



Article

Intermittent Hypoxia Increased the Expression of DBH and PNMT in Neuroblastoma Cells via MicroRNA-375-Mediated Mechanism

Shin Takasawa ^{1,*}, Ryogo Shobatake ^{1,2}, Yoshinori Takeda ^{1,3}, Tomoko Uchiyama ^{1,4}, Akiyo Yamauchi ¹, Mai Makino ¹, Sumiyo Sakuramoto-Tsuchida ¹, Keito Asai ¹, Hiroyo Ota ^{1,5} and Asako Itaya-Hironaka ¹

¹ Department of Biochemistry, Nara Medical University, 840 Shijo-cho, Kashihara 634-8521, Nara, Japan; rshobatake@naramed-u.ac.jp (R.S.); y-takeda@naramed-u.ac.jp (Y.T.); uchiyama0403@naramed-u.ac.jp (T.U.); yamauchi@naramed-u.ac.jp (A.Y.); m.makino@naramed-u.ac.jp (M.M.); ssumiyo@naramed-u.ac.jp (S.S.-T.); dc120002@naramed-u.ac.jp (K.A.); hiroyon@naramed-u.ac.jp (H.O.); iasako@naramed-u.ac.jp (A.I.-H.)

² Department of Neurology, Nara Medical University, 840 Shijo-cho, Kashihara 634-8522, Nara, Japan

³ Department of Obstetrics and Gynecology, Nara Medical University, 840 Shijo-cho, Kashihara 634-8522, Nara, Japan

⁴ Department of Diagnostic Pathology, Nara Medical University, 840 Shijo-cho, Kashihara 634-8522, Nara, Japan

⁵ Department of Respiratory Medicine, Nara Medical University, 840 Shijo-cho, Kashihara 634-8522, Nara, Japan

* Correspondence: shintksw@naramed-u.ac.jp; Tel.: +81-744-22-3051 (ext. 2227); Fax: +81-744-24-9525



Citation: Takasawa, S.; Shobatake, R.; Takeda, Y.; Uchiyama, T.; Yamauchi, A.; Makino, M.; Sakuramoto-Tsuchida, S.; Asai, K.; Ota, H.; Itaya-Hironaka, A. Intermittent Hypoxia Increased the Expression of DBH and PNMT in Neuroblastoma Cells via MicroRNA-375-Mediated Mechanism. *Int. J. Mol. Sci.* **2022**, *23*, 5868. <https://doi.org/10.3390/ijms23115868>

Academic Editor: David Gozal

Received: 2 May 2022

Accepted: 23 May 2022

Published: 24 May 2022

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Copyright: © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

Abstract: Sleep apnea syndrome (SAS), characterized by recurrent episodes of oxygen desaturation and reoxygenation (intermittent hypoxia (IH)), is a risk factor for hypertension and insulin resistance. We report a correlation between IH and insulin resistance/diabetes. However, the reason why hypertension is induced by IH is elusive. Here, we investigated the effect of IH on the expression of catecholamine-metabolizing enzymes using an in vitro IH system. Human and mouse neuroblastoma cells (NB-1 and Neuro-2a) were exposed to IH or normoxia for 24 h. Real-time RT-PCR revealed that IH significantly increased the mRNA levels of *dopamine β-hydroxylase (DBH)* and *phenylethanolamine N-methyltransferase (PNMT)* in both NB-1 and Neuro-2a. Western blot showed that the expression of DBH and PNMT in the NB-1 cells was significantly increased by IH. Reporter assays revealed that promoter activities of *DBH* and *PNMT* were not increased by IH. The miR-375 level of IH-treated cells was significantly decreased relative to that of normoxia-treated cells. The IH-induced up-regulation of DBH and PNMT was abolished by the introduction of the miR-375 mimic, but not by the control RNA. These results indicate that IH stress increases levels of DBH and PNMT via the inhibition of miR-375-mediated mRNA degradation, potentially playing a role in the emergence of hypertension in SAS patients.

Keywords: intermittent hypoxia; sleep apnea syndrome; miR-375; neuroblastoma cells; hypertension; catecholamines; dopamine β-hydroxylase; phenylethanolamine N-methyltransferase

1. Introduction

Sleep apnea syndrome (SAS) is a highly prevalent sleep disorder characterized by the repetitive partial or complete collapse of the pharynx during sleep. It is estimated that nearly one billion adults aged 30–69 in the world may suffer from SAS [1]. It induces apnea and hypopnea, which often result in a decreased oxygen saturation. A growing body of evidence suggests that SAS acts through recurrent episodes of oxygen desaturation and reoxygenation (intermittent hypoxia (IH)), which cause hypertension [2]. The pathophysiology of hypertension in cases of SAS is complex and dependent on various factors. The hypertension rate in SAS patients is two- to three-fold higher than that in the general population. Furthermore, habitual snoring, one of the hallmark symptoms of SAS, has been reported to be associated with hypertension in several epidemiologic studies [3,4]. Since

SAS and hypertension share certain risk factors, i.e., the male gender, obesity, middle age, and a sedentary lifestyle, questions have been raised as to how much of this association may be attributable to these confounding variables. For the control of blood pressure, the sympathetic nervous system (catecholamine system), renin–angiotensin–aldosterone system and sodium reabsorption, and the endothelial system are important factors. We investigated how IH causes hypertension and report that IH up-regulates *renin* mRNA in juxtaglomerular cells via the down-regulation of microRNA (miR)-203 [5].

In the present study, we used catecholamine-producing human and mouse neuroblastoma cells and an in vitro IH system to investigate the direct effect of IH, a hallmark of SAS. An in vitro IH system is a controlled gas delivery system that regulates the flow of nitrogen and oxygen to generate IH [6,7]. We investigated the direct effect of IH on the gene expression(s) of catecholamine-synthesizing enzymes in neuroblastoma cells, and found significant increases in the mRNA levels of *dopamine β -hydroxylase (DBH)* and *phenylethanolamine N-methyltransferase (PNMT)* in response to IH treatment via the down-regulation of microRNA (miR)-375.

2. Results

2.1. Gene Expression Levels of DBH and PNMT in Human and Mouse Neuroblastoma Cells Were Increased by IH

We exposed catecholamine-producing mouse Neuro-2a and human NB-1 neuroblastoma cells to normoxia or IH for 24 h. Following the IH treatment, we prepared cellular RNA and measured the mRNA levels of catecholamine biosynthesis enzymes such as *tyrosine hydroxylase (Th)*, *L-3,4-dihydroxyphenylalanine (DOPA) decarboxylase (Ddc)*, *dopamine β -hydroxylase (Dbh)*, and *phenylethanolamine N-methyltransferase (Pnmt)* by means of a real-time reverse transcription-polymerase chain reaction (RT-PCR). We found that the mRNA levels of *Th*, *Ddc*, *Dbh*, and *Pnmt* were up-regulated by IH in mouse Neuro-2a cells (Figure 1).

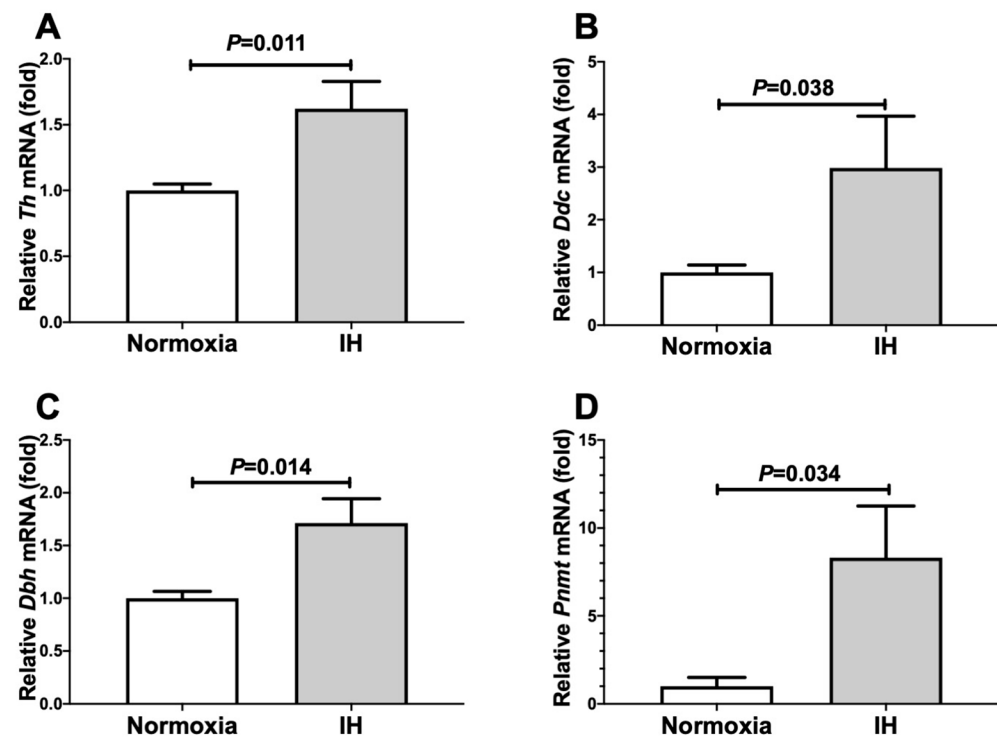


Figure 1. The mRNA levels of *Th* (A), *Ddc* (B), *Dbh* (C), and *Pnmt* (D) in mouse Neuro-2a cells subjected to normoxia or IH for 24 h. The levels of the catecholamine-synthesizing enzyme mRNAs were measured by means of a real-time RT-PCR using *rat insulinoma gene (Rig)/ribosomal protein S15 (Rps15)* as an endogenous control. The data are expressed as the mean \pm SE for each group of six independent experiments ($n = 6$). The statistical analyses were performed using Student's *t*-test.

Following the treatment, we prepared cellular RNA and measured the mRNA levels of *TH*, *DDC*, *DBH*, and *PNMT* by means of a real-time RT-PCR. As shown in Figure 2, the mRNA levels of *DBH* and *PNMT* were significantly increased in the NB-1 cells in response to IH, although the *TH* and *DDC* mRNA levels did not increase.

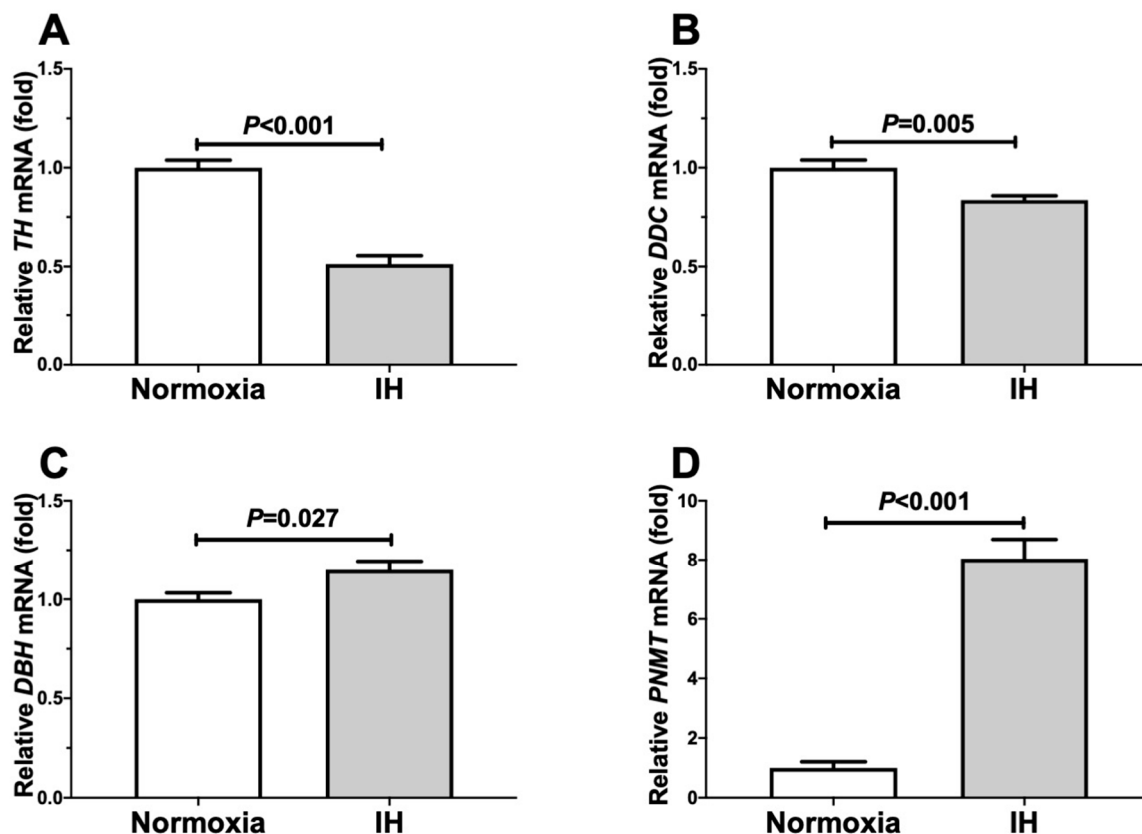


Figure 2. The mRNA levels of *TH* (A), *DDC* (B), *DBH* (C), and *PNMT* (D) in human NB-1 cells subjected to normoxia or IH for 24 h. The levels of the mRNAs were measured by means of a real-time RT-PCR using β -actin as an endogenous control. The data are expressed as the mean \pm SE for each group of six independent experiments ($n = 6$). The statistical analyses were performed using Student's *t*-test.

We further measured cellular DBH and PNMT levels by means of immunoblot analyses. As shown in Figures 3 and 4, IH significantly increased the cellular DBH and PNMT levels in NB-1 cells.

2.2. The Promoter Activities of DBH and PNMT Were Not Increased by IH

To determine whether the IH-induced increases in *DBH* and *PNMT* mRNAs were caused by the activation of transcription, a 1028 bp fragment containing 1018 bp of the human *DBH* promoter, and a 667 bp fragment containing 600 bp of the human *PNMT* promoter, were fused to the luciferase gene of pGL4.17 and transfected into NB-1 cells. After IH stimulation, we measured promoter activities and found that *DBH* and *PNMT* promoter activities were not increased by IH in NB-1 cells (Figure 5: $p = 0.694$ in *DBH* promoter and $p = 0.242$ in *PNMT* promoter). These results suggested that the gene expression of *DBH* and *PNMT* in response to IH was not regulated by transcription.

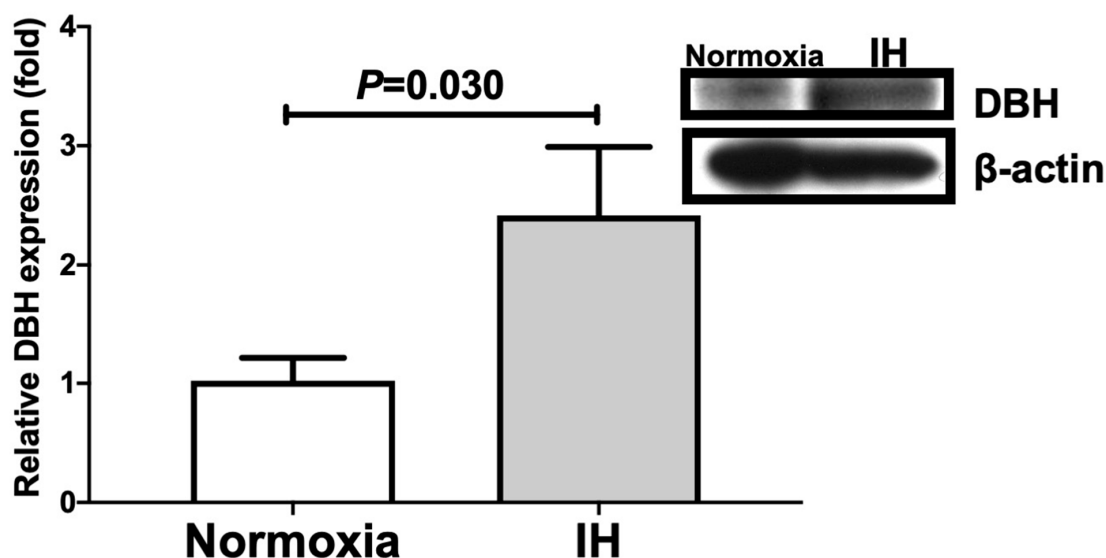


Figure 3. Relative protein expression of DBH in human NB-1 neuroblastoma cells subjected to IH. A representative immunoblot is shown in the right panel. The relative expression of DBH is arbitrarily presented. The DBH band densities were quantified using an image analysis and then normalized to β -actin, as measured in the same blot. Each bar represents the mean values of six independent experiments. The results are expressed as the mean \pm SE in arbitrary units.

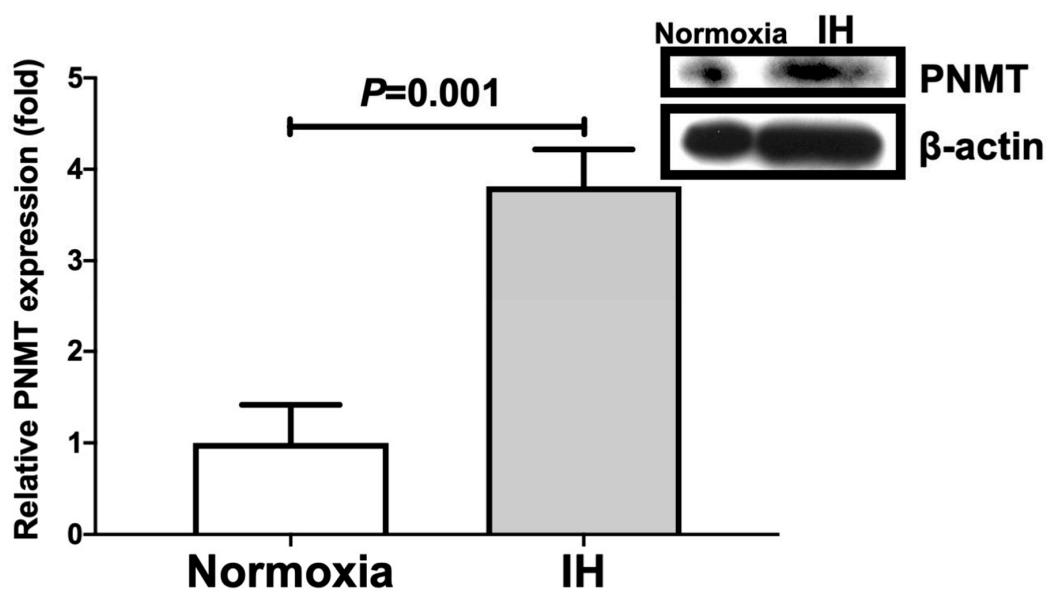


Figure 4. Relative protein expression of PNMT in human NB-1 neuroblastoma cells subjected to IH. A representative immunoblot is shown in the right panel. The relative expression of PNMT is arbitrarily presented. The PNMT band densities were quantified using an image analysis and then normalized to β -actin, as measured in the same blot. Each bar represents the mean values of six independent experiments. The results are expressed as the mean \pm SE in arbitrary units.

2.3. The MiR-375 Level Was Significantly Decreased by IH

We considered a possible explanation that the IH-induced up-regulation of *DBH* and *PNMT* was controlled post-transcriptionally. Therefore, we searched for targeted miRNA using the MicroRNA.org program (<http://www.microrna.org/microrna/home.do>, 23 October 2021), which revealed that *DBH* and *PNMT* mRNAs had a potential target sequence for miR-375. There were no other miRNA candidates targeting both genes. We measured the miR-375 levels of IH-treated cells using real-time RT-PCR and found that the

level was significantly lower than that of normoxia-treated cells (Figure 6: 0.371 ± 0.108 folds vs. normoxia, $p = 0.022$). There are several possible reasons as to why the level of miR-375 was decreased by IH; one is that the mRNA levels of some enzymes involved in miRNA biosynthesis/degradation are influenced by IH; another is that the level of miR-375 was specifically decreased by IH, either via decreased biosynthesis or enhanced degradation. We measured the *endoribonuclease Dicer* (*DICER*), which is involved in the biosynthesis of miRNAs [8,9], and found that its expression was unchanged by IH (Figure 6: $p = 0.135$). These results suggest that miR-375 plays a key role in the post-transcriptional regulation of the mRNA levels of *DBH* and *PNMT*. To investigate whether *DBH* and *PNMT* expression in IH is regulated by miR-375, miR-375 mimic and non-specific control RNA (miR-375 mimic NC) were introduced into NB-1 cells with IH/normoxia exposure, and the mRNA levels of *DBH* and *PNMT* were measured using real-time RT-PCR.

As shown in Figure 7, we found that the IH-induced increases in *DBH* and *PNMT* mRNAs were abolished by the introduction of the miR-375 mimic, but not by the miR-375 mimic NC. These findings indicate that IH stress down-regulated the miR-375 level in human neuroblastoma cells (Figure 6) and that the levels of *DBH* and *PNMT* mRNAs were increased via the miR-375-mediated mechanism.

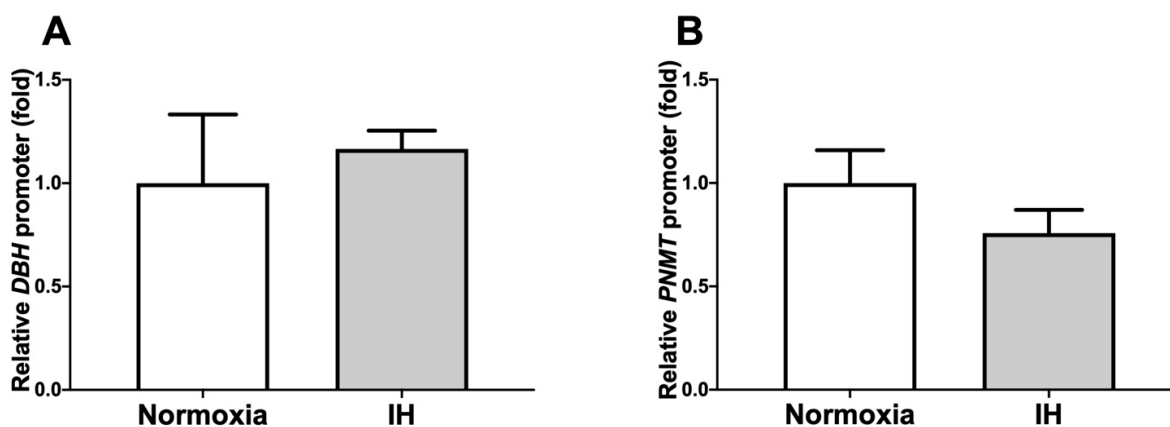


Figure 5. Promoter activities of *DBH* (A) and *PNMT* (B) in human NB-1 neuroblastoma cells. Reporter plasmids prepared by inserting the promoter fragments of *DBH* (−1018–+10) and *PNMT* (−600–+67) up-stream of a firefly luciferase reporter gene in a pGL4.17 vector were transfected into the NB-1 cells. After the cells were exposed to either IH or normoxia for 24 h, they were lysed, and the promoter activities of *DBH* and *PNMT* were measured. All data are presented as the mean \pm SE of the samples of six independent experiments ($n = 6$). The statistical analyses were performed using Student's *t*-test.

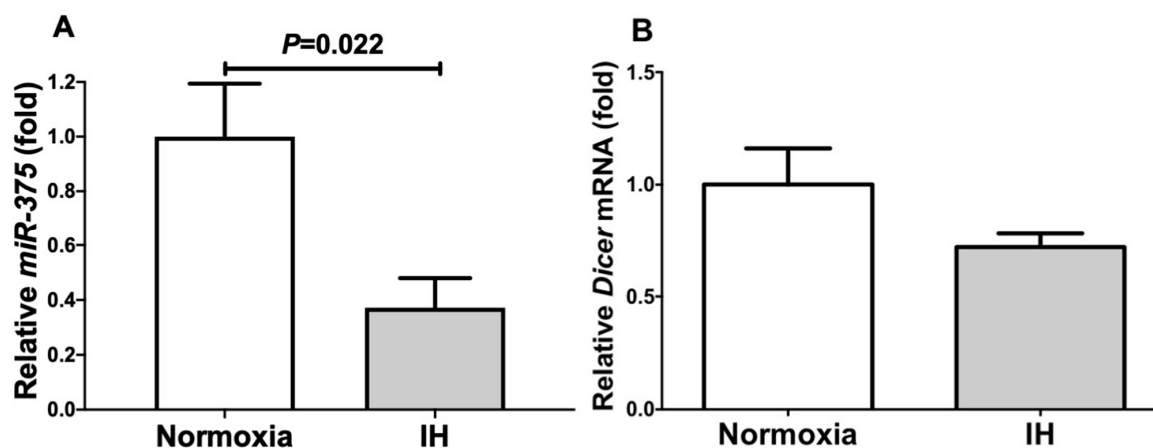


Figure 6. The levels of *miR-375* (A) and *Dicer* mRNA (B) in human NB-1 cells subjected to normoxia or IH for 24 h. The levels of *miR-375* and *Dicer* mRNA were measured by means of a real-time RT-PCR

using *U6* (for the miR-375) and β -actin (for the Dicer) as endogenous controls. The data are expressed as the mean \pm SE for each group of six independent experiments ($n = 6$). The statistical analyses were performed using Student's *t*-test.

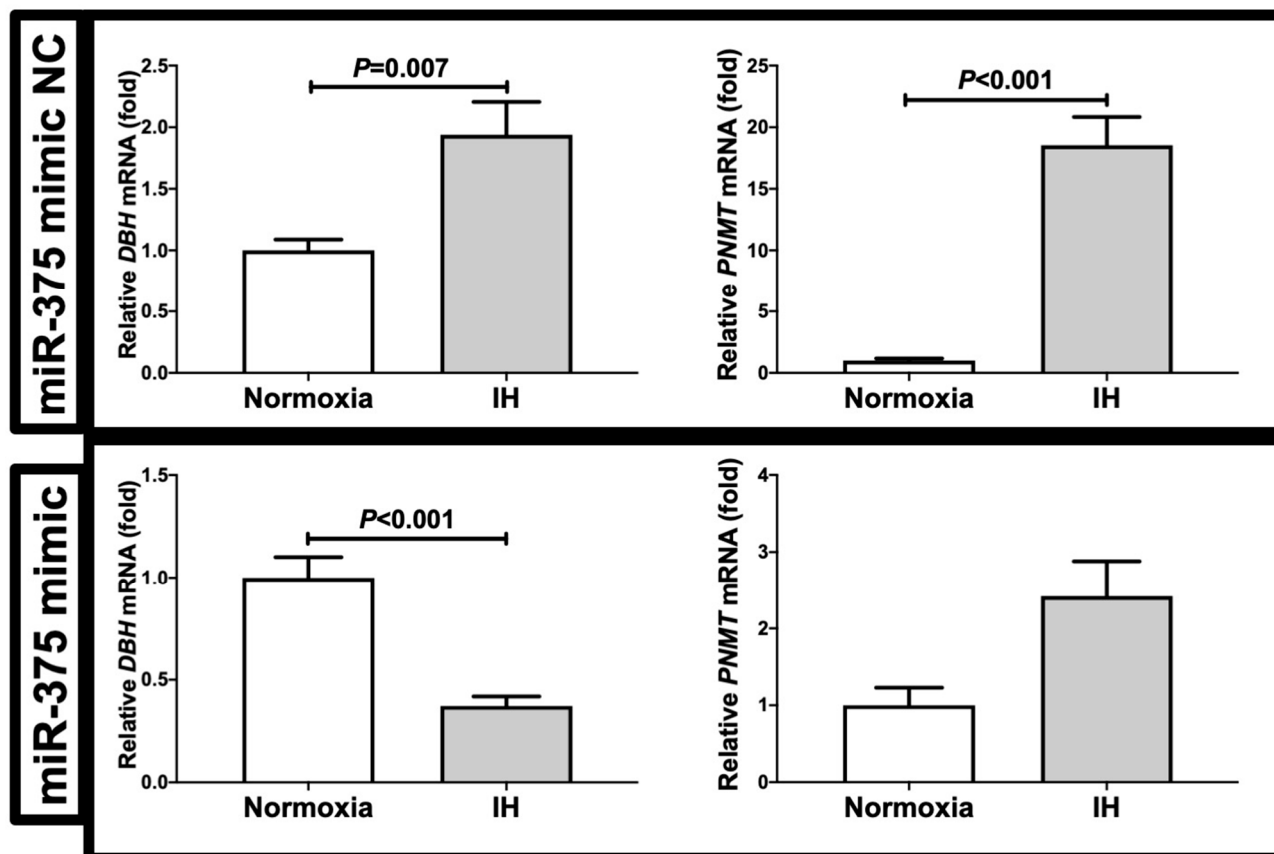


Figure 7. Effects of the miR-375 mimic transfection on *DBH* and *PNMT* expression. The miR-375 mimic and the non-specific control RNA (miR-375 mimic NC) were introduced into the NB-1 cells using Lipofectamine[®] RNAiMAX just before the IH/normoxia exposure, and the mRNA levels of *DBH* and *PNMT* were measured by means of a real-time RT-PCR, as described in the “Materials and Methods” section, using β -actin as an endogenous control. The data are expressed as the mean \pm SE for each group of six independent experiments ($n = 6$). The statistical analyses were performed using Student's *t*-test.

3. Discussion

SAS patients and their organs, tissues, and cells are exposed to IH. Thus, we exposed catecholamine-synthesizing neuroblastoma cells to IH in the present study and found that IH exposure induced increases in the *DBH* and *PNMT* mRNA levels in human neuroblastoma cells. We further studied the mechanisms by which IH up-regulates the mRNA levels of *DBH* and *PNMT* and found the possibility of post-transcriptional miRNA-regulated mechanisms. The expression of *DBH* and *PNMT* was up-regulated by IH via the miR-375-mediated mechanism. Hypertension in SAS patients could be caused by the IH-induced up-regulation of *DBH* and *PNMT*.

Recent epidemiological research demonstrated that SAS may be associated with various metabolic dysfunctions, including dyslipidemia, cardiovascular diseases, insulin resistance, and hypertension. The pathophysiology of hypertension in relation to SAS is dependent on various factors; for example, the sympathetic tone, peripheral vasoconstriction, altered baroreceptor reflexes, increased renin–angiotensin system activity [2,5], and increased plasma noradrenaline concentrations [10]. In particular, there are numerous reports concerning catecholamine synthesis in SAS patients and experimental models—more

specifically, increased catecholamine secretion [11,12] and the up-regulation of the *PNMT* gene by IH [13]. Regarding catecholamine secretion from neuronal/adrenal chromaffin cells in hypoxia, hypoxia stimulates catecholamine secretion/gene expression in vitro and in vivo [14–16].

Previous studies focusing on catecholamine biosynthetic enzyme activities in the adrenal medulla, which is derived from the neural crest of spontaneously hypertensive rats (SHRs), showed inconsistent results. This was reported to be decreased [17–19], unchanged [20], and increased [21,22] in young SHRs. *Ddc*, *Dbh*, and *Pnmt* activities were decreased [18,23] or increased [24,25] in young SHRs, but unchanged in adult SHRs [18,25]. Due to the apparent correlation of the catecholamine biosynthetic enzyme activities, some authors have suggested that these genes may be co-regulated by a single locus [26].

We also investigated the mechanisms by which IH up-regulates the mRNA levels of *DBH* and *PNMT*. We found that the promoter activities of the genes were not increased by IH, which suggested that the IH-induced up-regulation of the *DBH* and *PNMT* mRNAs is regulated during the post-transcriptional step. miRNAs are small non-coding RNAs (≈ 22 nucleotides in length) that modulate gene expression by either translational suppression or the degradation of the mRNA through binding to the 3'-untranslated regions of the target genes in a base-pairing manner [27]. They affect the stability of their target mRNAs, resulting in changes in the amount of target mRNA, which is one of the mechanisms associated with post-transcriptional regulation. To date, a number of studies concerning the role of miR-375 have been reported to be influenced in normal pancreatic genesis [28], the proinflammatory macrophage response [29], pituitary prolactin synthesis [30], neuroendocrine differentiation and tumorigenesis in lung carcinoid cells [31], inhibition in proliferation and invasion of nasopharyngeal carcinomas [32], and inhibition in the proliferation, migration, and invasion of esophageal squamous cell carcinomas [33]. Several such studies have indicated that miRNAs are involved in the regulation of many biological processes (migration, metastasis, cell proliferation, apoptosis, chemosensitivity, etc.) in these various types of cells.

A few studies have addressed the correlation between miRNAs and hypertension in patients with SAS. For example, three plasma miRNAs (miR-378a-3p, miR-100-5p, and miR-486-5p) have been found to predict blood pressure responses to the continuous positive airway pressure treatment in patients with resistant hypertension and SAS [34]. Several miRNAs with altered expression in catecholamine-producing pheochromocytomas and paragangliomas (such as miR-15, -16, -21-3p, -96, -101-, -133, -137, -139-3p, -183, -195, -210, -338-3p, -375, -382, -483-5p, -488, -508, -765, -855, and -1225-3p) were reported [35]. In particular, there are a few reports on the function of miR-375, and they suggested it to be involved in the regulation of cytokines, extracellular matrix–receptor interaction, focal adhesion, phosphatidylinositol-3 kinase–protein kinase B (Akt), amoebiasis, and protein-processing pathways [36]. Single nucleotide polymorphisms (rs11174811 and rs3803107) in miR-375 target sites of the 3'-untranslated region in the arginine vasopressin receptor 1a gene were reported to be associated with the risk of hypertension [37]. However, these studies did not indicate the involvement of miR-375 in SAS patients' hypertension. In the present study, the decline in miR-375 with a target sequence in the *DBH* and *PNMT* mRNAs could have contributed to the worsening hypertension in the IH condition induced by the up-regulation of the *DBH* and *PNMT* mRNAs. Therefore, it is quite important and necessary to clarify the relationships between miR-375, catecholamine-metabolizing enzyme expression, and hypertension using experimental animal models and/or clinical samples in the future. Additionally, since miRNA is easily measured in a clinical laboratory using clinically obtained blood samples of patients, it is possible that the measurement of miR-375 is used for the prediction and diagnosis of hypertension in SAS and/or habitual snoring patients.

In this study, the gene expression of *DBH* and *PNMT* was increased via the down-regulation of the miR-375 level in the IH-treated neuroblastoma cells. It is suggested that, in SAS patients, the up-regulation of *DBH* and *PNMT* in neural cells in the adrenal medulla

may induce hypertension, while miR-375 could play a crucial role in the regulation of such gene expressions.

4. Materials and Methods

4.1. Cell Culture

The mouse neuroblastoma Neuro-2a cells utilized were purchased from the Japanese Collection of Research Bioresources Cell Bank (Ibaraki, Japan). The Neuro-2a cells were grown in E-MEM medium (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) containing 10% (*v/v*) fetal calf serum (FCS), 100 units/mL penicillin G (FUJIFILM Wako), and 100 µg/mL streptomycin (FUJIFILM Wako), and human neuroblastoma NB-1 cells were grown in RPMI1640 medium (Nacalai Tesque, Inc., Kyoto, Japan) containing 10% (*v/v*) FCS, 100 units/mL penicillin G (FUJIFILM Wako), and 100 µg/mL streptomycin (FUJIFILM Wako), as described in prior studies [38–40]. The cells were exposed to either normoxia (21% O₂, 5% CO₂, and balanced N₂) or IH (70 cycles of 5 min sustained hypoxia (1% O₂, 5% CO₂, and balanced N₂) and 10 min normoxia) in a custom-designed, computer-controlled incubation chamber attached to an external O₂-CO₂-N₂ computer-driven controller (O₂ programmable control, 9200EX, Wakenyaku Co., Ltd., Kyoto, Japan), as described in previous studies [5,40–46]. We used this *in vitro* model of IH, which resulted in fluctuations in the pressure of oxygen similar to the IH condition observed in patients with severe SAS, to repeatedly expose the cells to severe hypoxemia followed by mild hypoxemia or normoxia (i.e., IH) [47]. We previously reported that the magnitude of the IH expressed by SpO₂ fluctuated between 75% and 98% and between 50% and 80% in patients with SAS [6,7,48], which was nearly equivalent to the medium condition in the present study.

4.2. RT-PCR

Total RNA was isolated using an RNeasy Plus Cell Mini Kit (Qiagen, Hilden, Germany) for Neuro-2a and NB-1 cells, and cDNA was synthesized from total RNA as a template using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA), as previously described [5,40–46,49–51]. A real-time polymerase chain reaction (PCR) was performed using an SYBR[®] Fast qPCR kit (KAPA Biosystems, Boston, MA) and a Thermal Cycler Dice Real Time [JP1] System (Takara Bio, 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 Tables 1 and 2. PCR was performed in 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*; 45 cycles of 10 s at 95 °C, 5 s at 60 °C, and 20 s at 72 °C for *Rig/RpS15*; and 45 cycles of 3 s at 95 °C and 20 s at 60 °C for *Th* (human and mouse), *Ddc* (human and mouse), *Dbh* (human and mouse), *Pnmt* (human and mouse), *endoribonuclease Dicer (DICER)*, and *microRNA-375 (miR-375)*. The mRNA expression levels were normalized to the mRNA level of *Rig/RpS15* in mouse samples or *β-actin* in human samples, and the *miR-375* level was normalized to the *U6* RNA level.

4.3. Construction of Reporter Plasmid and Luciferase Assay

Reporter plasmids were prepared by inserting the promoter fragments of human *DBH* (−1018→+10) and *PNMT* (−600→+67) up-stream of a firefly luciferase reporter gene in the pGL4.17 vector (Promega, Madison, WI, USA). The reporter plasmids were transfected into human NB-1 neuroblastoma cells using Lipofectamine[®] 3000 (Invitrogen, Waltham, MA, USA), as previously described [5,40,43,45,46,50], and the cells were exposed to either 70 cycles/24 h of IH or normoxia for 24 h. After the cells were exposed to IH, they 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, Carlsbad, CA, USA). To monitor the 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 [5,40,41,43,46,49,50].

Table 1. PCR primers (mouse) for the real-time RT-PCR.

Target mRNA	Primer Sequence
<i>Th</i> (NM_009377)	5'-GCCAAGGACAAGCTCAGGAAC-3'
	5'-ATCAATGGCCAGGGTGTACG-3'
<i>Ddc</i> (NM_001190448)	5'-CTGCAGGCTTACATCCGAA-3'
	5'-TTGATCTCTGAAGCAGCT-3'
<i>Dbh</i> (NM_138942)	5'-GACTCAACTACTGCCGGCACGT-3'
	5'-CTGGGTGCACTTGTCTGTGCAGT-3'
<i>Pnmt</i> (NM_008890)	5'-GTGAAGCGAGTCCCTGCCTATC-3'
	5'-AAGATGCCTTTGACATCATCTACC-3'
<i>Rig/RpS15</i> (NM_009091)	5'-ACGGCAAGACCTTCAACCAG-3'
	5'-ATGGAGAACTCGCCAGGTAG-3'

Table 2. PCR primers (human) for the real-time RT-PCR.

Target mRNA/MiR	Primer Sequence
<i>TH</i> (NM_199292)	5'-GGAGTTCGGGCTGTGTAAGCA-3'
	5'-GACTGGTACGTCTGGTCTTGGTAGG-3'
<i>DDC</i> (NM_001082971)	5'-GAACAGACTTAACGGGAGCCTTT-3'
	5'-AATGCCGGTAGTCAGTGATAAGC-3'
<i>DBH</i> (NM_000787)	5'-GTGCTACATTAAGGAGCTTCCAAAG-3'
	5'-GGCCTCATTGCCCTTGGT-3'
<i>PNMT</i> (NM_002686)	5'-CTGACTCGGCCCCGGGCCAG-3'
	5'-GGCCTCCCCAGCCAGGTACC-3'
<i>Dicer</i> (NM_177438)	5'-GAGCTGTCCTATCAGATCAGGG-3'
	5'-ACTTGTTGAGCAACCTGGTTT-3'
β -actin (NM_001101)	5'-GCGAGAAGATGACCCAGA-3'
	5'-CAGAGGCGTACAGGGATA-3'
miR-375 (NR_029867)	5'-AGCCGTTTGTTCGTTCCGGCT-3'
	5'-GTGCAGGGTCCGAGGT-3'
U6 (NR_004394)	5'-CTCGCTTCGGCAGCAC-3'
	5'-AACGCTTCACGAATTTGCGT-3'

4.4. Immunoblot Analysis

The immunoblot analysis was performed using an NB-1 cell extract (5×10^5 cells), as described in previous studies [5,39,52–54], an anti-DBH or -PNMT rabbit polyclonal antibody (Proteintech, Rosemont, IL, USA) and an anti- β -actin monoclonal antibody (Sigma, St. Louis, MO, USA) raised against Ac-Asp-Asp-Asp-Ile-Ala-Ala-Leu-Val-Ile-Asp-Asn-Gly-Ser-Gly-Lys, a SNAP id[®] 2.0 Protein Detection System (Merck Millipore, Burlington, MA, USA), and an ECL Select detection reagent (Cytiva, Marlborough, MA, USA). The band intensities were analyzed using ImageJ software (National Institute of Health, Bethesda, MD, USA), as previously described [5,53,55,56].

4.5. MiRNA Extraction, Reverse Transcription, and Real-Time Quantitative PCR

Total RNA, including miRNA, was isolated from NB-1 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 (Clontech

Laboratories, Inc., Mountain View, CA, USA) according to the manufacturer's protocol. The conditions for PCR were 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, as previously described [5,43,45,50]. The primers are listed in Table 2.

4.6. MiR-375 Mimic Transfection

MiR-375 mimic (5'-UUUGUUCGUUCGGCUCGCGUGAtt-3', 5'-UCACGCGAGCCG AACGAACAAAtt-3') and non-specific control RNA (miR-375 mimic NC) (5'-UUUGUACU ACACAAAAGUACUGtt-3', 5'-CAGUACUUUUGUGUAGUACAAAtt-3') were synthesized by NGRL and introduced into NB-1 cells using Lipofectamine[®] RNAiMAX (Invitrogen) [5,43,45,46,50] just before IH/normoxia exposure, and the mRNA levels of *DBH* and *PNMT* were measured by real-time RT-PCR, as previously described in Section 4.2.

4.7. Data Analysis

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

Author Contributions: S.T. contributed to the study design. S.T. and R.S. contributed to the data collection. S.T., R.S., Y.T. and A.I.-H. contributed to the data analysis. S.T., R.S., Y.T., T.U., A.Y., M.M., S.S.-T., K.A., H.O. and A.I.-H. contributed to the data interpretation. S.T. and R.S. contributed to the preparation of the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported in part by the Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan (grant numbers 08102003, 15K19425, and 21K16344), the Japan Society for the Promotion of Science, and the Japan Science and Technology Agency.

Acknowledgments: We are indebted to Satoshi Ueno (Nara Medical University) for encouragement.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

DBH	Dopamine β -hydroxylase
DDC	DOPA decarboxylase
DOPA	L-3,4-dihydroxyphenylalanine
Dicer	Endoribonuclease Dicer
FCS	Fetal calf serum
IH	Intermittent hypoxia
miRNA	MicroRNA
PNMT	Phenylethanolamine <i>N</i> -methyltransferase
Rig	Rat insulinoma gene
RpS15	Ribosomal protein S15
SAS	Sleep apnea syndrome
SHRs	Spontaneously hypertensive rats
TH	Tyrosine hydroxylase

References

1. Benjafeld, A.V.; Ayas, N.T.; Eastwood, P.R.; Heinzer, R.; Ip, M.S.M.; Morrell, M.J.; Nunez, C.M.; Patel, S.R.; Penzel, T.; Pépin, J.-L.; et al. Estimation of the global prevalence and burden of obstructive sleep apnoea: A literature-based analysis. *Lancet Respir. Med.* **2019**, *7*, 687–698. [[CrossRef](#)]
2. Ahmad, M.; Makati, D.; Akbar, S. Review of and updates on hypertension in obstructive sleep apnea. *Int. J. Hypertens.* **2017**, *2017*, 1848375. [[CrossRef](#)] [[PubMed](#)]
3. Gislason, T.; Aberg, H.; Taube, A. Snoring and systemic hypertension—an epidemiological study. *Acta Med. Scand.* **1987**, *222*, 415–421. [[CrossRef](#)] [[PubMed](#)]
4. Marrone, O.; Bonsignore, M.R.; Fricano, L.; Lo Coco, R.; Cerasola, G.; Bonsignore, G. Gender and the systemic hypertension-snoring association: A questionnaire-based case-control study. *Blood Press* **1998**, *7*, 11–17. [[CrossRef](#)]

5. Takeda, Y.; Itaya-Hironaka, A.; Yamauchi, A.; Makino, M.; Sakuramoto-Tsuchida, S.; Ota, H.; Kawaguchi, R.; Takasawa, S. Intermittent hypoxia upregulates the *renin* and *Cd38* mRNAs in renin-producing cells via the downregulation of miR-203. *Int. J. Mol. Sci.* **2021**, *22*, 10127. [[CrossRef](#)]
6. Ota, H.; Fujita, Y.; Yamauchi, M.; Muro, S.; Kimura, H.; Takasawa, S. Relationship between intermittent hypoxia and Type 2 diabetes in sleep apnea syndrome. *Int. J. Mol. Sci.* **2019**, *20*, 4756. [[CrossRef](#)]
7. Kimura, H.; Ota, H.; Kimura, Y.; Takasawa, S. Effects of intermittent hypoxia on pulmonary vascular and systemic diseases. *Int. J. Environ. Res. Public Health* **2019**, *16*, 3101. [[CrossRef](#)]
8. Francia, S.; Michelini, F.; Saxena, A.; Tang, D.; de Hoon, M.; Anelli, V.; Mione, M.; Carninci, P.; d'Adda di Fagagna, F. Site-specific DICER and DROSHA RNA products control the DNA-damage response. *Nature* **2012**, *488*, 231–235. [[CrossRef](#)]
9. Zhang, J.; Zhang, X.-H.; Wang, C.-X.; Liu, B.; Fan, X.-S.; Wen, J.-J.; Shi, Q.-L.; Zhou, X.-J. Dysregulation of microRNA biosynthesis enzyme Dicer plays an important role in gastric cancer progression. *Int. J. Clin. Exp. Pathol.* **2014**, *7*, 1702–1707. [[PubMed](#)]
10. Baylor, P.; Mouton, A.; Shamoon, H.H.; Goebel, P. Increased norepinephrine variability in patients with sleep apnea syndrome. *Am. J. Med.* **1995**, *99*, 611–615. [[CrossRef](#)]
11. Ziegler, M.G.; Nelesen, R.; Mills, P.; Ancoli-Israel, S.; Kennedy, B.; Dimsdale, J.E. Sleep apnea, norepinephrine-release rate, and daytime hypertension. *Sleep* **1997**, *20*, 224–231. [[CrossRef](#)] [[PubMed](#)]
12. Semenza, G.L.; Prabhakar, N.R. Neural regulation of hypoxia-inducible factors and redox state drives the pathogenesis of hypertension in a rodent model of sleep apnea. *J. Appl. Physiol.* **2015**, *119*, 1152–1156. [[CrossRef](#)] [[PubMed](#)]
13. Khurana, S.; Peng, S.; McDonald, E.; Yates, W.; Venkataraman, K.; Tai, T.C. Phenylethanolamine N-methyltransferase gene expression in PC12 cells exposed to intermittent hypoxia. *Neurosci. Lett.* **2018**, *666*, 169–174. [[CrossRef](#)] [[PubMed](#)]
14. Nurse, C.A.; Salman, S.; Scott, A.L. Hypoxia-regulated catecholamine secretion in chromaffin cells. *Cell Tissue Res.* **2018**, *372*, 433–441. [[CrossRef](#)]
15. Makarenko, V.V.; Peng, Y.J.; Khan, S.A.; Nanduri, J.; Fox, A.P.; Prabhakar, N.R. Long-term facilitation of catecholamine secretion from adrenal chromaffin cells of neonatal rats by chronic intermittent hypoxia. *J. Neurophysiol.* **2019**, *122*, 1874–1883. [[CrossRef](#)]
16. Gridina, A.; Su, X.; Khan, S.A.; Peng, Y.J.; Wang, B.; Nanduri, J.; Fox, A.P.; Prabhakar, N.R. Gaseous transmitter regulation of hypoxia-evoked catecholamine secretion from murine adrenal chromaffin cells. *J. Neurophysiol.* **2021**, *125*, 1533–1542. [[CrossRef](#)]
17. Grobecker, H.; Roizen, M.F.; Weise, V.; Saavedra, J.M.; Kopin, I.J. Sympathoadrenal medullary activity in young, spontaneously hypertensive rats. *Nature* **1975**, *258*, 267. [[CrossRef](#)]
18. Grobecker, H.; Saavedra, J.M.; Weise, V.K. Biosynthetic enzyme activities and catecholamines in adrenal glands of genetic and experimental hypertensive rats. *Circ. Res.* **1982**, *50*, 742–746. [[CrossRef](#)]
19. Moura, E.; Pinho Costa, P.M.; Moura, D.; Guimarães, S.; Vieira-Coelho, M.A. Decreased tyrosine hydroxylase activity in the adrenals of spontaneously hypertensive rats. *Life Sci.* **2005**, *76*, 2953–2964. [[CrossRef](#)]
20. Nakamura, K.; Nakamura, K. Enhanced sympathetic activity in young spontaneously hypertensive rats is not the trigger mechanism for genetic hypertension. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **1977**, *299*, 143–148. [[CrossRef](#)]
21. Teitelman, G.; Ross, R.A.; Joh, T.H.; Reis, D.J. Differences in utero in activities of catecholamine biosynthetic enzymes in adrenals of spontaneously hypertensive rats. *Clin. Sci.* **1981**, *61*, 227s–230s. [[CrossRef](#)]
22. Kumai, T.; Tanaka, M.; Watanabe, M.; Kobayashi, S. Elevated tyrosine hydroxylase mRNA levels in the adrenal medulla of spontaneously hypertensive rats. *Jpn. J. Pharmacol.* **1994**, *65*, 367–369. [[CrossRef](#)] [[PubMed](#)]
23. Yamada, H.; De Jong, W.; Lovenberg, W. Further studies on catecholamine synthesis in the spontaneously hypertensive rat: Catecholamine synthesis in the central nervous system. *Eur. J. Pharmacol.* **1973**, *22*, 91–98. [[CrossRef](#)]
24. Nagatsu, T.; Kato, T.; Numata, Y.; Ikuta, K.; Umezawa, H.; Matsuzaki, M.; Takeuchi, T. Serum dopamine β -hydroxylase activity in developing hypertensive rats. *Nature* **1974**, *251*, 630–631. [[CrossRef](#)] [[PubMed](#)]
25. Nagatsu, T.; Ikuta, K.; Numata, Y.; Kato, T.; Sano, M. Vascular and brain dopamine β -hydroxylase activity in young spontaneously hypertensive rats. *Science* **1976**, *191*, 290–291. [[CrossRef](#)]
26. Ciaranello, R.D.; Hoffman, H.J.; Shire, J.G.; Axelrod, J. Genetic regulation of the catecholamine biosynthetic enzymes. II. Inheritance of tyrosine hydroxylase, Dopamine- β -hydroxylase, and phenylethanolamine N-methyltransferase. *J. Biol. Chem.* **1974**, *249*, 4528–4536. [[CrossRef](#)]
27. Esteller, M. Non-coding RNAs in human disease. *Nat. Rev. Genet.* **2011**, *12*, 861–874. [[CrossRef](#)]
28. Poy, M.N.; Hausser, J.; Trajkovski, M.; Braun, M.; Collins, S.; Rorsman, P.; Zavolan, M.; Stoffel, M. miR-375 maintains normal pancreatic α - and β -cell mass. *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 5813–5818. [[CrossRef](#)]
29. Frank, A.-C.; Ebersberger, S.; Fink, A.F.; Lampe, S.; Weigert, A.; Schmid, T.; Ebersberger, I.; Syed, S.N.; Brüne, B. Apoptotic tumor cell-derived microRNA-375 uses CD36 to alter the tumor-associated macrophage phenotype. *Nat. Commun.* **2019**, *10*, 1135. [[CrossRef](#)]
30. Zhang, J.; Gao, J.; Zhang, D.; Liu, H.; Gou, K.; Cui, S. miR-375 acts as a novel factor modulating pituitary prolactin synthesis through *Rasd1* and *Esr1*. *J. Endocrinol.* **2021**, *250*, 25–35. [[CrossRef](#)]
31. Yang, X.; Nanayakkara, J.; Claypool, D.; Saghafinia, S.; Wong, J.J.M.; Xu, M.; Wang, X.; Nicol, C.J.B.; Michael, I.P.; Hafner, M.; et al. A miR-375/YAP axis regulates neuroendocrine differentiation and tumorigenesis in lung carcinoid cells. *Sci. Rep.* **2021**, *11*, 10455. [[CrossRef](#)] [[PubMed](#)]
32. Xu, J.-Y.; Song, W.; Lu, F.-F.; Dai, Z.-J.; Cao, L.-H.; Lin, S. miR-375 inhibits the proliferation and invasion of nasopharyngeal carcinoma cells by suppressing PDK1. *Biomed. Res. Int.* **2020**, *2020*, 9704245. [[CrossRef](#)] [[PubMed](#)]

33. Wu, W.; Zhang, Y.; Li, X.; Wang, X.; Yuan, Y. miR-375 inhibits the proliferation, migration and invasion of esophageal squamous cell carcinoma by targeting XPR1. *Curr. Gene Ther.* **2021**, *21*, 290–298. [[CrossRef](#)] [[PubMed](#)]
34. Sánchez-de-la-Torre, M.; Khalyfa, A.; Sánchez-de-la-Torre, A.; Martínez-Alonso, M.; Martínez-García, M.Á.; Barceló, A.; Lloberes, P.; Campos-Rodriguez, F.; Capote, F.; Diaz-de-Atauri, M.J.; et al. Precision medicine in patients with resistant hypertension and obstructive sleep apnea: Blood pressure response to continuous positive airway pressure treatment. *J. Am. Coll. Cardiol.* **2015**, *66*, 1023–1032. [[CrossRef](#)]
35. Bouça, B.; Bogalho, P.; Rizzo, M.; Silva-Nunes, J. The role of metabolome and non-coding RNA on pheochromocytomas and paragangliomas: An update. *Metabolites* **2022**, *12*, 131. [[CrossRef](#)]
36. Chen, X.; Li, B.; Luo, R.; Cai, S.; Zhang, C.; Cao, X. Analysis of the function of microRNA-375 in humans using bioinformatics. *Biomed. Rep.* **2017**, *6*, 561–566. [[CrossRef](#)]
37. Zhang, L.; Liu, J.; Cheng, P.; Lv, F. Correlation between miRNA target site polymorphisms in the 3' UTR of *AVPR1A* and the risk of hypertension in the Chinese Han population. *Biosci. Rep.* **2019**, *39*, BSR20182232. [[CrossRef](#)]
38. Nakazawa, T.; Takasawa, S.; Noguchi, N.; Nata, K.; Tohgo, A.; Mori, M.; Nakagawara, K.; Akiyama, T.; Ikeda, T.; Yamauchi, A.; et al. Genomic organization, chromosomal localization, and promoter of human gene for FK506-binding protein 12.6. *Gene* **2005**, *360*, 55–64. [[CrossRef](#)]
39. Takasawa, S.; Kuroki, M.; Nata, K.; Noguchi, N.; Ikeda, T.; Yamauchi, A.; Ota, H.; Itaya-Hironaka, A.; Sakuramoto-Tsuchida, S.; Takahashi, I.; et al. A novel ryanodine receptor expressed in pancreatic islets by alternative splicing from *type 2 ryanodine receptor* gene. *Biochem. Biophys. Res. Commun.* **2010**, *397*, 140–145. [[CrossRef](#)]
40. Shobatake, R.; Takasawa, K.; Ota, H.; Itaya-Hironaka, A.; Yamauchi, A.; Sakuramoto-Tsuchida, S.; Uchiyama, T.; Makino, M.; Sugie, K.; Takasawa, S.; et al. Up-regulation of *POMC* and *CART* mRNAs by intermittent hypoxia via GATA transcription factors in human neuronal cells. *Int. J. Biochem. Cell Biol.* **2018**, *95*, 100–107. [[CrossRef](#)]
41. Ota, H.; Tamaki, S.; Itaya-Hironaka, A.; Yamauchi, A.; Sakuramoto-Tsuchida, S.; Morioka, T.; Takasawa, S.; Kimura, H. Attenuation of glucose-induced insulin secretion by intermittent hypoxia via down-regulation of CD38. *Life Sci.* **2012**, *90*, 206–211. [[CrossRef](#)] [[PubMed](#)]
42. Ota, H.; Itaya-Hironaka, A.; Yamauchi, A.; Sakuramoto-Tsuchida, S.; Miyaoka, T.; Fujimura, T.; Tsujinaka, H.; Yoshimoto, K.; Nakagawara, K.; Tamaki, S.; et al. Pancreatic β cell proliferation by intermittent hypoxia via up-regulation of *Reg* family genes and *HGF* gene. *Life Sci.* **2013**, *93*, 664–672. [[CrossRef](#)] [[PubMed](#)]
43. Uchiyama, T.; Ota, H.; Itaya-Hironaka, A.; Shobatake, R.; Yamauchi, A.; Sakuramoto-Tsuchida, S.; Makino, M.; Kimura, H.; Takeda, M.; Ohbayashi, C.; et al. Up-regulation of *selenoprotein P* and *HIP/PAP* mRNAs in hepatocytes by intermittent hypoxia via down-regulation of miR-203. *Biochem. Biophys. Res. Commun.* **2017**, *11*, 130–137. [[CrossRef](#)]
44. Kyotani, Y.; Itaya-Hironaka, A.; Yamauchi, A.; Sakuramoto-Tsuchida, S.; Makino, M.; Takasawa, S.; Yoshizumi, M. Intermittent hypoxia-induced epiregulin expression by IL-6 production in human coronary artery smooth muscle cells. *FEBS Open Bio* **2018**, *8*, 868–876. [[CrossRef](#)] [[PubMed](#)]
45. Uchiyama, T.; Itaya-Hironaka, A.; Yamauchi, A.; Makino, M.; Sakuramoto-Tsuchida, S.; Shobatake, R.; Ota, H.; Takeda, M.; Ohbayashi, C.; Takasawa, S. Intermittent hypoxia up-regulates *CCL2*, *RETN*, and *TNF α* mRNAs in adipocytes via down-regulation of miR-452. *Int. J. Mol. Sci.* **2019**, *20*, 1960. [[CrossRef](#)] [[PubMed](#)]
46. Shobatake, R.; Itaya-Hironaka, A.; Yamauchi, A.; Makino, M.; Sakuramoto-Tsuchida, S.; Uchiyama, T.; Ota, H.; Takahashi, N.; Ueno, S.; Sugie, K.; et al. Intermittent hypoxia up-regulates gene expressions of *peptide YY (PYY)*, *glucagon-like peptide-1 (GLP-1)*, and *neurotensin (NTS)* in enteroendocrine cells. *Int. J. Mol. Sci.* **2019**, *20*, 1849. [[CrossRef](#)]
47. Murphy, A.M.; Thomas, A.; Crinion, S.J.; Kent, B.D.; Tambuwala, M.M.; Fabre, A.; Pepin, J.-L.; Roche, H.M.; Arnaud, C.; Ryan, S. Intermittent hypoxia in obstructive sleep apnoea mediates insulin resistance through adipose tissue inflammation. *Eur. Respir. J.* **2017**, *49*, 1601731. [[CrossRef](#)]
48. Ota, H.; Takasawa, S.; Yamauchi, M.; Yoshikawa, M.; Tomoda, K.; Kimura, H. Intermittent hypoxia in pancreatic beta cells. *Pancreat. Disord. Ther.* **2015**, *5*, S5-004. [[CrossRef](#)]
49. Tohma, Y.; Dohi, Y.; Shobatake, R.; Uchiyama, T.; Takeda, M.; Takasawa, S.; Tanaka, Y.; Ohgushi, H. *Reg* gene expression in periosteum after fracture and its in vitro induction triggered by IL-6. *Int. J. Mol. Sci.* **2017**, *18*, 2257. [[CrossRef](#)]
50. Takasawa, S.; Tsuchida, C.; Sakuramoto-Tsuchida, S.; Takeda, M.; Itaya-Hironaka, A.; Yamauchi, A.; Misu, M.; Shobatake, R.; Uchiyama, T.; Makino, M.; et al. Expression of human *REG* family genes in inflammatory bowel disease and their molecular mechanism. *Immunol. Res.* **2018**, *66*, 800–805. [[CrossRef](#)]
51. Akasaka, J.; Naruse, K.; Sado, T.; Uchiyama, T.; Makino, M.; Yamauchi, A.; Ota, H.; Sakuramoto-Tsuchida, S.; Itaya-Hironaka, A.; Takasawa, S.; et al. Involvement of receptor for advanced glycation endproducts in hypertensive disorders of pregnancy. *Int. J. Mol. Sci.* **2019**, *20*, 5462. [[CrossRef](#)] [[PubMed](#)]
52. Takasawa, S.; Tohgo, A.; Noguchi, N.; Koguma, T.; Nata, K.; Sugimoto, T.; Yonekura, H.; Okamoto, H. Synthesis and hydrolysis of cyclic ADP-ribose by human leukocyte antigen CD38 and inhibition of the hydrolysis by ATP. *J. Biol. Chem.* **1993**, *268*, 26052–26054. [[CrossRef](#)]
53. Yoshimoto, K.; Fujimoto, T.; Itaya-Hironaka, A.; Miyaoka, T.; Sakuramoto-Tsuchida, S.; Yamauchi, A.; Takeda, M.; Kasai, T.; Nakagawara, K.; Nonomura, A.; et al. Involvement of autoimmunity to REG, a regeneration factor, in patients with primary Sjögren's syndrome. *Clin. Exp. Immunol.* **2013**, *174*, 1–9. [[CrossRef](#)]

54. Kato, I.; Yamamoto, Y.; Fujimura, M.; Noguchi, N.; Takasawa, S.; Okamoto, H. CD38 disruption impairs glucose-induced increases in cyclic ADP-ribose, $[Ca^{2+}]_i$, and insulin secretion. *J. Biol. Chem.* **1999**, *274*, 1869–1872. [[CrossRef](#)] [[PubMed](#)]
55. Ikehata, F.; Satoh, J.; Nata, K.; Tohgo, A.; Nakazawa, T.; Kato, I.; Kobayashi, S.; Akiyama, T.; Takasawa, S.; Toyota, T.; et al. Autoantibodies against CD38 (ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase) that impair glucose-induced insulin secretion in noninsulin-dependent diabetes patients. *J. Clin. Investig.* **1998**, *102*, 395–401. [[CrossRef](#)] [[PubMed](#)]
56. Shervani, N.J.; Takasawa, S.; Uchigata, Y.; Akiyama, T.; Nakagawa, K.; Noguchi, N.; Takada, H.; Takahashi, I.; Yamauchi, A.; Ikeda, T.; et al. Autoantibodies to REG, a beta-cell regeneration factor, in diabetic patients. *Eur. J. Clin. Investig.* **2004**, *34*, 752–758. [[CrossRef](#)]