathway. The deep reduction of the expression levels of *Mst1*/*Mst2*/*Nf2* is critical to elucidate the role of p53 pathway in the control of liver growth.

Materials and methods

siRNA synthesis, screening and lipid nanoparticles (LNP) formulation

siRNAs targeting mouse *Mst1*, *Mst2*, *Nf2* and *Yap1* were designed and screened as previously described [24]. The sequences of each siRNA are provided in Supplementary Table 1. The RNA strands were synthesized, characterized and duplexed by Alnylam Pharmaceuticals as previously described [24]. Individual siRNAs were formulated into lipid nanoparticles and mixed prior to injections [23].

Cell culture

Hepa1-6, NIH3T3, AML-12 cells were obtained from ATCC and were propagated in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Cells were transfected with siRNA using either Lipofectamine RNAiMAX (Invitrogen) or LNP with siRNA as described elsewhere [24].

AML-12 cells obtained from ATCC were grown on BD BioCoat collagen I coated plasticware. Cells were treated with taurocholic acid (Sigma-Aldrich, time and dose as indicated). Cells were collected in RIPA buffers supplemented with proteases and phosphatases inhibitors (Pierce Bio) for western blot, or fixed with 2% buffered paraformaldehyde solution, followed by permeabilization with 1% Triton X-100 in PBS for immunocytochemistry.

Animals

C57BL/6 mice were purchased from Charles River laboratories. *p53*$^{LSL/LSL}$ mice were published elsewhere [29]. All animals received humane care, and animal protocols were approved by the Committee on Animal Care at MIT and the Institutional Animal Care and Use Committee of Alnylam Pharmaceuticals, certified by the American Association for Accreditation of Laboratory Animal Care. After 3 days of acclimatization in the animal facility, 7–9 week-old mice were injected via tail vein (intravenously) with either PBS or siRNA in LNP formulations at various concentrations. To restore p53 expression in *p53*$^{LSL/LSL}$ mice, animals were treated with tamoxifen (two intraperitoneal doses) before the siRNA treatment. Animals were sacrificed by CO_2 overdose; tissues were harvested at different time points as indicated. Hepatocytes, stellate cells and Kupffer cells were isolated from C57BL/6 mice via collagenase perfusion, density centrifugation and antibody selections as described previously [30–32].

Histological, immunohistological and immunocytochemical analysis

Mouse tissues were fixed in 4% paraformaldehyde. Tissue sections were stained according to standard immunohistochemistry protocols as previously described [33] or with secondary antibodies labelled with Alexa 488, Alexa 555 and Alexa 647 (Invitrogen) to visualize antigen localization. We have used the following primary antibodies: anti-Cytokeratin 18 (Progen), anti-Cytokeratin 19 (Abcam), anti-E-cadherin (BD Biosciences), anti-F4/80 (Biolegends), anti-glutamine synthase (BD Biosciences), anti-Ki67 (Neomarkers), anti-p21 (Santa Cruz), anti-p53 (Leica Biosystems), anti-Yap1 (Cell Signaling), phospho-pH2A.X (Cell Signaling). Phalloidin coupled with Alexa555 was used to visualize F-actin. Analysis of hematoxylin and eosin (H&E), immunohistochemistry and immunocytochemical images was performed using ImageJ package (NIH).

Western blots and quantification

The liver tissues were homogenized in RIPA buffer (Thermo Fisher) to harvest proteins. Total protein was resolved on TGX™ gradient gels (BioRad). The following primary antibodies were used: anti-β-actin (Sigma), anti-cyclin D1 (Millipore), anti-Mst1 (Cell Signaling), anti-Mst2 (Cell Signaling), anti-Nf2 (Sigma), anti-p21 (Santa Cruz), anti-pYap1 (Cell Signaling), anti-Yap1, phospho-pH2A.X (Abcam) Licor Odyssey Imaging system was used to visualize protein bands. Gray scale images were quantified with ImageJ as described previously [24].

Gene expression analysis

The levels of mRNAs were measured by branched DNA assay (Affymetrix) or quantitative PCR using TaqMan probes and Roche LightCycler 480. Levels of mRNA of the genes of interest were normalized to the levels of *Gapdh* mRNA and then to the average arbitrary value of the control group.

Serum chemistry

Albumin, total protein, glucose, high-density lipoprotein (HDL), low-density lipoprotein (LDL), direct and total bilirubin, aspartate transaminase (AST), alanine transaminase (ALT), total bile acids were measured in serum using Beckman Coulter reagents and Olympus Au400 autoanalyser.

Statistical analysis

Student's *t* tests and one-way ANOVA tests were used to calculate *p* values using Prism 5 (GraphPad).

Results

siRNA mediated knockdown of Mst1, Mst2 and Nf2 leads to hepatomegaly

We designed and screened sets of chemically modified siRNAs [34] targeting *Mst1* and *Mst2* (Supplementary Fig. 1). Specifically, 27 siRNAs targeting *Mst1* and 28 siRNAs targeting *Mst2* were synthesized. The gene silencing efficiency for each siRNA was examined in NIH3T3 cells at 5 nM (Supplementary Fig. 1A, C). We found that nine siRNAs targeting *Mst1* and nine siRNAs targeting *Mst2* can achieve deep knockdown at this dose (Supplementary Fig. 1A, C). A dose response study was performed using the 3 most potent siRNAs for each gene. The IC_{50} for the most potent siRNA targeting *Mst1* and *Mst2* were ~0.05 nM and 0.2 nM, respectively (Supplementary Fig. 1B, D). The most efficient siRNAs were chosen and formulated into lipid nanoparticles (LNP), which have shown hepatocyte specific targeting with high