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Inhibition of microRNA122 decreases SREBP1 expression by modulating suppressor of cytokine signaling 3 expression

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ABSTRACT

While inhibition of microRNA122 (miR122) function *in vivo* results in reduced serum cholesterol and fatty acid levels, the molecular mechanisms underlying the link between miR122 function and lipid metabolism remains unclear. Because the expression of SREBP1, a central transcription factor involved in lipid metabolism, is known to be increased by suppressor of cytokine signaling 3 (SOCS3) expression, and because we previously found that SOCS3 expression is regulated by miR122, in this study, we examined the correlation between miR122 status and the expression levels of SOCS3 and SREBP1. SREBP1 expression decreased when SOCS3 expression was reduced by miR122 silencing *in vitro*. Conversely, SREBP1 expression in miR122-silenced cells was restored by enforced expression of SOCS3. Such correlations were observed in human liver tissues with different miR122 expression levels. These signaling links may explain one of the molecular mechanisms linking inhibition of miR122 function or decreased expression of miR122 to decreased fatty acid and cholesterol levels, in the inhibition of miR122 function, or in pathological status in chronic liver diseases.

1. Introduction

MiRNA122 (miR122) is the most abundant and tissue-specific miRNA in the liver [1], and inhibition of miR122 *in vivo* was shown to greatly reduce serum cholesterol and fatty acid levels [2–6]. Recently, miravirsen, a locked nucleic acid-modified DNA phosphorothioate antisense oligonucleotide against miR122, was introduced practically to reduce hepatitis C virus (HCV) RNA levels in patients with HCV infection [7]. Although the primary purpose of the trial was to inhibit HCV replication, lipid levels were also found to decrease [7]. Despite consistent results concerning miR-122-mediated lipid metabolism, the underlying molecular mechanisms remain unclear, and although the expression levels of genes associated with lipid metabolism were affected by miR-122 inhibition, these genes were not direct targets of miR122, as judged by sequence similarities.

Members of the family of sterol regulatory element-binding proteins (SREBPs) are critical regulators of cholesterol and lipid homeostasis. The SBEBP family belongs to the basic helix–loop–helix–leucine zipper (bHLH-zipper) family of transcription factors and comprises SREBP-1a, SREBP-1c, and SREBP-2. The SREBF-1 gene on chromosome 17p11.2 encodes SREBP-1a and SREBP-1c, which are generated as alternatively spliced variants. Increased SREBP activity causes cholesterol and fatty acid accumulation by activating the expression of more than 30 genes dedicated to the synthesis and uptake of cholesterol, fatty acids, triglycerides, and phospholipids, as well as the NADPH cofactor required for synthesis of these molecules [8], which increases lipid levels.

The promoter activities of SREBP1 are potently inhibited by activated STAT3 [9]. In addition, STAT3-mediated inhibition of SREBP1 expression was shown to be antagonized by co-expression of the SOCS3 protein [9]. Conversely, SOCS3 inhibition in the liver in obese mice subjected to antisense treatment was reported to completely normalize the increased expression of SREBP1, leading to dramatic amelioration of hyperlipidemia. These results indicate the importance of SOCS3 in regulating SREBP expression and subsequent lipid metabolism [9].

We recently reported that silencing miR122 in hepatocytes leads to decreased SOCS3 expression accompanied by hypermethylation of the SOCS3 promoter in a Dnmt1-independent manner [10]. In this study, we first assessed the correlated expression levels of SREBP1 in miR122-silenced and miR122 precursor-overexpressing cells. In addition, SREBP1 was recovered by enforced expression or inhibition of SOCS3 in miR122-modulated cells. We next confirmed the correlations among miR122, SOCS3, and SREBP

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expression levels in human liver tissues in various pathological states with different miR122 expression levels [11]. Based on these analyses, we infer a molecular link between miR122 and lipid metabolism in miR122 inhibition and various liver pathological states.

2. Methods

2.1. Cells

The Huh7 and Hep3B human hepatocellular carcinoma cell lines were obtained from the Japanese Collection of Research Bioresources (JCRB, Osaka, Japan). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum.

2.2. Plasmids, viral production, and transduction

A miR122 precursor-expressing plasmid with a puromycin resistance gene (pCDH-miR122 with puro) and an H1 promoter-driven antisense miR122 stem–loop–stem RNA-expressing plasmid (pmiRZIP122 with puro) were constructed as described previously [10]. For double stable Hep3B cells with miR122 precursor and SOCS3 shRNA expression, a miR122 precursor with hygromycin resistance gene (pCDH-miR122 with hygro) was constructed by replacement of the puromycin resistance gene with a hygromycin resistant gene using the infusion method (Clontech, Mountain View, CA). SOCS3 shRNA-expressing lentiviral particles were purchased from Santa Cruz Biotechnology (Dallas, TX). An HA-SOCS3-expressing lentiviral construct with a neomycin resistance gene was constructed as described previously [10]. A pCDH control vector (System Biosciences, Mountain View, CA) was used as a negative control. Lentiviral particles, produced using pPACKH1 lentivector packaging plasmid mix (System Biosciences) according to the manufacturer's recommendations, were used as a negative control. Cells were transduced with lentiviruses using polybrene (EMD Millipore, Billerica, MA) and were then selected using puromycin.

2.3. Reporter plasmids, transient transfections, and luciferase assays

The reporter plasmids used for analysis of miR122 function were constructed as described previously [12]. Plasmid transfection was performed using FuGene6 Transfection Reagent (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's instructions. pGL4-TK, a control plasmid containing *Renilla reniformis* (sea pansy) luciferase under the control of the herpes simplex virus thymidine kinase promoter (Promega, Madison, WI), was used to determine transfection efficiency. Relative luciferase values were calculated by normalizing firefly luciferase activity values to sea pansy luciferase activity values to account for changes in transfection efficiency. Luciferase activity was measured using a Dual Luciferase Reporter Assay System (Promega) with a Lumat LB9507 luminometer (EG&G Berthold, Bad Wildbad, Germany).

2.4. Northern blotting of miRNAs

Northern blotting of miRNAs was performed as described previously [23]. Briefly, total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA). Ten micrograms of RNA was resolved in denaturing 15% polyacrylamide gels containing 7 M urea in 1× TBE and then transferred to a Hybond N+ membrane (GE Healthcare, Milwaukee, WI) in 0.25× TBE. Membranes were UV-cross-linked and prehybridized in hybridization buffer. Hybridization was performed overnight at 42 °C in ULTRAhyb-Oligo Buffer (Ambion, Austin, TX) containing a biotinylated probe specific for miR122 (tgg agt gtg aca atg gtg ttt g), antisense miR122 (caa aca cca ttg tca cac tcc a), or U6 (cac gaa ttt gcg tgt cat cct t), which had previously been heated at 95 °C for 2 min. Membranes were washed at 42 °C in 2× SSC containing 0.1% SDS, and the bound probe was visualized using a BrightStar BioDetect Kit (Ambion). A pre-stained RNA size marker for small RNA (BioDynamics Laboratory, Tokyo, Japan) was used to estimate band sizes. Blots were stripped by boiling in a solution containing 0.1% SDS and 5 mM EDTA for 10 min prior to rehybridization.

2.5. Western blot analysis and antibodies

Western blotting was performed as described previously [12]. Anti-SREBP was purchased from Santa Cruz Biotechnology. Anti-β-actin was acquired from Sigma–Aldrich (St. Louis, MO). An anti-HA antibody was obtained from Roche Applied Science (Penzberg, Germany). Other antibodies were purchased from Cell Signaling Technology (Danvers, MA).

2.6. Immunohistochemistry

Tissue arrays containing liver tissues (LV1504) were purchased from US Biomax (Rockville, MD). Immunohistochemistry was performed as described previously [23]. Briefly, after deparaffinization of the slides, endogenous peroxidase activity was blocked with 3% hydrogen peroxide buffer. Antigen retrieval was achieved by incubating the slides at 89 °C in 10 mM sodium citrate buffer (pH 6.0) for 30 min. To minimize nonspecific background staining, slides were blocked in 5% normal goat serum (Dako, Glostrup, Denmark). Tissues were labeled overnight at 4 °C with primary antibodies raised against SOCS3 or SREBP1. Slides were then incubated with an anti-mouse horseradish peroxidase-conjugated secondary antibody (Nichirei Bioscience, Tokyo, Japan) for 1 h. Bound antibody was visualized by incubation in 3,3′-diaminobenzidine (Nichirei Bioscience) for 5 min. The slides were counterstained with hematoxylin, dehydrated with ethanol, and mounted using Clarion mounting medium (Biomeda, Foster City, CA).

2.7. In situ hybridization to assess miR122

Locked nucleic acid (LNA)-scramble (negative control), LNA-anti-U6 (positive control), and LNA-anti-miR122 probes were obtained from EXIQON (Vedbæk, Denmark). The expression of miR122 in liver tissues was examined by *in situ* hybridization as described previously [12]. Briefly, after deparaffinization, tissue sections were treated with 10 μg/ml proteinase K for 5 min at 37 °C, refixed with 4% paraformaldehyde, and acetylated with 0.25% anhydrous acetic acid in 0.1 M Tris–HCl buffer (pH 8.0). Following prehybridization for 30 min at 48 °C, hybridization was performed overnight with each LNA probe (20 nM) in hybridization buffer (5× SSC buffer, 50% formamide, 500 μg/ml tRNA, 50 μg/ml Cot-1 DNA). After the completion of hybridization, the sections were washed with 0.1× SSC buffer for 10 min at 52 °C three times and blocked with DIG blocking buffer (Roche Diagnostics, Basel, Switzerland) for 30 min. Sections were then probed with anti-DIG (1:500; Roche Diagnostics) for 1 h at room temperature. Detection was performed by incubation in NBT/BCIP buffer (Promega) overnight. Nuclei were stained with Nuclear Fast Red (Sigma–Aldrich).

2.8. Histological scoring

Tissue staining was scored as described previously [23]. Briefly, staining intensity was semiquantitatively categorized into the

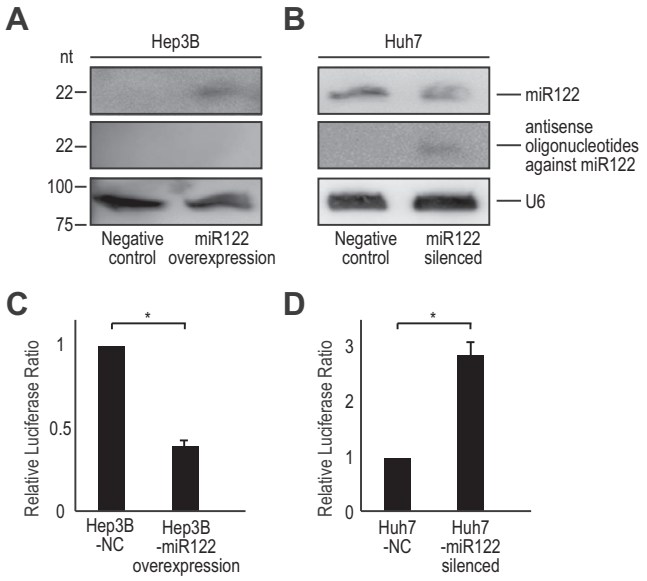

Fig. 1. Establishing of miR122-overexpressing and miR122-silenced cell lines. A, B, Northern blotting against miR122 and miR122 antisense oligonucleotides in Hep3B cells (A) and Huh7 cells (B). U6 levels were used as a loading control. Representative images from three independent experiments are shown. C, D, Luciferase expression from the reporter construct containing two tandem miR122 responsive elements in its 3'UTR, which are targeted by miR122, were examined. The suppressive effects of stably miR122 precursor-overexpressing Hep3B cells (C) and silencing effects on endogenous miR122 function in stably miR122 antisense-expressing Huh7 cells (D) are shown. Test values were normalized to those obtained from the cells transduced with a miRNA precursor-non-expressing negative control, which were set to 1 (nc). Data represent the mean ± standard deviations (SD) of three independent experiments.

following four categories by two independent investigators: −, no staining; +, weak staining; ++, moderate staining; and +++, intense staining. The color scale reflects staining intensity from green (no staining) to pink (intense staining).

2.9. Statistical analysis

Statistically significant differences between groups were determined using Student's *t*-test. *P*-values less than 0.05 were considered statistically significant.

3. Results

3.1. Establishment of miR122-overexpressing and -silenced cell lines

To determine the function of miR122, we modulated miR122 expression levels and function in liver cell lines by overexpressing an miR122 precursor construct or antisense sequences, respectively, against miR122, as previously reported [10,12]. Because Hep3B cells have relatively low miR122 expression and Huh7 cells have relatively high miR122 expression [13], we constructed miR122-overexpressing Hep3B cells and miR122-silenced Huh7 cells to examine the effects of modulating miR122. Overexpression of the miR122 precursor construct in Hep3B cells was confirmed by Northern blotting against miR122 sequences (Fig. 1A). Expression of the antisense construct against miR122 in Huh7 cells in Northern blotting appeared to be lower when using a probe to detect antisense miR122 (Fig. 1B). In these cells, miR122 levels were also lower, although miR122 was highly expressed in control Huh7 cells (Fig. 1B), suggesting that introduction of the antisense construct against miR122 may result not only in sequestration of endogenous miR122 by binding but also degradation of endogenous miR122 after formation of double-stranded RNA composed of sense and antisense miR122.

Next, we confirmed the changes in miR122 function using a luciferase reporter targeted by miR122. As predicted, in miR122-overexpressing Hep3B cells, luciferase activity decreased by more than half due to the ectopic miR122 expression (Fig. 1C). In contrast, in miR122-silenced Huh7 cells, luciferase activity increased about three times compared to control cells (Fig. 1D). These results suggest that modulation of miR122 by overexpressing exogenous constructs targeting miR122 was efficient.

3.2. SOCS3 and SREBP1 expression is decreased by miR122 silencing

Because we previously reported that miR122 silencing increased methylation in the promoter region of the SOCS3 gene and decreased its expression [10], we confirmed the expression levels of SOCS3 in miR122-overexpressing Hep3B cells and miR122-silenced Huh7 cells. Consistent with previous reports [10], SOCS3 expression increased and was downregulated in miR122-overexpressing Hep3B cells and miR122-silenced Huh7 cells, respectively (Fig. 2A and B). SOCS3 is a potent inhibitor of STAT3 activation [14]. Thus, we examined the phosphorylation status of STAT3. While total STAT3 expression levels remained unchanged, STAT3 phosphorylation decreased in miR122-overexpressing Hep3B cells and was higher in miR122-silenced Huh7 cells (Fig. 2A and B), suggesting that miR122 overexpression results in decreased STAT3 activation and that miR122 silencing has the opposite effect. Because a previous report revealed that STAT3 inhibits the promoter activity of SREBP1, a key regulator of fatty acid synthesis in the liver, we next examined the

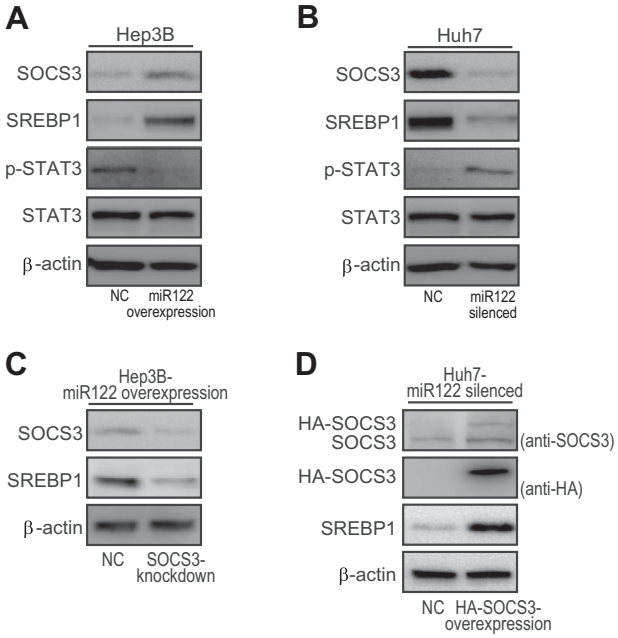

Fig. 2. SREBP1 expression is regulated by SOCS3. (A and B) SOCS3, SREBP1, and STAT3 protein expression levels and phosphorylation levels of STAT3 were determined by western blotting. Representative results from three independent experiments using Hep3B cells (A) and miR122-silenced Huh7 cells (B) are shown. (C) The effects of SOCS3 knockdown on SREBP expression in miR122 overexpressing Hep3B cell were stably transduced with SOCS3 shRNA. Representative results from three independent experiments are shown. (D) The effects of SOCS3 overexpression on SREBP expression in miR122-silenced Huh7 cells. Indicated protein expression levels were determined after miR122-silenced Huh7 cells were stably transduced with HA-SOCS3 expressing lentiviruses. HA-SOCS3 was visualized using anti-SOCS3 (the upper panel) and anti-HA (the second panel). Representative results from three independent experiments are shown.

expression levels of SREBP1 in miR122-modulated cells. The expression of SREBP1 was found to increase in miR122-overexpressing Hep3B cells and decrease in miR122-silenced Huh7 cells (Fig. 2A and B).

To confirm the role of OSCS3 in SREBP1 expression, we determined the effects of knockdown of SOCS3 expression in miR122-overexpresing Hep3B cells and enforced the expression of SOCS3 in miR122-silenced Huh7 cells (Fig. 2C and D). Knockdown of SOCS3 in miR122-overexpressing Hep3B cells reduced SREBP1 expression and enforced the expression of SOCS3 in miR122-silenced Huh7 cells increased SREBP1 expression (Fig. 2C and D). These results suggest that miR122 upregulates SOCS3 expression, resulting in the inhibition of STAT3 activation and subsequent upregulation of SREBP1 expression, and that inhibition of miR122 has the opposite effect.

3.3. Correlation of mIR122, SOCS3, and SREBP1 expression levels in human liver tissues

To confirm the above results in human clinical liver tissues, we examined 50 human liver tissues for the expression levels of miR122, SOCS3, and SREBP1 by *in situ* hybridization and immunohistochemistry (Fig. 3A and B). The expression levels of miR122 varied in liver tissues under various conditions (Fig. 3). When the expression levels of miR122 were reduced, the expression levels of SOCS3 and SREBP1 also decreased in more than 70% of cases (Fig. 3C). As previously reported [11,15], the expression levels of miR122 tended to decrease in liver cirrhosis, and SOCS3 and SREBP1 levels typically also decreased under such pathological conditions (Fig. 3C and Supplementary Table 1). These results may explain, at least in part, the decreased fatty acid and cholesterol synthesis observed clinically in the cirrhotic liver.

4. Discussion

In this study, we demonstrated that silencing miR122 function in liver cells resulted in decreased expression of SOCS3, and subsequently, decreased the expression of SREBP1. Because SREBP1 plays a key role in regulating fatty acid and cholesterol synthesis, reduced expression of miR122, which is frequently observed clinically in various chronic liver diseases [11,15], may be a cause of the decreased fatty acid and cholesterol synthesis in such pathological

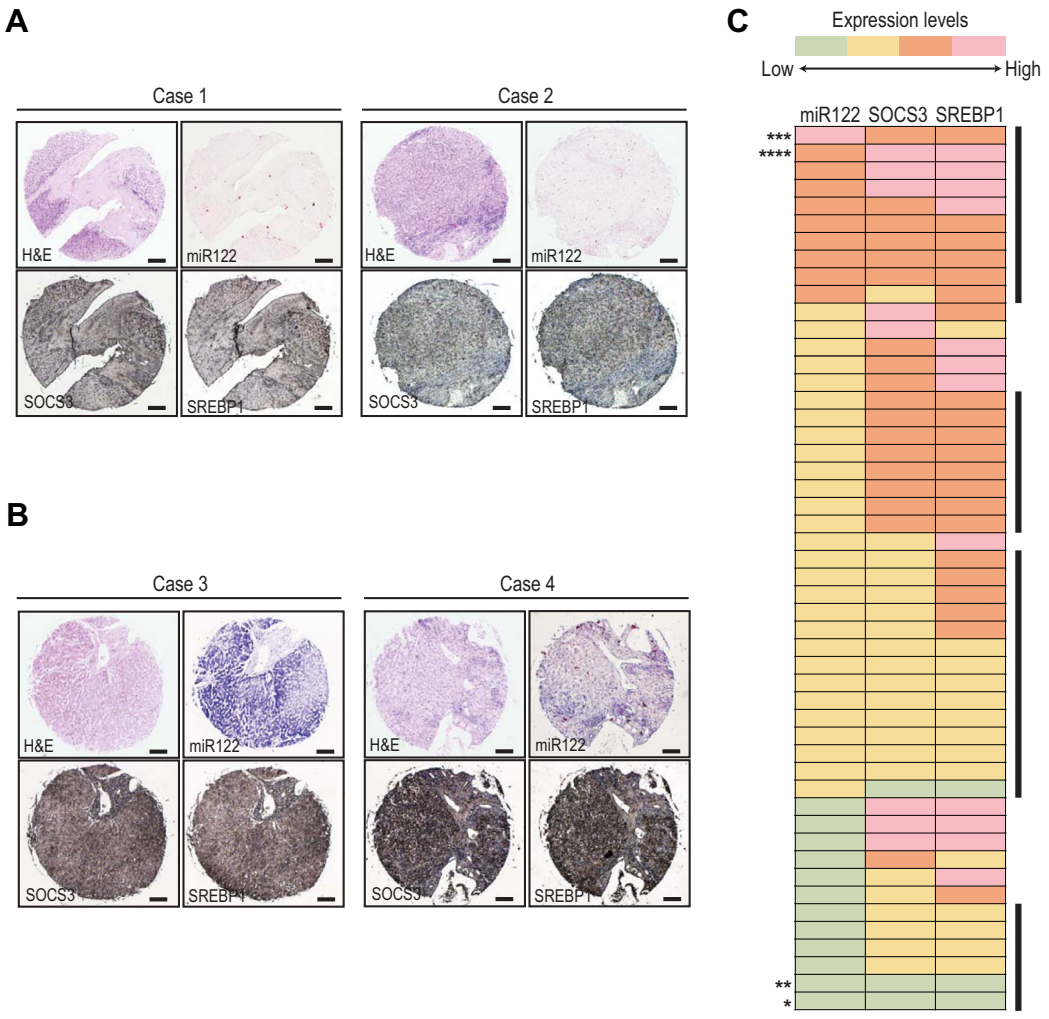

Fig. 3. Correlation of miR122, SOCS3, and SREBP expression levels in human liver tissues. (A and B), Representative liver tissues from four cases with correlated low (A) and high (B) expression levels of miR122, SOCS3, and SREBP. MiR122 was visualized by *in situ* hybridization (blue) and nuclei were stained with Nuclear Red (pink). SOCS3 and SREBP1 were stained by immunohistochemistry (brown). Bars, 500 μm. Hematoxylin and eosin (H&E)-stained tissues from each case are also shown as references. (C) Summarized expression levels of miR122, SOCS3, and SREBP1 in liver tissues from 50 cases. The color reflects the expression level of each parameter examined. Green denotes the lowest expression level and pink the highest, as the color scale bar at the top indicates. In 37 cases (indicated by black bars to the right), the differences between the expression scores of the three parameters are within one point on the color scale. ⁎, Case 1 (A); ⁎⁎, Case 2 (A); ⁎⁎⁎, Case 3 (B); ⁎⁎⁎⁎, Case 4 (B). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

states. In addition, silencing miR122 function decreases cholesterol and fatty acid levels [2–7]. While miR122 does not directly target known fatty acid-related molecules based on sequence similarities, the results of this study may explain the molecular mechanism linking miR122 silencing to decreased fatty acid and cholesterol levels.

We previously reported that silencing miR122 leads to decreased SOCS3 expression levels and increased SOCS3 promoter methylation in a Dnmt1-independent manner [10]. Such correlations in human clinical tissues were confirmed in most cases in this study. However, some cases did not show such a correlation, perhaps because while SOCS3 expression is mainly regulated by methylation of its promoter [16,17], such modifications are probably not mediated solely by miR122. Nonetheless, the decrease in SOCS3 expression that frequently accompanies decreased miR122 expression in chronic liver pathological states suggests that the SOCS3 expression in hepatocytes is largely regulated by miR122 expression or function.

The expression of SREBP1 is negatively regulated by activated STAT3, which inhibits SREBP1 promoter activities [9]. Decreased SREBP1 expression caused by increased STAT3 activity, which was itself due to decreased SOCS3 expression, was observed in miR122-silenced Huh7 cells and in liver tissues with decreased miR122 expression. Because miR122 expression levels tend to decrease as the pathological status of the liver progresses from chronic hepatitis to liver cirrhosis [11,15], observing a decreased synthesis of fatty acid and cholesterol in progressed chronic liver diseases such as liver cirrhosis may be reasonable.

One complex liver pathological situation from this point of view is obese subjects with fatty liver and insulin resistance. In these subjects, persistently elevated cytokine levels may have downregulated STAT3-mediated signaling by increasing SOCS3 protein levels in the liver [18]. Increased SOCS3 protein levels may in turn increase fatty acid synthesis by upregulating SREBP1 expression, presumably through suppression of STAT3 activation [18]. Overproduction of fatty acids and lipotoxicity result in further insulin resistance [19]. However, as these pathological conditions persist, liver cirrhosis gradually becomes apparent and fatty acid synthesis decreases. At this stage, miR122 expression in the liver becomes low [11]. Thus, in these cases, the effects of decreased miR122 expression may become apparent for the first time at the later stages of disease progression, which may be one of the reasons why not all cases showed an exact correlation between miR122 expression and SREBP levels in clinical samples.

Recently, miravirsen, an anti-miR122 oligonucleotide, was successfully applied as a novel therapeutic against HCV [7]. Although the main purpose of applying antisense miR122 *in vivo* is at present to inhibit HCV replication, decreased fatty acid and cholesterol levels were also reported [7]. In several *in vivo* experiments, antisense miR122 reduced serum lipid levels [3–5]. In addition, mice with a miR122 gene deletion in the liver showed reduced fatty acid and cholesterol levels [2,20], despite SREBP1 not being a direct target of miR122. The molecular pathway reported here may explain the reduced lipid levels observed in miR122 inhibition or gene deletion. Moreover, from these results, inhibiting miR122 may be a promising approach to controlling serum lipid levels, although the long-term inhibition of miR122 must be confirmed to be safe with no unfavorable consequences because miRNAs have pleiotropic effects [21,22].

Author contributions

C.S. and M.O. planned the research and wrote the paper. C.S., T.K., M.O., M.O., T.Y., and A.Y. performed the majority of the experiments. H.Y. supported several experiments. K.K. supervised the entire project.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.07.064.

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