Up-regulation of *selenoprotein P* and *HIP/PAP* mRNAs in hepatocytes by intermittent hypoxia via down-regulation of miR-203

Tomoko Uchiyama[a,b], Hiroyo Ota[a,c], Asako Itaya-Hironaka[a], Ryogo Shobatake[a], Akiyo Yamauchi[a], Sumiyo Sakuramoto-Tsuchida[a], Mai Makino[a], Hiroshi Kimura[c], Maiko Takeda[b], Chiho Ohbayashi[b], Shin Takasawa[a,*]

[a] *Department of Biochemistry, Nara Medical University, Kashihara 634-8521, Japan*
[b] *Department of Diagnostic Pathology, Nara Medical University, Kashihara 634-8522, Japan*
[c] *Second Department of Internal Medicine, Nara Medical University, 840 Shijo-cho, Kashihara 634-8522, Japan*

A B S T R A C T

Sleep apnea syndrome is characterized by recurrent episodes of oxygen desaturation and reoxygenation (intermittent hypoxia [IH]) and is a risk factor for insulin resistance/type 2 diabetes. However, the mechanisms linking IH stress and insulin resistance remain elusive. We exposed human hepatocytes (JHH5, JHH7, and HepG2) to experimental IH or normoxia for 24 h, measured mRNA levels by real-time reverse transcription polymerase chain reaction (RT-PCR), and found that IH significantly increased the mRNA levels of *selenoprotein P* (*SELENOP*) — a hepatokine — and *hepatocarcinoma-intestine-pancreas/pancreatitis-associated protein* (*HIP/PAP*) — one of *REG* (Regenerating gene) family. We next investigated promoter activities of both genes and discovered that they were not increased by IH. On the other hand, a target mRNA search of micro RNA (miRNA) revealed that both mRNAs have a potential target sequence for miR-203. The miR-203 level of IH-treated cells was significantly lower than that of normoxia-treated cells. Thus, we introduced miR-203 inhibitor and a non-specific control RNA (miR-203 inhibitor NC) into HepG2 cells and measured the mRNA levels of *SELENOP* and *HIP/PAP*. The IH-induced expression of *SELENOP* and *HIP/PAP* was abolished by the introduction of miR-203 inhibitor but not by miR-203 inhibitor NC. These results demonstrate that IH stress up-regulates the levels of *SELENOP* in human hepatocytes to accelerate insulin resistance and up-regulates the levels of *HIP/PAP* mRNAs to proliferate such hepatocytes, via the miR-203 mediated mechanism.

1. Introduction

Sleep apnea syndrome (SAS) is characterized by the narrowing or collapse of the upper airway during sleep that leads to a cessation of airflow. Apnea and hypopnea are often accompanied by a drop in oxygen saturation. Accumulating evidence suggests that recurrent episodes of oxygen desaturation and reoxygenation (intermittent hypoxia [IH]), which are typical features of SAS, contribute to the development of β cell dysfunction and impaired glucose tolerance [1].

Epidemiological and clinical evidence postulates that SAS may be a causal factor of type 2 diabetes. The increasing severity of SAS is associated with worsening insulin resistance [2,3]. The recent report by

Priou et al. suggested that an increase in the severity of SAS may worsen glucose control in patients with asymptomatic, untreated, or early stages of type 2 diabetes [4]. Nocturnal IH is associated with an increased risk of type 2 diabetes among community-dwelling Japanese people independent of its traditional risk factors such as age, sex, and body habitus [5]. However, the mechanisms by which IH induces insulin resistance in SAS patients are not well established.

Recently, several proteins that are exclusively or predominantly secreted from the liver, called hepatokines, were established as directly affecting glucose and lipid metabolism [6,7]. For example, fibroblast growth factor 21 (FGF21) has recently emerged as a novel hormone, leading to beneficial effects on glucose metabolism and lipid

homeostasis [8], while selenoprotein P is correlated positively with insulin resistance and could be a therapeutic target for type 2 diabetes [9]. The changes of these hepatokine expressions in hepatocytes by IH remain elusive.

The regenerating gene (*Reg*) was identified in regenerating islets [10,11] and a *Reg* gene product — Reg protein — acts as a growth factor and promotes cell proliferation and regeneration [11–13]. In humans, five functional *Reg* family genes (*REG Iα, REG Iβ, REG III, hepatocarcinoma-intestine-pancreas/pancreatitis-associated protein* [*HIP/PAP*] and *REG IV*) have been isolated. For several cells, *Reg* family proteins have been suggested to be involved in cellular proliferation [11]. We have reported that IH stress stimulates pancreatic β cell proliferation via up-regulation of *Reg* family mRNAs and may cause hyperinsulinemia, which makes patients more obese [1,14]. However, the direct effects of IH on hepatocyte proliferation and the IH-induced changes in *Reg* family gene expression in hepatocytes remain unknown.

In the present study, we investigated the changes of gene expression in hepatokines and *Reg* family genes, as well as their regulation mechanisms in response to IH stress in hepatocytes.

2. Materials and methods

2.1. Cell culture

Rat H4IIE hepatocytes were grown in DMEM medium (Wako Pure Chemical Industries, Ltd., Osaka, Japan) containing 10% (v/v) fetal calf serum (FCS), 100 units/mL penicillin G (Wako) and 100 μg/mL streptomycin (Wako) as described [15]. Human hepatocarcinoma JHH5 cells and JHH7 cells were purchased from Japanese Collection of Research Bioresources (Sennan, Japan) and human hepatocarcinoma HepG2 cells were purchased from RIKEN BRC CELL BANK (Tsukuba, Japan). JHH5 and JHH7 cells were grown in William's E medium (Sigma, St. Louis, MO) containing 10% (v/v) FCS, 100 units/mL penicillin G (Wako), and 100 μg/mL streptomycin (Wako), and HepG2 cells were grown in DMEM medium containing 10% (v/v) FCS, 100 units/mL penicillin G, and 100 μg/mL streptomycin as described [16]. Cells were exposed to either normoxia (21% O_2, 5% CO_2, and balance N_2) or intermittent hypoxia (IH: 64 cycles of 5 min sustained hypoxia [1% O_2, 5% CO_2, and balanced N_2] and 10 min normoxia) using a custom-designed, computer-controlled incubation chamber attached to an external O_2-CO_2-N_2 computer-driven controller (O_2 programmable control, 9200EX, Wakenyaku CO., Ltd, Kyoto, Japan), as described [1,16]. These conditions are similar to the conditions reported in patients with severe degree of SAS — in severe cases of SAS, patients are repeatedly exposed to severe hypoxemia followed by mild hypoxemia or normoxia (i.e., IH). We previously reported that the magnitude of IH expressed by SpO_2 fluctuated between 75–98% and 50–80% in SAS [1,17], which was almost equivalent to the medium condition in the present study.

2.2. Real-time reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated using a RNA protect cell mini kit (Qiagen, Hilden, Germany) from JHH5, JHH7, HepG2, and H4IIE cells, and cDNA was synthesized from total RNA as template using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) as described [14,16,18–20]. Real-time polymerase chain reaction (PCR) was performed using SYBR® Fast qPCR kit (KAPA Biosystems, Boston, MA) and a Thermal Cycler Dice Real Time System (Takara, Kusatsu, Japan). All the PCR primers were synthesized by Nihon Gene Research Laboratories, Inc. (NGRL; Sendai, Japan), and the primer sequences for each primer set are described in Table 1. PCR was performed with an initial step of 3 min at 95 °C followed by 40 cycles of 3 s at 95 °C and 20 s at 60 °C for *β-actin, rat insulinoma gene (Rig)/ribosomal protein S15 (RpS15), REG III,* and *HIP/PAP*, 40 cycles of 3 s at 95 °C and 20 s at 64 °C for *REG Iα, REG Iβ*, and *REG IV,* 45 cycles of 3 s at 95 °C and 20 s at 60 °C for *α2 HS-glycoprotein (AHSG), angiopoietin-*

Table 1
Primers used for real-time RT-PCR.

Target mRNA/miR	Primer sequence
Rat *Selenop* (NM_001082911)	5′-GACAGTGGTTGCTCTTCTTCAA-3′
	5′-TCGCAGGTCTTCCAATCTG-3′
Rat *Rig/RpS15* (NM_017151)	5′-ACGGCAAGACCTTCAACCAG-3′
	5′-ATGGAGAACTCGCCCAGGTAG-3′
Human *SELENOP* (NM_001093726)	5′-TCATCAAGGAATCTCTTCTCG-3′
	5′-CAAGACGGCCACATCTATCA-3′
Human *ANGPTL6* (NM_031917)	5′-CTGTGGTTCCGGTCCGTCTT-3′
	5′-GCTGCTCACACCTACTGACACT-3′
Human *SHBG* (NM_001040)	5′-TCAATCTCCGAGACATTCCC-3′
	5′-TGGTGTCCCAAGAGCAAG-3′
Human *LIPASIN* (NM_018687)	5′-GGCCGCACAATAGAACTCC-3′
	5′-CAGCGTGAGCCTTTAAGACC-3′
Human *LECT2* (NM_002302)	5′-GTGTTCGAATATCTGGAAGAGGT-3′
	5′-AAGGGCAATAGAGTTCCAAGT-3′
Human *FGF21* (NM_019113)	5′-ACCTGGAGATCAGGGAGGAT-3′
	5′-AGTGGAGCGATCCATACAGG-3′
Human *AHSG* (NM_001622)	5′-CCCCGGAAAACACGCACA-3′
	5′-GTGCCAAACCTCCTCATCTCT-3′
Human *REG Iα* (NM_002909)	5′-AGGAGAGTGGCACTGATGACTT-3′
	5′-TAGGAGACCAGGGACCCACTG-3′
Human *REG Iβ* (NM_006507)	5′-GCTGATCTCCTCCCTGATGTTC-3′
	5′-GGCAGCTGATTCGGGGATTA-3′
Human *REG III* (AB161037)	5′-GAATATTCTCCCCAAACTG-3′
	5′-GAGAAAAGCCTGAAATGAAG-3′
Human *HIP/PAP* (NM_138937)	5′-AGAGAATATTCGCTTAATTCC-3′
	5′-AATGAAGAGACTGAAATGACA-3′
Human *REG IV* (AY007243)	5′-ATCCTGGTCTGGCAAGTC-3′
	5′-CGTTGCTGCTCCAAGTTA-3′
Human *SOCS3* (NM_003955)	5′-CCACTCTTCAGCATCTCTGT-3′
	5′-ATCGTACTGGTCCAGGAACT-3′
Human *TP63* (NM_003722)	5′-TGTATCCGCATGCAGGACT-3′
	5′-CTGTGTTATAGGGACTGGTGGAC-3′
Human *DICER* (NM_177438)	5′-GAGCTGTCCTATCAGATCAGGG-3′
	5′-ACTTGTTGAGCAACCTGGTTT-3′
Human *DROSHA* (NM_013235)	5′-GGCCCGAGAGCCTTTTATAG-3′
	5′-TGCACACGTCTAACTCTTCCAC-3′
Human *MCPIP1* (NM_025079)	5′-TGCCTATCACAGACCAGCAC-3′
	5′-CTCACCTTCGCGAAGTAGCTC-3′
Human *β-actin* (NM_001101)	5′-GCGAGAAGATGACCCAGA-3′
	5′-CAGAGGCGTACAGGGATA-3′
Human *miR-203* (NR_029620)	5′-GCCGGTGAAATGTTTAGGAC-3′
	5′-GTGCAGGGTCCGAGGT-3′
Human *U6* (NR_004394)	5′-CTCGCTTCGGCAGCACA-3′
	5′-AACGCTTCACGAATTTGCGT-3′

related growth factor 6 (*ANGPTL6*), *FGF21, leukocyte cell-derived chemotaxin 2* (*LECT2*), *LIPASIN, sex hormone-binding globulin* (*SHBG*), *Selenoprotein P* (*SELENOP*), *ribonuclease type III* (*DROSHA*), *endoribonuclease Dicer* (*DICER*), *monocyte chemotactic protein-induced protein 1* (*MCPIP1*), *transformation-related protein p63* (*TP63*), and *miR-203*. The mRNA expression levels were normalized to the mRNA level of *Rig/RpS15* in rat samples or *β-actin* in human samples, and the *miR-203* level was normalized to the *U6* RNA level.

2.3. Measurement of Sepp1 in culture medium by enzyme-linked immunosorbent assay (ELISA)

Cells were exposed either normoxia or IH for 24 h, culture medium was collected, and the concentration of Sepp1 was measured by using a Human Selenoprotein P (SELENOP) ELISA kit (Cusabio, Wuhan, China) for human cells and Rat Selenoprotein P (Selenop) ELISA kit (Cusabio) according to the instructions of supplier.

2.4. Measurement of viable cell numbers by tetrazolium salt cleavage

HepG2 cells (2.5×10^4 cells/100 μL in 96-well plate) were incubated at 37 °C over night and the medium was replaced with DMEM +10% FCS just before normoxia/IH exposure. After a 24-h treatment of normoxia or IH, the viable cell numbers were determined by a Cell
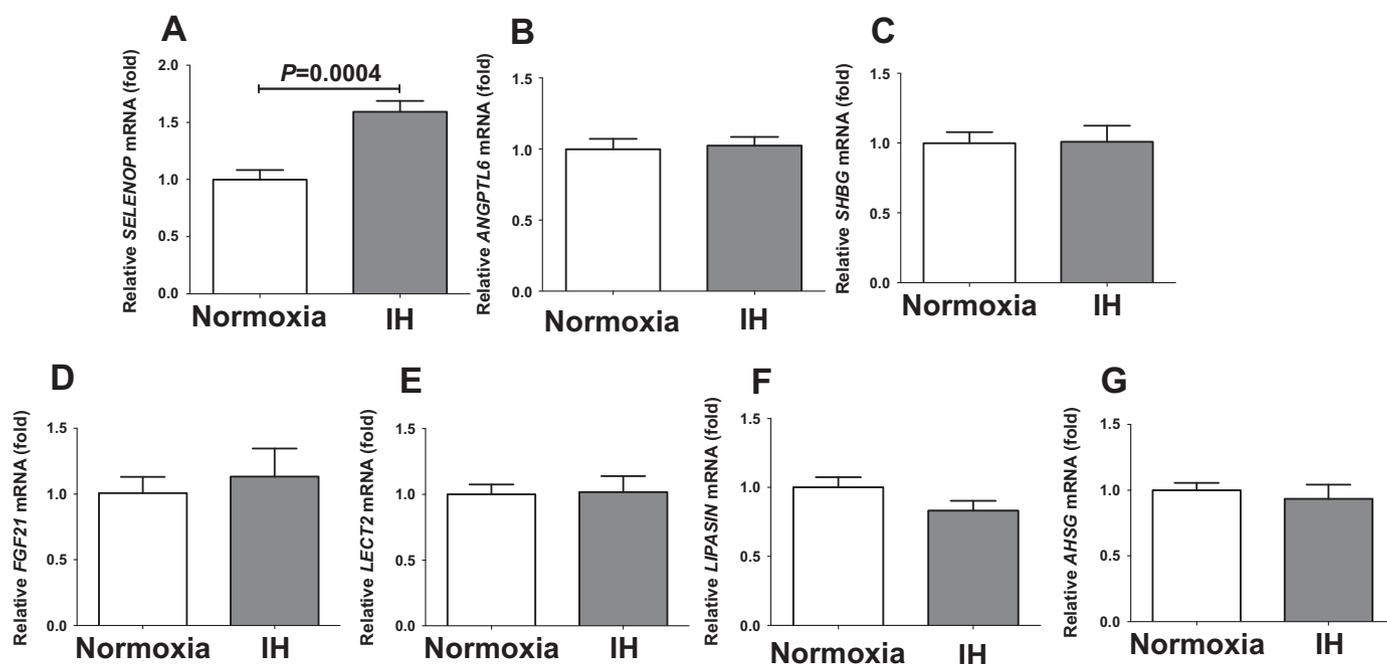
Fig. 1. The mRNA levels of *SELENOP* (A), *ANGPTL6* (B), *SHBG* (C), *FGF21* (D), *LECT2* (E), *LIPASIN* (F), and *AHSG* (G) in HepG2 cells treated by normoxia or IH for 24 h. The levels of the hepatokine mRNAs were measured by real-time RT-PCR using *β-actin* as an endogenous control. Data is expressed as means ± SE for each group (n = 4). The statistical analyses were performed using Student's *t*-test.

Counting kit-8 (Dojindo Laboratories, Mashiki-machi, Japan) according to the manufacturer's instructions. Briefly, WST-8 (2-(2-methoxy-4-nitrophenyl) − 3-(4-nitrophenyl) − 5-(2,4-disulfophenyl) − 2H-tetrazolium monosodium salt) solution was added to cells in 96-well plates, and the cells were incubated at 37 °C for 30 min. The optical density of each well was read at 450 nm (reference wave length at 650 nm) using a Sunrise™ microplate reader (Tecan, Männedorf, Switzerland), as described [14,20].

2.5. RNA interference

Small interfering RNA (siRNA) directed against human *HIP/PAP* and *REG Iα* were synthesized by NGRL. The sense sequences of siRNA for human *HIP/PAP* and *REG Iα* were 5'-GUGAAGAGCAUUGGUAACAGCUAtt-3' (corresponding 335–357 of NM_002580) and 5'-GCAAUUACAACGGAGUCAAtt-3' (corresponding 640–658 of NM_002909), respectively. The Silencer® Select human scrambled siRNA was purchased from Ambion® and used as a control. Transfection of siRNAs into HepG2 cells was carried out using Lipofectamine® RNAiMAX Transfection Reagent (Life Technologies). Cells were transfected with 1 pmol each of siRNA in a 96-well culture dish as described [14,18–20], and viable cell numbers were analyzed by WST-8 assay as described above.

2.6. Construction of reporter plasmid and luciferase assay

Reporter plasmids were prepared by inserting the promoter fragments of human *SELENOP* (−2989~+10) and *HIP/PAP* (−4030~ +27) upstream of a firefly luciferase reporter gene in the pGL4.17 vector (Promega, Madison, WI). The reporter plasmids were transfected into human HepG2 hepatocytes using Lipofectamine® 3000 (Invitrogen), as described [19,20], and the cells were exposed to either 64 cycles/24 h of IH, mimicking hepatocytes of SAS patients, or normoxia for 24 h. After cells were exposed to IH, cells were lysed and promoter activities were measured. The cells were harvested and cell extracts were prepared in Extraction Buffer (0.1 M potassium phosphate, pH 7.8/0.2% Triton X-100; Life Technologies). To monitor transfection efficiency, pCMV-SPORT-βgal plasmid (Life Technologies)

was co-transfected in all experiments at a 1:10 dilution. Luciferase activity was measured using a PicaGene luciferase assay system (Toyo-ink, Tokyo, Japan) and was normalized by the β-galactosidase activity as described previously [15,16,18–23].

2.7. MiRNA extraction, reverse transcription, and real-time quantitative PCR

Total RNA including miRNA was isolated from HepG2 cells using the miRNeasy mini kit (Qiagen) according to the manufacturer's instructions. An equal amount of DNase-treated RNA was Poly-A tailed using a Mir-X™ miRNA first strand synthesis kit (Clonetech) according to the manufacturer's protocol. The condition for PCR was 95 °C for 10 s, followed by 45 cycles of amplification (95 °C, 5 s, 60 °C, 20 s). U6 small nuclear RNA was used as an endogenous control for miRNA. The primers are listed in Table 1.

2.8. MiR-203 inhibitor transfection

MiR-203 inhibitor (5′-CUAGUGGUCCUAAACAUUUCAC-3′) and non-specific control RNA (miR-203 inhibitor NC) (5′-CAGUACUUUUGUGUAGUACAA-3′) were synthesized by NGRL and introduced into HepG2 cells using Lipofectamine® RNAiMAX (Invitrogen) [18–20] just before IH/normoxia exposure, and the mRNA levels of *SELENOP* and *HIP/PAP* were measured by real-time RT-PCR, as described [14,16,18–20].

2.9. Data analysis

Results are expressed as mean ± SE. Statistical significance was determined by Student's *t*-test using GraphPad Prism software (GraphPad Software, La Jolla, CA).

3. Results

3.1. SEPP1 gene expression was increased by IH

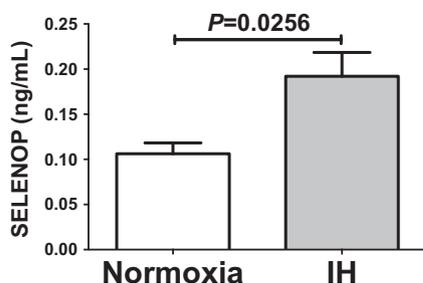Hepatokines, secretory proteins mainly produced by the liver, were

Fig. 2. Concentrations of SELENOP in HepG2 cell culture medium were measured by ELISA. HepG2 cells were treated by normoxia or IH conditions for 24 h. Data is expressed as means ± SE for each group (n = 4).

reported to correlate with glucose homeostasis and insulin resistance [6,7]. We analyzed the mRNA levels of several hepatokines affecting glucose metabolism (*SELENOP, ANGPTL6, SHBG, FGF21, LIPASIN, LECT2,* and *AHSG*) by real-time RT-PCR in IH and normoxia-treated hepatocytes. The mRNA levels of *Selenop* were significantly increased by IH in all the analyzed hepatocytes: HepG2 ($P = 0.0004$; Fig. 1), JHH5 ($P = 0.0477$; Supplementary Fig. 1), JHH7 ($P = 0.0027$; Supplemental Fig. 2), and H4IIE ($P = 0.0057$; Supplementary Fig. 3). However, there was no hepatokine other than *Selenop* that increased in all the four tested cells in response to IH stimulation (Fig. 1 and Supplementary Figs. 1–3). These results indicate that IH stress specifically up-regulates expression of *SELENOP* in hepatocytes.

We further measured Selenop protein in the culture medium by ELISA and found that the levels of Selenop were significantly increased by IH in all the analyzed hepatocytes: HepG2 ($P = 0.0256$; Fig. 2), JHH5 ($P = 0.0370$; Supplementary Fig. 4 A), JHH7 ($P = 0.0434$; Supplementary Fig. 4B), and H4IIE ($P = 0.048$; Supplementary Fig. 4 C).

3.2. HIP/PAP gene expression was increased by IH

The *Reg* gene was originally identified in regenerating pancreatic islets, and the *Reg* gene and its related genes (*Reg* family genes) were revealed to encode growth factors for several cells [10–12]. We have reported that IH stress induced β cell proliferation via up-regulation of *Reg* family genes [1,14]. In this study, we analyzed the changes of *REG* family gene expression in human hepatocytes by IH. The mRNA levels of *HIP/PAP* were significantly increased in IH-treated human hepatocytes: HepG2 ($P = 0.0226$: Fig. 3), JHH5 ($P = 0.0022$: Supplementary Fig. 5), and JHH7 ($P = 0.0016$: Supplementary Fig. 6). Meanwhile, mRNA levels of the other *REG* family members (*REG Iα, REG Iβ, REG III,* and *REG IV*) were not increased (Fig. 3). Except for *REG IV* in JHH5 (Supplementary Fig. 5), the mRNA levels of other *REG* family genes were not increased by IH (Supplementary Figs. 5 and 6).

3.3. HIP/PAP acts as an autocrine/paracrine growth factor for hepatocytes in IH condition

In order to evaluate direct effects of IH on hepatocyte proliferation, HepG2 cells were exposed to normoxia or IH for 24 h. After the treatment, cell viability was determined by WST-8 assay. HepG2 cell proliferation was significantly increased by IH (Fig. 4). Furthermore, RNA interference of *HIP/PAP* inhibited HepG2 cell proliferation measured by WST-8 assay, whereas interference of *REG Iα* did not (Fig. 5). These results indicate that HIP/PAP, up-regulated by IH, works as an autocrine/paracrine growth factor in hepatocyte proliferation.

3.4. The promoter activities of SELENOP and HIP/PAP were not increased by IH

To determine whether the IH-induced increases in *SELENOP* and *HIP/PAP* mRNA were caused by activation of transcription, a 2999 bp

fragment containing 2989 bp of the *SELENOP* promoter and a 4057 bp fragment containing 4030 bp of *HIP/PAP* promoter were fused to the luciferase gene of pGL4.17 and transfected into HepG2 cells. After IH stimulation, we measured promoter activities and found that both *SELENOP* and *HIP/PAP* promoter activities actually decreased due to IH in HepG2 cells (Fig. 6). In addition, promoter activities of *SELENOP* and *HIP/PAP* did not increased due to IH in JHH5 and JHH7 cells (Supplementary Fig. 7). These results strongly suggest that the gene expression of both *SELENOP* and *HIP/PAP* in response to IH was not regulated by transcription.

3.5. The miR-203 level was significantly decreased by IH

As the IH-induced up-regulation of *SELENOP* and *HIP/PAP* was considered to be regulated post-transcriptionally, we searched targeted miRNA using the MicroRNA.org program (http://www.microrna.org/microrna/home.do), which revealed that both *SELENOP* and *HIP/PAP* mRNAs have a potential target sequence for miR-203. There were no other miRNA candidates targeting both genes. There are some controversial reports concerning occurrence of miR-203 in mammalian liver cells: Sonkoly et al. reported miR-203 was abundant in skin but scarcely detected in liver [24]. Szele et al. reported miR-203 occurrence in liver was 0.614–0.616 using 5 S RNA as an internal control [25]. We measured the miR-203 level of IH-treated cells by RT-PCR and found that the level was significantly lower than that of normoxia-treated cells (0.0270 ± 0.02058 fold vs normoxia, $P = 0.0391$). There are two possibilities as to why the level of miR-203 was decreased by IH. One possibility is that IH influences mRNA levels of some enzymes for miRNA biosynthesis/degradation. Another is that the level of miR-203 was specifically decreased by IH either via decreased biosynthesis or enhanced degradation in response to IH. We measured the mRNA levels of *DROSHA, DICER, MCPIP1,* and *TP63*, which are involved in the biosynthesis and degradation of miRNAs [26–28] and found that their expression was unchanged by IH (Fig. 7). These results suggest that miR-203 plays a key role in post-transcriptional regulation of mRNA levels of *SELENOP* and *HIP/PAP*. To investigate whether *SELENOP* and *HIP/PAP* expression in IH is regulated by miR-203, miR-203 inhibitor and non-specific control RNA (miR-203 inhibitor NC) were introduced into HepG2 cells just before IH/normoxia exposure, and the mRNA levels of *SELENOP* and *HIP/PAP* were measured by real-time RT-PCR. As shown in Fig. 8, the IH-induced increases in *SELENOP* and *HIP/PAP* mRNAs were abolished by the introduction of miR-203 inhibitor but not by miR-203 inhibitor NC. We also introduced miR-203 mimic into HepG2 cells, exposed the cells with IH, measured mRNA levels, and found that the IH-induced increases in *SELENOP* and *HIP/PAP* mRNAs were abolished by the introduction of miR-203 mimic but not by non-specific control RNA (miR-203 mimic NC) (Supplementary Fig. 8). These findings indicate that IH stress down-regulates the miR-203 level in human hepatocytes and that the levels of *SELENOP* and *HIP/PAP* mRNAs are increased via the miR-203 mediated mechanism.

4. Discussion

Recent epidemiological research demonstrates that SAS may be associated with hypertension, dyslipidemia, cardiovascular diseases and insulin resistance, suggesting that SAS patients may be susceptible to various metabolic dysfunctions. In particular, SAS is closely associated with type 2 diabetes, independent of obesity and family history [2,29]. Kendzerska et al. reported that the initial SAS severity predicted risk for diabetes [3] and Punjabi and Beamer reported that mild, moderate, and severe SAS patients displayed a 26.7%, 36.5%, and 43.7% reduction in insulin sensitivity, respectively, after adjusting for age, sex, race, and body fat percentage [29]. Moreover, the intervention with continuous positive airway pressure for SAS treatment could improve not only SAS itself but also diabetes [2,30,31].

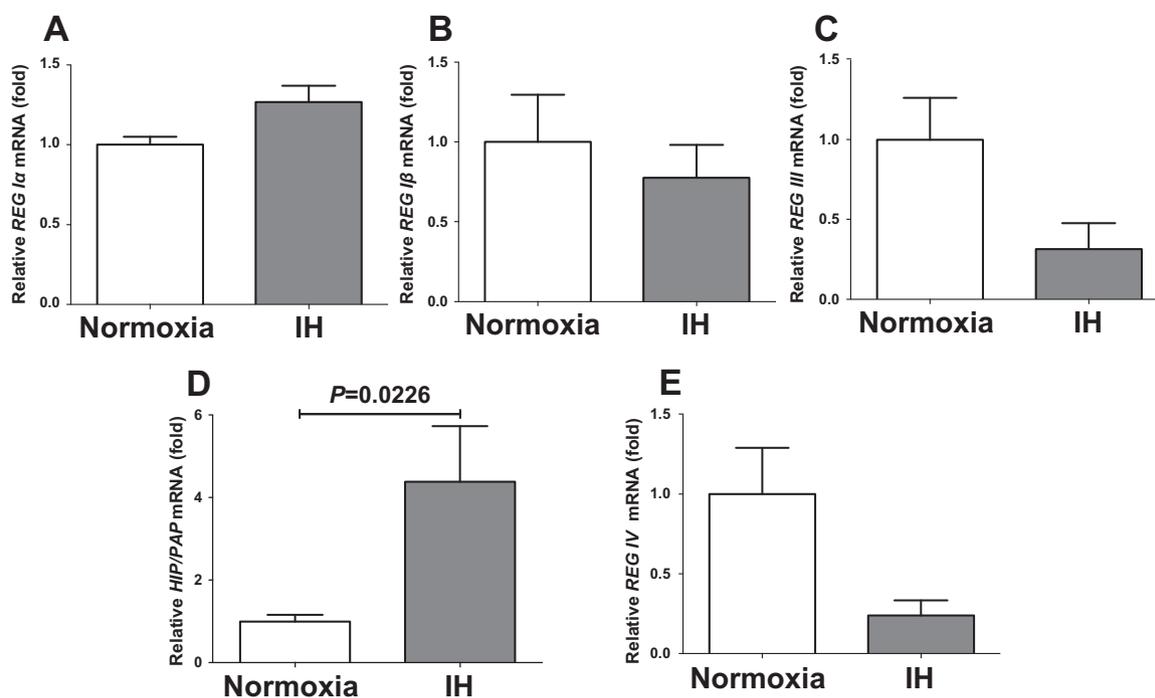The repeated hypoxia and reoxygenation cycles, IH, which are

Fig. 3. The mRNA levels of *REG Iα* (A), *REG Iβ* (B), *REG III* (C), *HIP/PAP* (D), and *REG IV* (E) in HepG2 cells treated by normoxia or IH for 24 h were measured by real-time RT-PCR. Data is expressed as means ± SE for each group (n = 4). The statistical analyses were performed using Student's *t*-test.
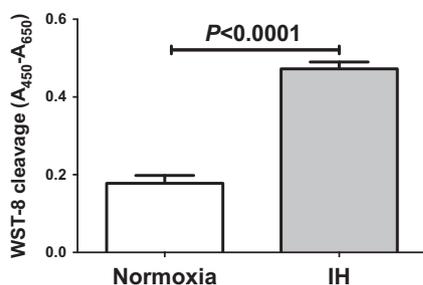

Fig. 4. Effects of IH on cellular proliferation of HepG2 cells. They were exposed to normoxia or IH conditions for 24 h, and cellular proliferation was measured by WST-8 assay. Data is expressed as means ± SE for each group (n = 12). The statistical analyses were performed using Student's *t*-test.

induced by upper airway collapse, are a characteristic feature of SAS patients. Recent studies revealed that the nocturnal IH is correlated with an increased risk of developing type 2 diabetes [5]. Although the mechanisms linking IH stress to insulin resistance remain to be elusive, several explanations have been proposed, such as activating the sympathetic nervous system, inducing the release of catecholamines, producing reactive oxygen species, and activating inflammatory

pathways [2,31–33]. Hyperglycemia in type 2 diabetes patients is the result of impaired insulin secretion and increased insulin resistance in target tissues — liver, skeletal muscle, adipose tissue, etc. Recently, like adipose tissue and skeletal muscle, the liver is known to affect glucose metabolism by releasing proteins into circulation, which are termed hepatokines [6]. In the present study, to investigate the direct effect of IH on the expression of hepatokines in hepatocytes, we measured several mRNAs for hepatokine (*ANGPTL6*, *SHBG*, *FGF21*, *LIPASIN*, *LECT2*, *AHSG*, and *SELENOP*) that were reported to play critical roles in glucose homeostasis [8,34–38]. We found that the *SELENOP* mRNA level was significantly increased in all the hepatocytes, whereas the other hepatokines were not (Fig. 1, Supplementary Figs. 1–3). Furthermore, the concentrations of selenoprotein P in all the hepatocyte culture media were significantly increased by IH. Selenoprotein P was first characterized to contain selenium as selenocysteine, and it plays a central role in selenium homeostasis [39]. Recently, Misu et al. revealed that the serum levels of selenoprotein P and gene expression of *SELENOP* in human liver were increased in type 2 diabetes patients [6] and that *Selenop* knockout mice showed increases responsiveness to insulin/exercise [9]. Our results indicated that IH stress up-regulates the mRNA levels of *selenoprotein P* in hepatocytes, suggesting the possible contribution of selenoprotein P in SAS patients to aggravate insulin
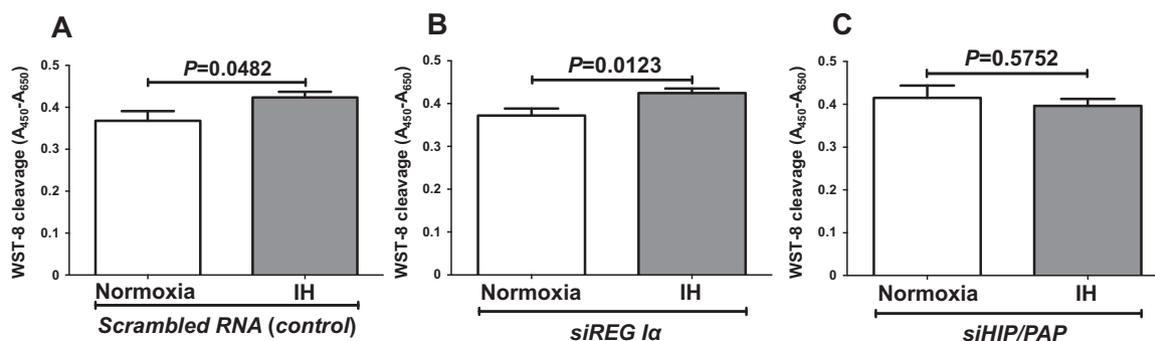

Fig. 5. Effects of siRNA transfection on cell proliferation. After transfection of siRNA against *REG Iα* or *HIP/PAP* into HepG2 cells, cells were exposed to normoxia or IH conditions for 24 h, and cellular proliferation was measured by WST-8 assay. Data is expressed as means ± SE for each group (n = 12). The statistical analyses were performed using Student's *t*-test.
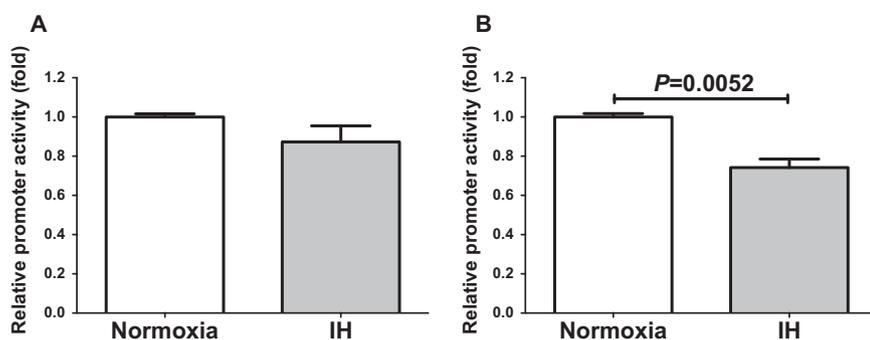
Fig. 6. Luciferase assays in HepG2 cells. Reporter plasmids prepared by inserting the promoter fragments of *SELENOP* ($-2989\sim +10$) and *HIP/PAP* ($-4030\sim +27$) upstream of a firefly luciferase reporter gene in the pGL4.17 vector were transfected into HepG2 cells. After cells were exposed to 24 h of either IH or normoxia, the cells were lysed, and promoter activities of *SELENOP* (A) and *HIP/PAP* (B) were measured. All data is represented as the mean ± SE of the samples (n = 3). The statistical analyses were performed using Student's *t*-test.

resistance.

HIP/PAP is originally found as a gene overexpressed in liver carcinoma [40] and the pancreas during the acute phase of pancreatitis [41]. Recent *HIP/PAP* transgenic/knockout mouse experiments [42–44] revealed that it is an autocrine/paracrine mitogenic factor, accelerating liver regeneration. HIP/PAP promotes cell proliferation in hepatocytes by protecting them from apoptosis and acting as a mitogenic factor [42,44]. Based on the primary structure of encoded protein, *HIP/PAP* is considered to be a member of the *REG* family gene [11]. We evaluated cellular proliferation by WST-8 assay and found that HepG2 cell proliferation was significantly increased by IH ($P < 0.0001$) compared to that by normoxia (Fig. 4). Furthermore, we measured the mRNA levels of all the *REG* family genes (*REG Iα, REG Iβ, REG III, HIP/PAP* and *REG IV*) and found that *HIP/PAP* was significantly increased by IH (Fig. 3, Supplementary Fig. 5). This result suggests that IH stress up-regulates the mRNA levels of *HIP/PAP* in human hepatocytes and may stimulate the proliferation of liver cells with high levels of *SELENOP* mRNA. In fact, the knockdown experiment using siRNA against *HIP/PAP* mRNA showed that HIP/PAP acts as an autocrine/paracrine growth factor for hepatocytes stimulated by IH (Fig. 5). Recently, putative links between SAS and increased cancer incidence were reported [45,46], suggesting possible involvement of up-regulation of HIP/PAP as a growth factor for cancer cells. In addition, HIP/PAP has recently been recognized as an obesogenic factor [47] in addition to a growth factor. It may be possible that HIP/PAP in addition to SELENOP is involved in insulin resistance via obesity.

Next, we investigated the mechanisms by which IH up-regulates the mRNA levels of *SELENOP* and *HIP/PAP* and found that the promoter activities of *SELENOP* and *HIP/PAP* were not increased by IH (Fig. 6, Supplementary Fig. 7). This suggests that IH-induced up-regulation of *SELENOP* and *HIP/PAP* mRNAs (Figs. 1 and 3, Supplementary Figs. 1–3, 5 and 6) was regulated by post-transcriptional step. MiRNAs are short non-coding RNAs that modulate gene expression via post-transcriptional regulation in many cellular processes [48]. They promote RNA degradation and inhibit transcription by binding to mRNA and affecting its stability, resulting in the regulation of the amount of mRNAs. Some miRNAs have specific individual targets, while others regulate hundreds of mRNA levels simultaneously [49,50]. The role of miRNA in the regulation of numerous biological processes (angiogenesis, apoptosis, cell proliferation, migration, etc.) for various types of cancer has been well-established. Additionally, accumulating evidence indicates the importance of the roles of miRNAs in metabolism, such as regulating cholesterol, lipid metabolism, and controlling insulin signaling [51–54]. In the current study, miR-203 with common target sequence in *SELENOP* and *HIP/PAP* mRNAs could contribute to worsening glucose intolerance in IH-conditions by up-regulation *SELENOP* and *HIP/PAP* mRNA.

In conclusion, the present study revealed that the gene expression of *SELENOP* and *HIP/PAP* were increased via down-regulation of miR-203 level in IH-treated hepatocytes. In SAS patients, it is suggested that the up-regulation of SELENOP plays a role in worsening insulin resistance. In addition, overexpression of HIP/PAP could proliferate such hepatocytes in SAS patients, leading to decreased insulin sensitivity and much more.
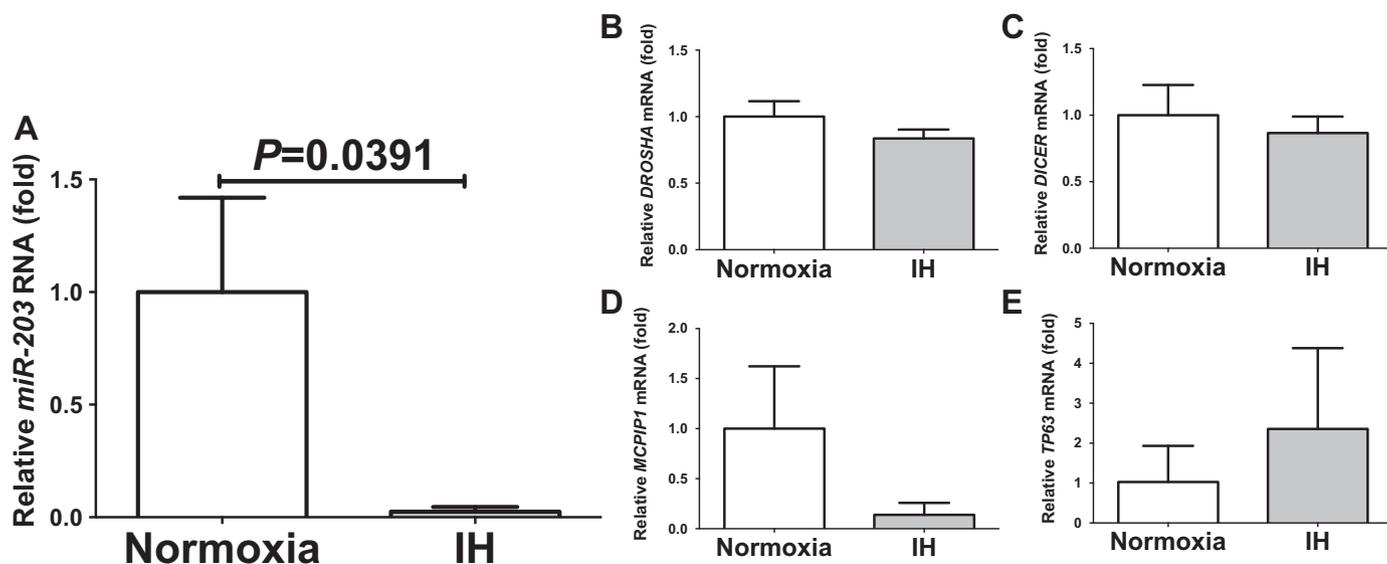

Fig. 7. The mRNA levels of *miR-203* (A), *DROSHA* (B), *DICER* (C), *MCPIP1* (D), and *TP63* (E) in HepG2 cells treated by normoxia or IH for 24 h. The levels of mRNAs were measured by real-time RT-PCR using *β-actin* as an endogenous control. Data is expressed as means ± SE for each group (n = 4). The statistical analyses were performed using Student's *t*-test.
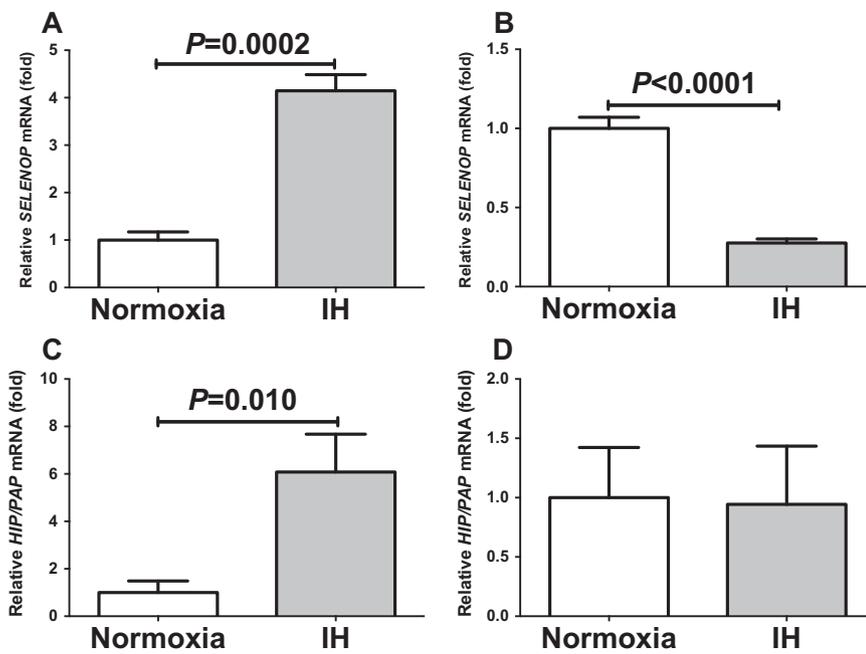
Fig. 8. Effects of miR-203 inhibitor transfection on *SELENOP* and *HIP/PAP* expression. The miR-203 inhibitor and non-specific control RNA (miR-203 inhibitor NC) were introduced into HepG2 cells just before normoxia or IH exposure. The expression of *SELENOP* and *HIP/PAP* mRNA was measured by real-time RT-PCR using *β-actin* as an endogenous control. The figure represents (A) *SELENOP* mRNA expression in miR-203 inhibitor NC-introduced cells, (B) *SELENOP* mRNA expression in miR-203 inhibitor-introduced cells, (C) *HIP/PAP* mRNA expression in miR-203 inhibitor NC-introduced cells and (D) *HIP/PAP* mRNA expression in miR-203 inhibitor-introduced cells. Data is expressed as means ± SE for each group (n = 4). The statistical analyses were performed using Student's *t*-test.

Acknowledgements

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2017.07.005.

References

[1] H. Ota, S. Takasawa, M. Yamauchi, M. Yoshikawa, K. Tomoda, H. Kimura, Intermittent hypoxia in pancreatic beta cells, Pancreat. Disord. Ther. 5 (2015) S5–004.

[2] E. Tasali, M.S.M. Ip, Obstructive sleep apnea and metabolic syndrome: alterations in glucose metabolism and inflammation, Proc. Am. Thorac. Soc. 5 (2008) 207–217.

[3] T. Kendzerska, A.S. Gershon, G. Hawker, G. Tomlinson, R.S. Leung, Obstructive sleep apnea and incident diabetes. A historical cohort study, Am. J. Respir. Crit. Care Med. 190 (2014) 218–225.

[4] P. Priou, M. Le Vaillant, N. Meslier, S. Chollet, T. Pigeanne, P. Masson, A. Bizieux-Thaminy, M.P. Humeau, F. Goupil, P.H. Ducluzeau, F. Gagnadoux, The IRSR sleep control group, association between obstructive sleep apnea severity and glucose control in patients with untreated versus treated diabetes, J. Sleep Res. 24 (2015) 425–431.

[5] I. Muraki, T. Tanigawa, K. Yamagishi, S. Sakurai, T. Ohira, H. Imano, A. Kitamura, M. Kiyama, S. Sato, T. Shimamoto, M. Konishi, H. Iso, CIRCS investigators, Nocturnal intermittent hypoxia and the development of type 2 diabetes: the circulatory risk in communities study (CIRCS), Diabetologia 53 (2010) 481–488.

[6] H. Misu, T. Takamura, H. Takayama, H. Hayashi, N. Matsuzawa-Nagata, S. Kurita, K. Ishikura, H. Ando, Y. Takeshita, T. Ota, M. Sakurai, T. Yamashita, E. Mizukoshi, T. Yamashita, M. Honda, K. Miyamoto, T. Kubota, N. Kubota, T. Kadowaki, H.J. Kim, I.K. Lee, Y. Minokoshi, Y. Saito, K. Takahashi, Y. Yamada, N. Takakura, S. Kaneko, A liver-derived secretory protein, selenoprotein P, causes insulin resistance, Cell Metab. 12 (2010) 483–495.

[7] S.J. Yang, S.Y. Hwang, H.Y. Choi, H.J. Yoo, J.A. Seo, S.G. Kim, N.H. Kim, S.H. Baik, D.S. Choi, K.M. Choi, Serum selenoprotein P levels in patients with type 2 diabetes and prediabetes: Implications for insulin resistance, inflammation, and atherosclerosis, J. Clin. Endocrinol. Metab. 96 (2011) E1325–E1329.

[8] J. Yu, B. Yu, H. Jiang, D. Chen, Conjugated linoleic acid induces hepatic expression of fibroblast growth factor 21 through PPAR-α, Br. J. Nutr. 107 (2012) 461–465.

[9] H. Misu, H. Takayama, Y. Saito, Y. Mita, A. Kikuchi, K. Ishii, K. Chikamoto, T. Kanamori, N. Tajima, F. Lan, Y. Takeshita, M. Honda, M. Tanaka, S. Kato, N. Matsuyama, Y. Yoshioka, K. Iwayama, K. Tokuyama, N. Akazawa, S. Maeda, K. Takekoshi, S. Matsugo, N. Noguchi, S. Kaneko, T. Takamura, Deficiency of the hepatokine selenoprotein P increases responsiveness to exercise in mice through upregulation of reactive oxygen species and AMP-activated protein kinase in

[10] muscle, Nat. Med. 23 (2017) 508–516.

[10] K. Terazono, H. Yamamoto, S. Takasawa, K. Shiga, Y. Yonemura, Y. Tochino, H. Okamoto, A novel gene activated in regenerating islets, J. Biol. Chem. 263 (1988) 2111–2114.

[11] S. Takasawa, Regenerating gene (REG) product and its potential clinical usage, Exp. Opin. Ther. Targets 20 (2016) 541–550.

[12] T. Watanabe, Y. Yonemura, H. Yonekura, Y. Suzuki, H. Miyashita, K. Sugiyama, S. Moriizumi, M. Unno, O. Tanaka, H. Kondo, A.J. Bone, S. Takasawa, H. Okamoto, Pancreatic beta-cell replication and amelioration of surgical diabetes by Reg protein, Proc. Natl. Acad. Sci. USA 91 (1994) 3589–3592.

[13] N.J. Shervani, S. Takasawa, Y. Uchigata, T. Akiyama, K. Nakagawa, N. Noguchi, H. Takada, I. Takahashi, A. Yamauchi, T. Ikeda, Y. Iwamoto, K. Nata, H. Okamoto, Autoantibodies to REG, a beta-cell regeneration factor, in diabetic patients, Eur. J. Clin. Invest. 34 (2004) 752–758.

[14] H. Ota, A. Itaya-Hironaka, A. Yamauchi, S. Sakuramoto-Tsuchida, T. Miyaoka, T. Fujimura, H. Tsujinaka, K. Yoshimoto, K. Nakagawara, S. Tamaki, S. Takasawa, H. Kimura, Pancreatic β cell proliferation by intermittent hypoxia via up-regulation of *Reg* family genes and *HGF* gene, Life Sci. 93 (2013) 664–672.

[15] S. Murakami-Kawaguchi, S. Takasawa, T. Onogawa, K. Nata, A. Itaya-Hironaka, S. Sakuramoto-Tsuchida, A. Yamauchi, H. Ota, M. Takeda, M. Kato, H. Okamoto, Expression of *Ins1* and *Ins2* genes in mouse fetal liver, Cell Tissue Res. 355 (2014) 303–314.

[16] H. Ota, S. Tamaki, A. Itaya-Hironaka, A. Yamauchi, S. Sakuramoto-Tsuchida, T. Morioka, S. Takasawa, H. Kimura, Attenuation of glucose-induced insulin secretion by intermittent hypoxia via down-regulation of CD38, Life Sci. 90 (2012) 206–211.

[17] M. Niijima, H. Kimura, H. Edo, T. Shinozaki, J. Kang, S. Masuyama, K. Tatsumi, T. Kuriyama, Manifestation of pulmonary hypertension during REM sleep in obstructive sleep apnea syndrome, Am. J. Respir. Crit. Care Med. 159 (1999) 1766–1772.

[18] A. Yamauchi, A. Itaya-Hironaka, S. Sakuramoto-Tsuchida, M. Takeda, K. Yoshimoto, T. Miyaoka, T. Fujimura, H. Tsujinaka, C. Tsuchida, H. Ota, S. Takasawa, Synergistic activations of *REG Iα* and *REG Iβ* promoters by IL-6 and glucocorticoids through JAK/STAT pathway in human pancreatic β cells, J. Diabetes Res. 2015 (2015) 173058.

[19] T. Fujimura, T. Fujimoto, A. Itaya-Hironaka, T. Miyaoka, K. Yoshimoto, A. Yamauchi, S. Sakuramoto-Tsuchida, S. Kondo, M. Takeda, H. Tsujinaka, M. Azuma, Y. Tanaka, S. Takasawa, Interleukin-6/STAT pathway is responsible for the induction of gene expression of REG Iα, a new auto-antigen in Sjögren's syndrome patients, in salivary duct epithelial cells, Biochem. Biophys. Rep. 2 (2015) 69–74.

[20] H. Tsujinaka, A. Itaya-Hironaka, A. Yamauchi, S. Sakuramoto-Tsuchida, H. Ota, M. Takeda, T. Fujimura, S. Takasawa, N. Ogata, Human retinal pigment epithelial cell proliferation by the combined stimulation of hydroquinone and advanced glycation end-products via up-regulation of *VEGF* gene, Biochem. Biophys. Rep. 2 (2015) 123–131.

[21] T. Akiyama, S. Takasawa, K. Nata, S. Kobayashi, M. Abe, N.J. Shervani, T. Ikeda, K. Nakagawa, M. Unno, H. Okamoto, Activation of *Reg* gene, a gene for insulin-producing β-cell regeneration: Poly (ADP-ribose) polymerase binds *Reg* promoter and regulates the transcription by autopoly(ADP-ribosyl)ation, Proc. Natl. Acad. Sci. U.S.A. 98 (2001) pp. 48–53.

[22] T. Nakazawa, S. Takasawa, N. Noguchi, K. Nata, A. Tohgo, M. Mori, K. Nakagawara, T. Akiyama, T. Ikeda, A. Yamauchi, I. Takahashi, T. Yoshikawa, H. Okamoto, Genomic organization, chromosomal localization, and promoter of human gene for

FK506-binding protein 12.6, Gene 360 (2005) 55–64.

[23] S. Takasawa, T. Ikeda, T. Akiyama, K. Nata, K. Nakagawa, N.J. Shervani, N. Noguchi, S. Murakami-Kawaguchi, A. Yamauchi, I. Takahashi, T. Tomioka-Kumagai, H. Okamoto, Cyclin D1 activation through ATF-2 in Reg-induced pancreatic β-cell regeneration, FEBS Lett. 580 (2006) 585–591.

[24] E. Sonkoly, T. Wei, P.C. Janson, A. Sääf, L. Lundeberg, M. Tengvall-Linder, G. Norstedt, H. Alenius, B. Homey, A. Scheynius, M. Ståhle, A. Pivarcsi, MicroRNAs: novel regulators involved in the pathogenesis of psoriasis? PLoS ONE 2 (2007) e610.

[25] E. Szele, K. Gombos, K. Juhász, V. Wohler, A. Kovács, I. Ember, Effects of purified glycerol from biodiesel on miRNAs compared to the expression profile of selected mRNAs in Balb/c mice, In Vivo 27 (2013) 107–111.

[26] J. Zhang, X.H. Zhang, C.X. Wang, B. Liu, X.S. Fan, J.J. Wen, Q.L. Shi, X.J. Zhou, Dysregulation of MicroRNA biosynthesis enzyme Dicer plays an important role in gastric cancer progression, Int. J. Clin. Exp. Pathol. 7 (2014) 1702–1707.

[27] S. Francia, F. Michelini, A. Sexena, D. Tang, M. de Hoon, V. Anelli, M. Mione, P. Carninci, F. d'Adda di Fagagna, Site-specific DICER and DROSHA RNA products control the DNA-damage response, Nature 488 (2012) 231–235.

[28] S. Mattiske, K. Ho, J.E. Noll, P.M. Neilsen, D.F. Callen, R.J. Suetani, TAp63 regulates oncogenic miR-155 to mediate migration and tumour growth, Oncotarget 4 (2013) 1894–1903.

[29] N.M. Punjabi, B.A. Beamer, Alterations in glucose disposal in sleep-disordered breathing, Am. J. Respir. Crit. Care Med. 179 (2009) 235–240.

[30] K. Rasche, T. Keller, B. Tautz, C. Hader, G. Hergenç, J. Antosiewicz, C. Di Giulio, M. Pokorski, Obstructive sleep apnea and type 2 diabetes, Eur. J. Med. Res. 15 (Suppl. 2) (2010) 152–156.

[31] W. Zhong, Y.G. Tang, X. Zhao, F.Y. Go, R.M. Harper, H. Hui, Treating obstructive sleep apnea with continuous positive airway pressure benefits type 2 diabetes management, Pancreas 43 (2014) 325–330.

[32] M. Yamauchi, H. Kimura, Oxidative stress in obstructive sleep apnea: putative pathways to the cardiovascular complications, Antioxid. Redox Signal. 10 (2008) 755–768.

[33] S. Tamaki, M. Yamauchi, A. Fukuoka, K. Makinodan, N. Koyama, K. Tomoda, M. Yoshikawa, H. Kimura, Nocturnal hypoxic stress activates invasive ability of monocytes in patients with obstructive sleep apnoea syndrome, Respirology 14 (2009) 689–694.

[34] Y. Oike, M. Akao, K. Yasunaga, T. Yamauchi, T. Morisada, Y. Ito, T. Urano, Y. Kimura, Y. Kubota, H. Maekawa, T. Miyamoto, K. Miyata, S. Matsumoto, J. Sakai, N. Nakagata, M. Takeya, H. Koseki, Y. Ogawa, T. Kadowaki, T. Suda, Angiopoietin-related growth factor antagonizes obesity and insulin resistance, Nat. Med. 11 (2005) 400–408.

[35] K. Chikamoto, H. Misu, H. Takayama, A. Kikuchi, K. Ishii, F. Lan, N. Takata, N. Tajima-Shirasaki, Y. Takeshita, H. Tsugane, S. Kaneko, S. Matsuugo, T. Takamura, Rapid response of the steatosis-sensing hepatokine LECT2 during diet-induced weight cycling in mice, Biochem. Biophys. Res. Commun. 478 (2016) 1310–1316.

[36] G. Andersen, K.S. Burgdorf, T. Sparsø, K. Borch-Johnsen, T. Jørgensen, T. Hansen, O. Pedersen, *AHSG* tag single nucleotide polymorphisms associate with type 2 diabetes and dyslipidemia: Studies of metabolic traits in 7,683 white Danish subjects, Diabetes 57 (2008) 1427–1432.

[37] Z. Fu, F. Berhane, A. Fite, B. Seyoum, A.B. Abou-Samra, R. Zhang, Elevated

[38] circulating lipasin/betatrophin in human type 2 diabetes and obesity, Sci. Rep. 4 (2014) 5013.

[38] E.L. Ding, Y. Song, J.E. Manson, D.J. Hunter, C.C. Lee, N. Rifai, J.E. Buring, J.M. Gaziano, S. Liu, Sex hormone-binding globulin and risk of type 2 diabetes in women and men, N. Engl. J. Med. 361 (2009) 1152–1163.

[39] R.F. Burk, K.E. Hill, Selenoprotein, P. An, extracellular protein with unique physical characteristics and a role in selenium homeostasis, Annu. Rev. Nutr. 25 (2005) 215–235.

[40] C. Lasserre, L. Christa, M.T. Simon, P. Vernier, C. Bréchot, A novel gene (HIP) activated in human primary liver cancer, Cancer Res. 52 (1992) 5089–5095.

[41] B. Orelle, V. Keim, L. Masciotra, J.C. Dagorn, J.L. Iovanna, Human pancreatitis-associated protein. Messenger RNA cloning and expression in pancreatic diseases, J. Clin. Invest. 90 (1992) 2284–2291.

[42] H.T. Lieu, F. Batteux, M.T. Simon, A. Cortes, C. Nicco, F. Zavala, A. Pauloin, J.G. Tralhao, O. Soubrane, B. Weill, C. Bréchot, L. Christa, HIP/PAP accelerates liver regeneration and protects against acetaminophen injury in mice, Hepatology 42 (2005) 618–626.

[43] M. Gironella, E. Folch-Puy, A. LeGoffic, S. Garcia, L. Christa, A. Smith, L. Tebar, S.P. Hunt, R. Bayne, A.J.H. Smith, J.-C. Dagorn, D. Closa, J.L. Iovanna, Experimental acute pancreatitis in PAP/HIP knock-out mice, Gut 56 (2007) 1091–1097.

[44] M.T. Simon, A. Pauloin, G. Normand, H.T. Lieu, H. Mouly, G. Pivert, F. Carnot, J.G. Tralhao, C. Brechot, L. Christa, HIP/PAP stimulates liver regeneration after partial hepatectomy and combines mitogenic and anti-apoptotic functions through the PKA signaling pathway, FASEB J. 17 (2003) 1441–1450.

[45] M.Á. Martínez-García, F. Campos-Rodríguez, I. Almendros, R. Farré, Relationship between sleep apnea and cancer, Arch. Bronconeumol. 51 (2015) 456–461.

[46] D. Gozal, R. Farré, F.J. Nieto, Putative links between sleep apnea and cancer: From hypotheses to evolving evidence, Chest 148 (2015) 1140–1147.

[47] V. Secq, C. Mallmann, M. Gironella, B. Lopez, D. Closa, S. Garcia, L. Christa, G. Montalto, N. Dusetti, J.L. Iovanna, PAP/HIP protein is an obesogenic factor, J. Cell. Physiol. 229 (2014) 225–231.

[48] M. Esteller, Non-coding RNAs in human disease, Nat. Rev. Genet. 12 (2011) 861–874.

[49] B. Bao, Z. Wang, Y. Li, D. Kong, S. Ali, S. Banerjee, A. Ahmad, F.H. Sarkar, The complexities of obesity and diabetes with the development and progression of pancreatic cancer, Biochim. Biophys. Acta 2011 (1815) 135–146.

[50] I. Slezak-Prochazka, S. Durmus, B.-J. Kroesen, A. van den Berg, MicroRNAs, macrocontrol: Regulation of miRNA processing, RNA 16 (2010) 1087–1095.

[51] X. Zhou, W. Liu, M. Gu, H. Zhou, G. Zhang, *Helicobacter pylori* infection causes hepatic insulin resistance by the c-Jun/miR-203/SOCS3 signaling pathway, J. Gastroenterol. 50 (2015) 1027–1040.

[52] V. Rottiers, A.M. Näär, MicroRNAs in metabolism and metabolic disorders, Nat. Rev. Mol. Cell Biol. 13 (2012) 239–250.

[53] C. Latouche, A. Natoli, M. Reddy-Luthmoodoo, S.E. Heywood, J.A. Armitage, B.A. Kingwell, MicroRNA-194 modulates glucose metabolism and its skeletal muscle expression is reduced in diabetes, PLoS One 11 (2016) e0155108.

[54] I.G.M. Kolfschoten, E. Roggli, V. Nesca, R. Regazzi, Role and therapeutic potential of microRNAs in diabetes, Diabetes Obes. Metab. 11 (2009) 118–129.