Regulation of INSIG2 by microRNA-96

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ABSTRACT
Mature forms of the microRNAs miR-96, -182, and -183 originate from a single genomic locus and have been shown to be elevated approximately 50-fold in the livers of sterol regulatory element-binding protein-1a and -2 (SREBP-1a and -2) transgenic mice. Our study attempted to identify the possible targets of these microRNAs using miRNA target prediction software. This revealed putative sites in insulin-induced genes (INSIGs). The 3′ untranslated region (UTR) of insulin-induced gene 1 (INSIG1) contained sites corresponding to miR-182, and -183, while the 3′ UTR of INSIG2 featured an miR-96 site. Among these putative sites, only miR-96 demonstrated an inhibitory effect that was specific to the 3′ UTR of INSIG2. As INSIG proteins are the main components of SREBP cleavage complexes that act to release active SREBPs, we assessed the effects of miR-96 on INSIG and SREBP levels and activities. We found that miR-96 reduced the levels of INSIG2 in INSIG1 knockout human fibroblasts, resulting in an increase in SREBP-1 and -2 nuclear forms and a subsequent increase in the abundance of the mRNA of their target genes. These results suggest that miR-96, an miRNA induced by SREBP-2 activation, regulates downstream targets of SREBPs and may increase the abundance of active SREBP.

Introduction
Cholesterol and fatty acids are the main components of cell membranes and are primarily synthesized from acetyl-CoA. The enzymes responsible for de novo cholesterol and fatty acid synthesis are regulated by sterol regulatory element-binding proteins (SREBPs). Three isoforms of SREBPs have been identified in mammals, SREBP-1a, -1c, and -2. SREBP-1c and SREBP-2 are the most prevalent isoforms found in adult tissue and are produced as inactive precursors. These are typically found as integral membrane proteins in the endoplasmic reticulum (ER). SREBPs contain an N-terminal transcription factor region that is released in the Golgi apparatus by two proteases, site-1 (S1P) and site-2 protease (S2P). This cleaved region moves to the nucleus where it activates target genes by binding sterol response elements (SREs). SREBP-1c preferentially regulates genes involved in fatty acid and triglyceride synthesis, while SREBP-2 activates genes involved in cholesterol synthesis, low-density lipoprotein receptors, and PCSK9 (Brown & Goldstein 1997; Horton & Shimomura 1999; Horton et al. 2002).

N-terminal cleavage of the SREBPs is regulated by several accessory proteins, such as SREBP cleavage activating protein (SCAP), insulin-induced gene 1 (INSIG1), and INSIG2. In situations where there is sufficient ER cholesterol, INSIGs bind to SCAP and prevent the SCAP-SREBP complex from moving to the Golgi apparatus (Yabe et al. 2002; Yang et al. 2002). However, when cholesterol is low in the ER, SCAP undergoes a conformational change that results in dissociation from INSIG proteins. This enables SCAP-SREBP to be incorporated into COP-I-coated vesicles that move to the Golgi apparatus, where the N-terminal region of SREBP is released (Sun et al. 2005). Free INSIG1 that dissociates from SCAP undergoes rapid ubiquitin-mediated proteosomal degradation, while INSIG2 has a longer half-life and is not regulated by sterols (Gong et al. 2006; Lee et al. 2006).

The target genes of each SREBP isoform have been identified through the study of livers from three types of mice that either overexpress nSREBP-1a (TgSREBP-1a), overexpress nSREBP-2 (TgSREBP-2), or are liver-specific SCAP knockouts (KO) (Scap\textsuperscript{-/-}) that have reduced expression for all SREBPs in liver (Shimano et al. 1996; Horton et al. 1998; Matsuda et al. 2001). In particular, genes that encode enzymes required for fatty acid and cholesterol synthesis were identified as the likely targets for SREBP-1 and -2, respectively (Horton et al. 2002; Horton et al. 2003). Additionally, SREBPs can...
also regulate non-coding RNAs. For example, a polycis-tronic microRNA (miRNA) locus that contains miR-96, -182, and -183 was identified as a non-coding RNA region that is directly activated by SREBP-2 (Jeon et al. 2013). In our study, we have identified a predicted binding site for miR-96 in the 3’ untranslated region (UTR) of INSIG2 and determined its role in the regulation of INSIG2 protein and SREBPs.

Experimental procedures

RNA isolation and miRNA qPCR

C57BL/6J mice were fed a normal chow diet ad libitum until the start of the experiment (Teklad Mouse/Rat Diet 2018, Harlan Teklad Premier Laboratory Diets). One group of mice (n = 5) were fasted for 12 h and then their livers were collected and frozen (low insulin group). A second group (n = 5) was fed a high carbohydrate diet (MP Biomedicals, Cat. No. 960238) for 12 h after the initial 12 h fasting period (high insulin group). Livers from the mice described above and livers from Tg-albumin-Cre;Scapf/f mice (n = 5) (liver-specific Scap KO mice) were obtained from Dr. Jay Horton in University Southwestern Medical Center. RNA was isolated from frozen livers as indicated in the manufacturer’s manual with minor modification. RNA was precipitated overnight in 70% isopropanol at –20°C. Reverse transcription reactions were performed using a TaqMan microRNA Reverse Transcription Kit (Life Technologies) and the quantity of each miRNA was measured using TaqMan microRNA Assays (Life Technologies). miR-96 levels were normalized to U6 RNA levels.

Generation of pFOXO1, -INSIG1, and -INSIG2 clones

The 3’ UTR regions of INSIG1, INSIG2, and FOXO1 were amplified using genomic DNA isolated from HepG2 cells and the primers FOXO1, 5’-TCTAGAGGTTTCTGAGCAGTTACACTTAA-3’ and 5’-GTCGACAGGTTCGAAGCCTTCAACTGAACT-3’; INSIG1A, 5’-TCTAGGAAGCTGGCTGACTGTA CAAATGC-3’ and 5’-GTCGACAGGTTTCTGAGCAGTTACACTTAA-3’; INSIG1B, 5’-TCTAGAGGTTTCTGAGCAGTTACACTTAA-3’ and 5’-GTCGACAGGTTTCTGAGCAGTTACACTTAA-3’; INSIG2, 5’-TCTAGAGGTTTCTGAGCAGTTACACTTAA-3’ and 5’-GTCGACAGGTTTCTGAGCAGTTACACTTAA-3’. PCR products were digested using XbaI and SalI and inserted into the XbaI and SalI sites of a pmirGLO dual–luciferase plasmid (Promega). Resulting plasmids were designated pFOXO1, pINSIG1A, pINSIG1B, and pINSIG2. The putative binding sites of miR-183 in INSIG1A and miR-96 in INSIG2 were deleted from pINSIG1A and pINSIG2 plasmid using a Quick Change Lightning Multi Site Directed Mutagenesis Kit (Agilent) and 5’-CATGTTATTAAACAAAGTTTTCAAGCTTGAAC-3’, and 5’-TGATCATCAGTGATATGTTGTCCTGTAG-3’ primers, respectively. The putative sites for miR-182 and -96 in FOXO1 were deleted from pFOXO1 using the primer 5’-AAATTTCATTACAATAAGCTACCTACACTACATATA-3’, and for miR-183 using primer 5’-CTGCTGTAGATAAGCTTGGAAAATTTACACTTAA-3’.

Luciferase assays using pmirGLO clones

CHO-K1 cells were obtained from the Korean Cell Line Bank (KCLB No. 10061) and cultured in Ham’s F-12 medium containing 10% fetal bovine serum and 1 x Antibiotic-Antimycotic (Life Technologies). Human mirVANA miRNA mimics were obtained from Life Technologies. CHO-K1 cells were plated at a density of 2.5 x 10^5 in 48-well plates on day 0. On day 2, pGLO plasmids (0.3 μg/well) and mirVANA miRNA mimics (0, 1, 3, 10, and 20 nM) were transfected into cells using DharmaFECT-Duo Transfection Reagent (GE Healthcare). Cell lysates were prepared in 1× lysis buffer (Promega). Firefly luciferase and Renilla luciferase activities were measured using a Dual-luciferase System (Promega). Firefly luciferase activity was normalized to Renilla luciferase activity.

Transfection of miRNAs and analysis of protein and mRNA levels

INSIG1 (TR4145, INSIG1-KO) or INSIG2 (TR4148, INSIG2-KO) KO human fibroblasts were kind gifts from Dr. Russell DeBose-Boyd at the University of Texas Southwestern Medical Center. On day 0, INSIG1-KO and INSIG2-KO cells were plated onto 100 mm dishes at densities of 5 x 10^5 and 6 x 10^5, respectively. On days 1 and 2, cells were transfected with 20 nM mirVANA miRNA mimics and 20 μL Lipofectamine RNAiMAX (Life Technologies) per plate. On day 3, cells were harvested and the membrane and nuclear proteins, and total RNA, were prepared as described previously (Jo, Sguigna et al. 2011). A 30 μl aliquot of protein from each sample was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblots using antibodies raised against INSIG1 (13F5), INSIG2 (17H1), SREBP-1 (20B12), SREBP-2 (22D5), SCAP, cAMP response element–binding protein, and calnexin (Jo, Lee et al. 2011; Moon et al. 2012; Jo et al. 2013; Rong et al. 2017). Total RNA was isolated using an RNA STAT solution and treated with DNasel
from a DNA-free kit (Ambion). cDNA was synthesized using a TaqMan Reverse Transcription Kit (Life Technologies). Real-time qPCR was performed using 2xSYBR Green PCR Master Mix (Life Technologies), as previously described (Liang et al. 2002).

Results
miR-96 levels were not regulated by insulin
miR-96 was previously identified as a miRNA originating from the polycistronic miRNA locus that also contains miR-182 and -183. This miRNA has previously been shown to be highly elevated (approximately 50-fold) in the livers of TgSREBP-1a, and TgSREBP-2 transgenic mice when compared to wild-type (WT) (Jeon et al. 2013). To determine whether insulin also regulates miR-96, levels of the miRNA were measured in livers of mice fasted for 12 h (low insulin) and mice refed a high carbohydrate diet for 12 h after 12 h of fasting (high insulin). No significant changes were detected in the levels of mature miR-96 between the fasting and refeeding periods (Figure 1(A)). The basal expression level of miR-96 was extremely low in WT mice but there was no further reduction in miR-96 levels in Scap−/− mice compared to WT. This suggests that SREBPs are not critical to the maintenance of basal levels of miR-96 (Figure 1(B)). These results also indicate that short-term changes in insulin level do not play a significant role in regulating miR-96.

The 3′ UTR of INSIG2 is inhibited by miR-96
Targets can and miRDB software were used to search for the target genes of miR-96, -182, and -183. This identified putative binding sites for miR-96 in the 3′ UTR of INSIG1 and for miR-183 in the 3′ UTR of INSIG2. Furthermore, these sites were found to be conserved in the mouse and rat genomes (Figure 2(A)). Putative binding sites for miR-182 were also identified in the 3′ UTRs of INSIG1 and INSIG2 were regulated by miRNAs, we cloned a 500 bp region from INSIG1 and INSIG2 containing these binding sites into a pmirGLO plasmid downstream of a firefly luciferase reporter gene. The 3′ UTR of FOXO1 was used as a positive control (Guttila & White 2009; Myatt et al. 2010). CHO-K1 cells were co-transfected with each plasmid and miR-96, -182, and -183 mimics. The firefly luciferase activity of each cell extract was then measured. This revealed that the luciferase activity of pFOXO1 was significantly inhibited by the presence of miR-96, -182, and -183. The luciferase activity of the plnSISG2 plasmid was inhibited by approximately 50% in cells transfected with miR-96, even when using concentrations as low as 1 nM. The inhibitory effects of miR-96 were abolished when the putative binding site for miR-96 was deleted from the 3′ UTR of INSIG2 (Figure 2(C)). The luciferase activities of plasmids containing the 3′ UTR regions of INSIG1 and INSIG2 with the putative binding sites for miR-182 and miR-183 were unaffected by the addition of miR-182 or miR-183 mimics (data not shown). These results suggest that miR-96 may inhibit INSIG2 by binding to the predicted site we identified in the 3′ UTR of INSIG2. Finally, we confirmed that the miR-96 binding site found in the 3′ UTR of mouse Insig2 behaved similarly.

Endogenous INSIG2 is inhibited by miR-96
To establish whether miR-96 can regulate endogenous INSIG1 or INSIG2 protein and affect SREBP processing in cells, human fibroblasts were transfected with miR-96 and the abundances of INSIG1 and INSIG2 were compared to cells transfected with a negative control miRNA (miR-NC) and non-transfected controls. Human fibroblasts in which INSIG1 or INSIG2 had individually been deleted (designated INSIG1-KO and INSIG2-KO) were used to assess the changes to each protein and any downstream effects more clearly. In INSIG1-KO cells, INSIG2 levels were significantly decreased in the presence of miR-96. The mature forms of SREBP-1 and SREBP-2 were also increased in the INSIG1-KO cells transfected with miR-96, suggesting an association with INSIG2 and miR-96. No changes were detected in the...
levels of SCAP (Figure 3). However, we found that adding miR-96 did not affect INSIG1 levels in INSIG2-KO cells and there was no subsequent effect on SREBP activation. Changes in the expression of the target genes of SREBP-1 and SREBP-2 were also assessed in INSIG1-KO cells transfected with miR-96 or miR-NC (Figure 3). These genes are primarily involved in the cholesterol synthesis pathway and include 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) synthase and HMG-CoA reductase. We found that the transcript levels of these genes were increased fourfold to sixfold in INSIG1-KO cells exposed to miR-96. Additionally, transcripts for genes involved in the fatty acid synthesis pathway, such as FAS, SCD-1, and ELOVL6, were increased twofold to fourfold in cells transfected with miR-96, alongside an increase in SREBP activation (Figure 4). These results suggested that miR-96 can inhibit endogenous INSIG2 and consequently enhance SREBP processing, leading to increased expression of SREBP target genes.

**Discussion**

The roles of SREBPs in the regulation of enzymes required for fatty acid and cholesterol metabolism have been extensively investigated using various transgenic and KO mice models. This detailed analysis has contributed to a better understanding of how lipid metabolism is regulated (Shimano et al. 1996; Shimano et al. 1997; Matsuda et al. 2001; Liang et al. 2002). Regulation of
non-coding genes by SREBPs, particularly miRNAs, has also been investigated (Jeon et al. 2013). For example, SREBP-2 was found to induce transcription of miR-96, -182, and -183, from a single region. Our study has shown that miR-96 targets the 3′ UTR of INSIG2, reducing its expression and thereby accelerating SREBP processing. This results in an increase in the abundance of the nuclear forms of SREBP-1 and -2, both of which have further roles in SREBP regulation. Another miRNA from the same transcript, miR-182 was shown to increase SREBP levels by inhibiting Fbxw7 (Jeon et al. 2013). We were unable to determine a role for miR-183 in our study and found no effect on INSIG or SREBP activation.

miR-96 and miR-183 both have an additional role in controlling FOXO1, a major transcription factor in the liver that regulates glucose production genes, such as glucose 6-phosphatase and phosphoenolpyruvate carboxykinase. The FOXO1 3′ UTR was used as a positive control in our study of regulation by miR-96 and miR-183. Although FOXO1 does not directly regulate SREBPs, a reduction in FOXO1 may increase the flow of substrates into the lipid synthesis pathway. By reducing the flow of glucose 6-phosphate into glucose production, it may be more available for fatty acid and cholesterol synthesis. The expression and subsequent regulation of miRNAs transcribed together may be both a way to rapidly activate SREBPs and a method to increase fatty acid and cholesterol synthesis. In concert with other regulation methods, including transcriptional and post-translational mechanisms, our study reveals that miRNAs act to control SREBP levels and also play a role in directing metabolic flow.

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Disclosure statement
No potential conflict of interest was reported by the authors.

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