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The induction of miR-96 by mitochondrial dysfunction causes impaired glycogen synthesis through translational repression of IRS-1 in SK-Hep1 cells

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ABSTRACT

MicroRNA (miRNA) is a class of endogenous small noncoding RNA that negatively regulates gene expression at the post-transcriptional level and plays an important role in the pathogenesis of various diseases. However, the identity and role of miRNAs involved in the development of insulin resistance resulting from mitochondrial dysfunction are largely unknown. In this study, mitochondrial dysfunction by genetic or metabolic inhibition induced an impairment of insulin signaling in SK-Hep1 cells via a reduction in the expression of IRS-1 protein. Significant up-regulation of miR-96, which was presumed to target *IRS-1* 3′UTR, was found in SK-Hep1 cells with mitochondrial dysfunction. Using reporter gene assay we confirmed that miR-96 authentically targeted *IRS-1* 3′UTR. Furthermore, the ectopic expression of miR-96 caused a substantial decrease in IRS-1 protein expression, and a consequent impairment in insulin signaling. These findings suggest that the up-regulation of miR-96 by mitochondrial dysfunction contributes to the development of insulin resistance by targeting IRS-1 in SK-Hep1 cells.

1. Introduction

Mitochondria play a pivotal role in the cellular energy metabolism, Ca^{2+} homeostasis, production of reactive oxygen species, and activation of apoptosis [1,2]. As the appropriate functions of mitochondria are critical for cell survival and homeostasis, mitochondrial dysfunction is intimately involved in the pathogenesis of various diseases, such as neurodegenerative diseases and cancer [2,3]. Over the last decade, an increasing number of studies have revealed the implications of mitochondrial dysfunction in the pathogenesis of metabolic diseases, particularly in insulin resistance and diabetes [1,4]. Insulin resistance is defined as a decreased responsiveness of the target tissues to ordinary levels of insulin, and is believed to be a central feature of type 2 diabetes and metabolic syndrome [5,6]. Cellular oxidative capacity, which depends mostly on mitochondrial function, is related directly to insulin sensitivity in skeletal muscles [7,8], and reduced mitochondria activity has been observed in patients with obesity and type 2 diabetes [9,10]. Previously, we demonstrated that mitochondrial dysfunction induced by inhibitors of mitochondrial function or depletion of mitochondrial DNA (mtDNA) caused insulin resistance in myocytes and hepatocytes through a reduction in the expression of insulin receptor substrate (IRS)-1 [11].

IRS-1 plays important roles in both metabolic and mitogenic signal transduction, and is a key molecule in the insulin signaling cascade by transmitting the signals from insulin receptor to intracellular phosphoinositide 3-kinase (PI3K) [12]. Although inappropriate post-translational modifications of IRS-1 proteins are major factors involved in the development of insulin resistance, a number of studies have suggested that the decreased expression of IRS-1 protein also leads to insulin resistance and type 2 diabetes [1]. The level of IRS-1 is lower in the liver and skeletal muscle from animal models of type 2 diabetes, such as *ob*/*ob* mice [13] and obese Zucker rats [14]. Moreover, a decrease in IRS-1 expression was reported in the skeletal muscle from diabetic patients, suggesting that this could represent a marker for the risk of insulin resistance and diabetes [15,16]. However, the molecular mechanism for how mitochondrial dysfunction reduces the expression of IRS-1 remains largely unknown.

MicroRNAs (miRNAs) are endogenous small non-coding RNAs that act as post-transcriptional inhibitors of protein expression by base-pairing with the 3' untranslated regions (3′UTR) of their target mRNAs [17]. Upon binding, miRNA initiates a pathway that either degrades the transcripts or suppresses their translation [17,18]. Although the target genes and biological functions of the individual miRNAs need to be elucidated, it has been suggested that miRNAs have a range of functions in both normal and pathological states [18]. In 2005, Mersey et al. [19] provided the first evidence that miRNAs were involved in complex metabolic processes, such as the energy metabolism. After the first discovery that miRNAs are involved in metabolic regulation, growing evidence has

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revealed a large number of miRNAs involved in the pathogenesis of insulin resistance and diabetes [20]. However, the involvement of miRNAs in insulin resistance, in particularly under the condition of mitochondrial dysfunction, still remains unexplored.

Here, we have shown that mitochondrial dysfunction resulting from genetic or metabolic inhibition significantly increased the expression of miR-96, which targets to *IRS-1* 3′UTR directly, resulting in impaired insulin signaling and glycogen synthesis through the translational repression of IRS-1. These findings suggest a novel mechanism for the development of insulin resistance in mitochondrial dysfunction by providing the first evidence that miR-96 mediates the repression of IRS-1 expression.

2. Materials and methods

2.1. Cell culture and induction of mitochondrial dysfunction

SK-Hep1 cells (ATCC HTB-52), originated in human hepatocellular carcinoma, were cultured in DMEM with 10% FBS. Although cancer cells exhibits a metabolic phenotype characterized by high rates of glycolysis relative to oxidative metabolism [21], SK-Hep1 cells are widely used to study cellular metabolism, signal transduction and apoptosis. For mtDNA depletion, the cells were incubated with ethidium bromide (EtBr, 0.2 μg/ml) and uridine (50 μg/ml) for 2 weeks in DMEM with 10% FBS. Under these experimental conditions, mtDNA was depleted to less than 10% of the normal levels. For mitochondrial metabolic inhibition, the cells were treated with Rotenone (0.1 μM) or Antimycin A (20 μM) for 18 h. The cells were deprived of serum for 5 h before all experimental analysis. The quantitation of mtDNA, staining of functional mitochondria, and ATP assay were performed as described previously [11].

2.2. RNA preparation and quantitative real-time RT-PCR (qRT-PCR)

Total cellular RNA was extracted using miRNeasy Mini kit (Qiagen, Valencia, CA). For *q*RT-PCR, total RNAs were reverse transcribed into cDNAs with miScript Reverse Transcription kit (Qiagen) and amplified with specific primers (Bionics, Seoul, Korea) as described previously [22]. *q*RT-PCR was carried out in LightCycler®480 (Roche-Applied Science, Mannheim, Germany) using SYBR-Green PCR Master Mix according to the manufacturer's instructions (Qiagen).

2.3. Transfection of miR-96 and reporter gene assay

miRIDIAN miR-96 mimic and miRIDIAN miR-96 mimic negative control oligonucleotides were purchased from Dharmacon (Lafayette, CO, USA), and transfected to SK-Hep1 cells with Lipofectamine 2000 (Invitrogen). For luciferase assay, DNA fragments (414 nt) of *IRS-1* 3′UTR containing predicted miR-96 binding site (IRS1-3U*wt*) were cloned into the pGL3-promoter plasmid (Promega) and the miR-96 binding sites were replaced with an 5 nt fragment to produce mutated 3′UTR pGL3 report plasmids (IRS1-3U*mut*). Luciferase assays were performed using a Dual luciferase reporter system (Promega) in accordance with the manufacturer's instructions. Briefly, SK-Hep1 cells were plated one day before transfection onto the 12-well plates and grown to an approximately 70% confluence. The cells were cotransfected with miR-96 mimic/negative control oligonucleotides, pRL-SV40 *Renilla* luciferase plasmid, and pGL3-control Firefly luciferase vectors containing empty, wild-type (IRS1-3U*wt*), or mutant (IRS1-3U*mut*) *IRS-1* 3′UTR sequence. At 48 h post-transfection, the cells were lysed and the Firefly and *Renilla* luciferase activities were measured on a Lumat LB 9507 Luminometer (EGG, Stuttgart, Germany). Relative luciferase

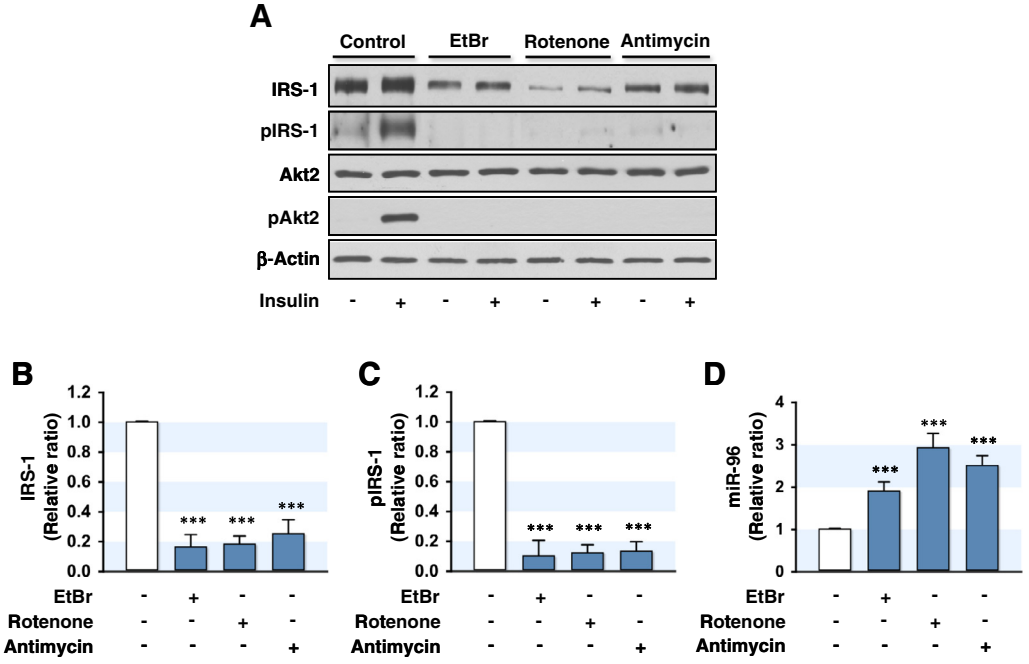

Fig. 1. Up-regulation of miR-96 in the impaired insulin signaling by mitochondrial dysfunction. SK-Hep1 cells depleted of mtDNA were prepared by EtBr treatment for 2 weeks. For mitochondrial metabolic inhibition, the cells were treated with Rotenone (0.1 μM) or Antimycin A (20 μM) for 18 h. The cells were then preincubated in the presence or absence of insulin (100 nM for 20 min) and the total cell lysates (15 μg protein) were subjected to 8% SDS–PAGE. (A) The expression (IRS-1, and Akt2) and phosphorylation (pIRS-1, and pAkt2) of the insulin signaling intermediates were analyzed by immunoblot. (B and C) The immunoblot intensities for IRS-1/β-Actin (IRS-1) and pIRS-1/β-Actin (pIRS-1) were quantified by densitometry and are expressed as the relative ratio, where the intensity of the normal control was set to one. (D) The expression of miR-96 in the cells was measured by *q*RT-PCR. The fold changes are expressed in the relative ratio where U6 intensity is set to one. The values are expressed as the means ± SEM from six independent experiments. $^{***}P < 0.001$.

activity was calculated by normalizing the ratio of Firefly/*Renilla* luciferase to that of negative control-transfected cells.

2.4. Glycogen assay

The glycogen contents in the cultured cells were analyzed using Glycogen Assay Kit (BioVision, Mountain View, CA) by spectrophotometry (570 nm) with an OxiRed probe according tothe manufacturer's suggestion.

2.5. Gel electrophoresis and immunoblotting

Cell lysates solubilized in Laemmli solution were subjected to SDS–PAGE on 8% or 10% resolving gels and immunoblotted, as described elsewhere [23]. The antibody against IRS-1 was purchased from Upstate (Lake Placid, NY) and the antibody against phospho-IRS-1 was purchased from Invitrogen (Carlsbad, CA). All other antibodies were obtained from Cell Signaling (Beverly, MA). The proteins were visualized using an enhanced chemiluminescent substrate kit (NEN Life Science Products). The immunoblot intensities were quantified by densitometry using an analytical scanning system (Molecular Dynamics Inc., Sunnyvale, CA).

2.6. Database and statistical analysis

The miRNAs targeting *IRS-1* 3′UTR were screened computationally with TargetScan (http://www.targetscan.org/index.html) and miRBase (http://microrna.sanger.ac.uk). The values were expressed as the means ± SEM from at least four independent experiments. Where applicable, the significance of the difference was analyzed using Student's *t* test for unpaired data.

3. Results

3.1. Up-regulation of miR-96 in mitochondrial dysfunction-induced insulin resistance

The mitochondrial dysfunction resulted from genetic (0.2 μg/ml of EtBr for 2 weeks) or chemicals (0.1 μM of Rotenone or 20 μM of Antimycin A for 18 h) inhibitors impaired insulin signaling in myocytes and hepatocytes [11,22]. The depletion of mtDNA by EtBr reduced the level of IRS-1 expression considerably in SK-Hep1 cells (Fig. 1A and B). Similarly, Rotenone or Antimycin A caused a significant decrease in the expression of IRS-1, whereas the expression in both Akt2 and GSK3β remained unaffected as compared to control (Fig. 1A and B). As expected, mitochondrial dysfunction induced by EtBr, Rotenone or Antimycin A significantly reduced the insulin-stimulated phosphorylation of IRS-1 and its downstream substrate, Akt2 (Fig. 1A and C). These findings clearly show that mitochondrial dysfunction induced with genetic and chemical inhibition leads to the impairment of insulin signaling through a reduction in IRS-1 expression. Because mitochondrial dysfunction reduced IRS-1 expression in SK-Hep1 cells, we analyzed the differential expression of miRNAs in mtDNA-depleted cells with Affymetrix's GeneChip miRNA analysis. Interestingly, the expressions of several miRNAs presumably targeting a segment of *IRS-1* 3′UTR, such as miR-96, miR-30e, miR-27a and miR-126, were more than twofold increased by mtDNA depletion as compared with normal control (data not shown). For this reason, the authentic expression of

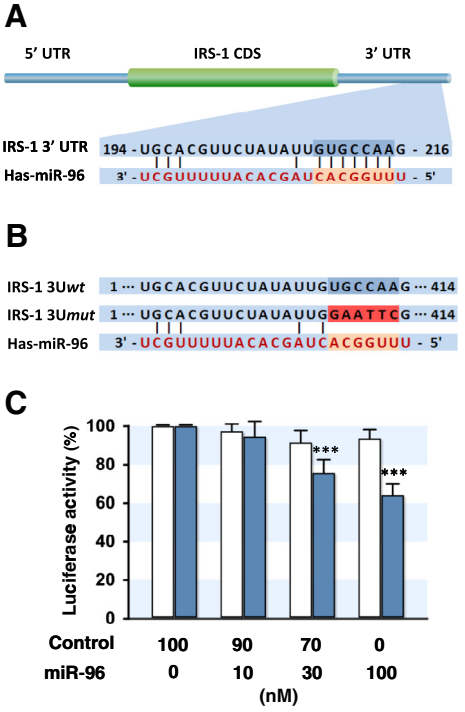

Fig. 2. Targeting site of miR-96 in the 3′UTR of *IRS-1* and assessment of its binding by reporter gene assay. (A) The seed sequence of miR-96 was predicted to target *IRS-1* 3′UTR. (B) For the reporter gene assay, 414 nt-long 3′UTR of the *IRS-1* gene was inserted downstream of a luciferase open reading frame (IRS1-3U*wt*). As a control, a mutated 3′UTR of the *IRS-1* gene (IRS1 3U*mut*) lacking the miR-96 binding site was used. (C) IRS1-3U*wt* (closed column) or IRS1-3U*mut* (open column) construct was cotransfected with the indicated concentrations of the negative control (Control) or miR-96 mimic (miR-96) oligonucleotides into SK-Hep1 cells, as described in the Section 2. Reporter gene assay was performed using a Dual-luciferase assays kit. The relative luciferase activities were plotted against that of the negative control (Control 100 nM), which was set as one. The values are expressed as the means ± SEM from four independent experiments. $^{***}P < 0.001$ vs. IRS1-3U*mut*.

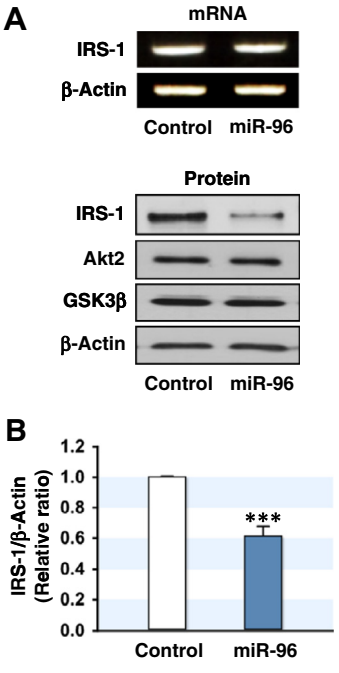

Fig. 3. Effect of miR-96 on the expression of IRS-1. SK-Hep1 cells were transfected with 200 nM of the negative control (Control) or miR-96 mimics (miR-96) oligonucleotides. (A) The mRNA level was analyzed after 24 h of transfection, whereas immunoblot analysis was conducted after 72 h of transfection. These are representative results from five independent experiments. (B) The expression of IRS-1 was analyzed by immunoblotting. The immunoblot intensities of IRS-1/β-Actin were quantified by densitometry, and are expressed as the relative ratio where the intensity of the negative control was set to one. The values are expressed as the means ± SEM from five independent experiments. $^{***}P < 0.001$.

miR-96, which is one of the most drastically up-regulated miRNAs, was verified by *q*RT-PCR in SK-Hep1 cells with mitochondrial dysfunction (Fig. 1D). Mitochondrial dysfunction induced by EtBr, Rotenone or Antimycin A up-regulated miR-96 expression in SK-Hep1 cells significantly. This suggest that mitochondrial dysfunction increases the level of miR-96, which is predicted to target *IRS-1* 3′UTR, indicating the involvement of miR-96 in the impaired insulin signaling.

3.2. Validation of miR-96 targeting in IRS-1 3′UTR

We have postulated that miR-96 inhibits IRS-1 expression by repressing its translation. Although *in silico* target analysis showed that the potential binding site for miR-96 is located within 3′UTR of *IRS-1*, extending from bp 209–215 (Fig. 2A), it is unclear whether miR-96 authentically binds *IRS-1* 3′UTR. To determine if miR-96 represses the expression of IRS-1 directly, we constructed a pGL3-promoter-based 3′UTR reporter vector consisting of a luciferase cDNA followed by the 414 nt 3′UTR of the *IRS-1* gene, which contains a potential miR-96 binding site (IRS1 3U*wt*) (Fig. 2B). A mutated 3′UTR of the *IRS-1* gene (IRS1 3U*mut*), which lacks a miR-96 binding site, was used as a control (Fig. 2B). The plasmid DNA of each reporter (IRS1-3U*wt* or IRS1-3U*mut*) was cotransfected with the indicated concentration of miR-96 mimic or negative control (scrambled oligonucleotides) into SK-Hep1 cells (Fig. 2C). The luciferase activity with IRS1-3U*wt* was reduced significantly by miR-96 mimic in a dose-dependent manner, whereas the luciferase activity with IRS1-3U*mut* was not decreased (Fig. 2C). These results clearly suggest that miR-96 targets *IRS-1* 3′UTR directly and represses IRS-1 expression.

3.3. Repression of IRS-1 by ectopic expression of miR-96

According to the luciferase reporter assays, the ectopic expression of miR-96 should reduce IRS-1 protein level without altering mRNA level in SK-Hep1 cells. To examine this further, SK-Hep1 cells were transfected with the negative control or miR-96 mimic, and IRS-1 expression was analyzed. As shown in Fig. 3A and B, the ectopic expression of miR-96 resulted in a drastic reduction of IRS-1 protein level with no apparent change in *IRS-1* mRNA, indicating the translational repression of IRS-1 by miR-96. On the other hand, the protein levels of Akt2 and GSK3β remained unaffected (Fig. 3A). Taken together, these results suggest that miR-96 represses the expression of IRS-1 at the translational level by binding directly to the responsive element in *IRS-1* 3′UTR.

3.4. Impaired insulin signaling and glycogen synthesis by miR-96

miR-96 was confirmed to be a translational repressor of IRS-1 expression. Hence, the up-regulation of miR-96 might be involved in the development of impaired insulin signaling. Therefore, the insulin-stimulated phosphorylation of insulin signaling intermediates and glycogen synthesis in the presence or absence of the ectopic expression of miR-96 was next analyzed (Fig. 4). The expression of IRS-1 was reduced significantly by 75% after treatment of miR-96 mimic (Fig. 4A and B), whereas the expression of Akt2 and

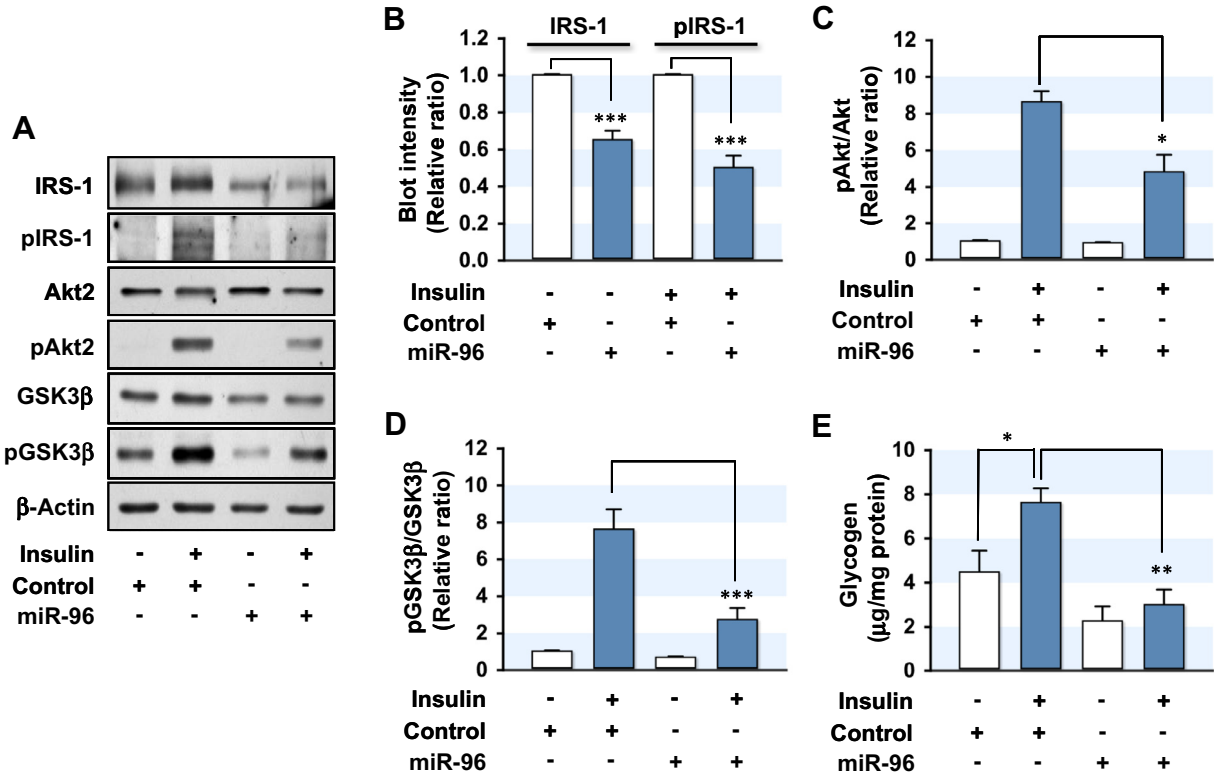

Fig. 4. Impaired insulin signaling and insulin-stimulated glycogen synthesis by the ectopic expression of miR-96. SK-Hep1 cells were transfected with negative control (Control) or miR-96 mimic (miR-96) oligonucleotides. After 72 h of transfection, the cells were preincubated in the presence or absence of insulin (100 nM for 20 min) and the total cell lysates (15 μg protein) were subjected to 8% SDS–PAGE. (A) The expression (IRS-1, Akt2, and GSK3β) and phosphorylation (pIRS-1, pAkt2, and pGSK3β) levels of the insulin signaling intermediates were analyzed by immunoblot. (B) The immunoblot intensities for IRS-1/β-Actin (IRS-1) and insulin-stimulated pIRS-1/β-Actin (pIRS-1) were quantified by densitometry, and are expressed as the relative ratio, where the intensity of the normal control was set to one. (C and D) The immunoblot intensities of pAkt2/Akt2 and pGSK3β/GSK3β were quantified by densitometry, and are expressed as the relative ratio. The intensity of the normal control was set to one. (E) The cellular glycogen contents were analyzed by spectrophotometry (570 nm) with OxiRed probe. The values were expressed as the means ± SEM from at least five independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

GSK3β was unaffected (Fig. 4A). As expected, the ectopic expression of miR-96 reduced significantly the insulin-stimulated phosphorylation of IRS-1 and its downstream kinases, Akt2 and GSK3β in SK-Hep1 cells (Fig. 4A–D). This effect was attributed mainly to a decrease in the expression of IRS-1. In addition, we examined how miR-96 affects the cellular glycogen contents in the presence or absence of insulin (Fig. 4E). In the control cells, insulin increased glycogen contents significantly, whereas the glycogen content in miR-96-overexpressed cells was reduced significantly as compared with the control. Moreover, the ectopic expression of miR-96 abolished insulin-stimulated glycogen synthesis. Therefore, the ectopic expression of miR-96 impairs insulin signaling and glycogen synthesis in SK-Hep1 cells through the translational repression of IRS-1.

4. Discussion

Mitochondrial dysfunction induced by inhibitors of mtDNA replication or mitochondrial electron transport causes insulin resistance in myocytes and hepatocytes through a decrease in IRS-1 [11,22,24]. Although the expression of IRS-1 under these conditions is regulated mainly at the transcriptional level, the post-transcriptional regulation of IRS-1 by miRNAs also plays animportant role [4,25]. In this study, we characterized the expression of miR-96 in mitochondrial dysfunction and its functional relevance to the pathogenesis of insulin resistance. The important finding of this study was that the expression of miR-96 is up-regulated by mitochondrial dysfunction, and miR-96 regulates IRS-1 expression directly by translational repression, and thereby leading to impaired insulin signaling in SK-Hep1 cells.

Previously, we have found that a significant increase in the expression of several miRNAs presumed to targeting IRS-1 under the condition of mitochondrial dysfunction [22]. Among those, miR-96 was up-regulated drastically by mitochondrial dysfunction in SK-Hep1 cells (Fig. 1D). Moreover, our recent result showed that miR-96 was up-regulated more than twofold by mitochondrial dysfunction induced with Rotenone or Antimycin A in L6 myocytes (data not shown). miR-96 belongs to the miR-183 family (miR-96, miR-182 and miR-183), which are transcribed coordinately from a single genetic locus residing on an intergenic region of human chromosome 7q. However, we did not observe any significant increase of miR-182 (1.1-fold) or miR-183 (1.09-fold) in the mtDNA-depleted SK-Hep1 cells. This suggests that there might be a different regulation process of these miRNAs at the post-transcription level. The mechanism involved in the up-regulation of miR-96 by mitochondrial dysfunction remains unclear at this moment. Recently, Biswas et al. reported that stress signaling resulting from mitochondrial dysfunction activates calcineurin A and a number of Ca^{2+} responsive transcription factors including NFAT, NF-κB, C/EBP and HNF4, etc. [26]. Interestingly, PROMO analysis showed that the promoter region of miR-96 has multiple responsive sites for transcription factors, such as NFAT, NF-κB, CEBP and HNF4. Therefore, the up-regulation of miR-96 in mitochondrial dysfunction can be attributed to the activation of transcription factors involved in retrograde signaling from the mitochondria to the nucleus.

The miR-183 family are involved in a range of biological processes, such as cell proliferation [27], migration [28], metastasis [29], and senescence [30]. Moreover, recent studies have shown that miR-96 is up-regulated in various cancers, such as bladder [31], prostate carcinoma [32], and myeloid leukemia [33]. In a recent study, it was reported that miR-96 targets the tumor suppressor gene forkhead box O3 (FOXO3) and FOXO1 by targeting their mRNA 3′UTR directly [27,34]. For this reason, an increase in miR-96 results in the down-regulations of transcriptional factor FOXO3 and FOXO1, and induces cell proliferation in human breast cancer [27,34]. Regarding the endocrine function of miR-96, Lovis et al. demonstrated that miR-96 modulates the expression of proteins involved in insulin exocytosis and affects insulin secretion from pancreatic β-cells [35]. However, the implication of miR-96 with insulin resistance has not been investigated. *In silico* analysis suggested that miR-96 targets several molecules associated with insulin signaling or sensitivity, such as IRS-1, PIK3R1, FOXO1 and VAMP3. IRS-1 is a key component of the insulin signaling cascade, which was observed to be reduced in type 2 diabetes [1]. miR-96 repressed the expression of IRS-1 by targeting *IRS-1* 3′UTR directly (Fig. 2) and the ectopic expression of miR-96 impaired insulin signaling and insulin-stimulated glycogen synthesis by down-regulating the expression of IRS-1 in SK-Hep1 cells (Fig. 4). The discovery that miR-96 regulates insulin signaling provides novel insights into the molecular basis underlying the mitochondrial dysfunction-derived pathogenesis of insulin resistance, and implicates miRNAs in metabolic diseases. Mitochondrial dysfunction generates a range of retrograde signals to the nucleus, which activates several kinases and transcriptional factors [24,26,36]. Certain transcriptional factors affected by this retrograde signaling might activate the production and processing of miR-96, thereby leading to IRS-1 post-transcriptional repression and subsequent insulin resistance. Although further *in vivo* investigation is required, miR-96 could be an important regulator of insulin sensitivity. In conclusion, we have demonstrated that expression of miR-96 is increased by mitochondrial dysfunction in SK Hep-1 cells. We confirmed that miR-96 is a crucial inhibitory factor in insulin signal transduction under conditions of mitochondrial dysfunction. Further analysis of the potential metabolic signals and transcriptional factors involved and how they work in miRNA-mediated insulin resistance will be the subject of future research.

Acknowledgments

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