R E S E A R C H   A R T I C L E

Role of HIF-1, Siah-1 and SKN-1 in Inducing Adiposity for *Caenorhabditis elegans* under Hypoxic Conditions

I Gede Widhiantara[1], Anak Agung Ayu Putri Permatasari[1], I Wayan Rosiana[1],
I Wayan Putu Sutirtayasa[2], Ferbian Milas Siswanto[1,*]

[1]Department of Biology, Faculty of Health and Science, Universitas Dhyana Pura, Jl. Raya Padangluwih, Badung, Indonesia
[2]Department of Clinical Pathology, Faculty of Medicine, Universitas Udayana, Jl. P.B. Sudirman, Denpasar, Indonesia

*Corresponding author. E-mail: ferbianms@undhirabali.ac.id

Abstract

BACKGROUND: Hypoxia has been shown to be able to induce adiposity. However, the mechanism and factors involved in this effect still remains unclear. Hence, we sought to investigate the role of oxygen-sensitive factors regarding hypoxia-induced adiposity in nematode *Caenorhabditis elegans*.

METHODS: The *C. elegans* were grown on nematode growth medium (NGM) agar plates seeded with *Escherichia coli* OP50 at 20°C. The ratio of width/body length was measured using the morphometry analysis. Fat accumulation was examined using Sudan Black methods. Protein levels of sterol binding protein (SBP)-1 were assessed by immunoblotting. Lifespan assay was performed at 20°C and was monitored every two days.

RESULTS: The results showed that of all mutant used, only *hif-1* mutant which did not experience an increase in the ratio of width/body length ($p>0.05$) and fat accumulation ($p>0.05$), indicating that hypoxia-inducible factors (HIF)-1 plays an important role in the pathogenesis of hypoxia-induced adiposity. Both *siah-1* and *skn-1* mutants experienced SBP-1 protein elevation ($p<0.05$), and increased *fat-6* mRNA expression ($p<0.05$) which were not experienced by a *hif-1* mutant ($p>0.05$) further supporting that HIF-1 acts as an upstream regulator from SBP-1.

CONCLUSION: In general, the results of this study provide evidences of the involvement of the transcription factor HIF-1 in inducing adiposity under the hypoxic conditions. However, we did not find the involvement of seven in absentia homolog-1 (Siah-1) and skinhead-1 (SKN-1).

KEYWORDS: hypoxia, adiposity, fat, HIF-1, Siah-1, SKN-1, *C. elegans*

Indones Biomed J. 2020; 12(1): 51-6

Introduction

Excess body adiposity including abdominal obesity and ectopic fat accumulation, is one of the major health problems worldwide and has shown an increasing trend in its prevalence over time.(1-3) Adiposity is a significant risk factor and contributor to increased morbidity and mortality of several diseases including cardiovascular disease (CVD), diabetes, cancer and chronic diseases, including osteoarthritis, liver and kidney disease, sleep apnea, and depression.(4) Recently, it has been found in addition to dietary factors, adiposity might be induced by a condition of lack of oxygen or hypoxia.(5,6) Hypoxia has been shown experimentally to induce adiposity in worms and mice (7,8), but it likely has not been shown in humans. However, epidemiologic study shows that people who suffer from diseases such as chronic obstructive pulmonary disease, sleep apnea, and asthma are known to be at high risk for obesity.(9) Another research shows that populations living at high altitudes with low oxygen concentration have a high risk of obesity.(10)

Study in *Caenorhabditis elegans* revealed that hypoxia induces fat accumulation and causes increasing body width/length ratio which is mediated by transcription factors sterol binding protein response element (SBP-1).(7,11) SBP-1 is a transcription factor that is responsible for regulating the expression of genes involved in the synthesis of fatty acids (including *elo-2, fat-2, fat-5, fat-6,* and *fat-7*) and inhibits the expression of genes involved in starvation response such as *acs-2*.(12) However, the mechanism by which SBP-1 is activated during hypoxia remains unidentified.

In this study, we examined the possible role of hypoxia-inducible factor 1 (HIF-1), seven in absentia homolog-1 (Siah-1) and skinhead-1 (SKN-1) to the hypoxia-induced adiposity. All those factors are strongly influenced by oxygen concentration and cellular redox status (13,14), so these factors are very likely an upstream regulator of SBP-1 which induces adiposity caused by hypoxia.

Methods

C. elegans Strains and Maintenance

All strains were incubated at the appropriate temperature on the nematode growth medium (NGM) as described previously.(15,16) The following strains used in this study were from Caenorhabditis Genetic Center (Minneapolis, USA) Wild-type reference strains (Bristol N2), VC1772 (skn-1(ok2315)IV/nT1[qIs51] (IV; V)), ZG31 (hif-1(ia4) V). The Siah-1 strain (tm1968) was from the National BioResource Project (Tokyo, Japan). All strains used in this study have been outcrossed at least 4 times.

Body Width/Length Ratios Measurement

Examination of worm body width/length ratios was carried out on images observed with a microscope connected with Optilab connected to computers equipped with Image Raster software under 25x magnification. The length of the animal was observed from the head to the tip of the tail and the width of the animal traced to the diameter of the body's largest body perpendicular to the longitudinal axis of the worm. Body width/length ratios was observed in 10 worms for each experimental group with 3 times replication.

Total Lipid Examination

All worms were initially stained for lipid content with Sudan Black followed by extraction modified according to what had been done and described previously.(7) Fluorescence of worm fraction dissolved in chloroform-methanol solvent was measured in 96 well plates at 598 nm emission wavelength using SpectraMax plate readers (Molecular Devices LLC, San Jose, USA). Total lipid was observed in 3 plates containing 100 worms/plate for each experimental group with 3 times replication.

Immunoblotting

SBP-1 and β-actin protein levels were examined by immunoblotting techniques as described in previous studies. (16,17) In brief, the protein is blotted to the nitrocellulose membrane (Bio-Rad, California, USA). Then, the membrane was incubated with primary antibodies against SBP-1 and β-actin (Santa Cruz Biotechnology, Santa Cruz, USA), and visualized using horseradish peroxidase-conjugate to goat IgG anti-rabbit (secondary antibody) (Bio-Rad) and with 4-chloro-1-naphthol (Bio-Rad). The band intensity was quantified using the National Institute of Health (NIH) Image software and normalized to the intensity of the β-actin band. The protein levels of SBP-1 was observed in 3 plates containing 100 worms/plate for each experimental group with 2 times replication.

Isolation of RNA and Reverse Transcription-Polymerase chain reaction (RT-PCR)

About 300 worms were collected and washed from *Escherichia coli* OP50 contamination using M9 buffer (22 mM KH_2PO_4, 22 mM Na_2HPO_4, 85 mM NaCl, 1 mM $MgSO_4$). RNA isolation was carried out by the Trizol method (13), then RT-PCR was examined to determine the amount of *fat-6* mRNA.

The results of RNA extraction were then used for the One-Step RT-PCR process. In each microtube 5 μL, R-Mix was added consisting of buffer, MgCl and deoxyribonucleotide triphosphate (dNTP), 0.6 μL primary forward, 0.6 μL primary backward, 2.55 μL aquabidest, 0.25 μL enzyme, and 1 μL RNA sample template. The primers used for amplification of *fat-6* (NCBI Reference Sequence, GenBank accession No.: NM_001268666.1) were 5′-CGGCGGCCCAGAGACGCAAT-3′ (forward primer) and 5′-CCTCCCTCTCCGACGGCAGC-3′ (reverse primer). Whereas *act-1* primers (NCBI Reference Sequence, GenBank accession No.: NM_073418.5) as an internal control were 5′-ACGACGAGTCCGGCCCATCC-3′ (forward primer) and 5′-GAAAGCTGGTGGTGACGATGGTT-3′ (reverse primer).

The reaction used in the PCR program was broadly divided into three stages, namely denaturation at 94°C for 45 seconds, annealing for 45 seconds at 55°C and elongation at 72°C for one minute. The entire PCR process lasts 30 cycles. Each 10 μL of PCR product obtained was then mixed with

DOI: 10.18585/inabj.v12i1.1007

The Role of Oxygen-Sensitive Factors in Hypoxia-Induced Adiposity (Widhiantara IG, *et al.*)
Indones Biomed J. 2020; 12(1): 51-6

2 µL 6x loading dye then electrophoresis for 25 minutes in a 2% agarose gel with 1x Tris-acetate-EDTA (TAE) running buffer, then visualized with ethidium bromide solution (0.5 µg/mL). The interpretation of the results was done by the calibrator method. The *fat-6* mRNA levels were observed in 3 plates containing 100 worms/plate for each experimental group with 2 times replication.

Statistical Analysis
Body width/length ratio, total fat concentration, SBP-1 protein level, and *fat-6* expression level were presented in the graph as mean±standard deviation and tested using one-way ANOVA followed by Bonferroni post hoc test. Differences were considered significant when $p < 0.05$ (*) or $p < 0.01$ (**).

Results

Body Width/Length Ratios
The results of an examination of body width/length ratios in wild-type N2 strains showed that hypoxic conditions induced an increase in body width/length ratio, which indicates that hypoxia caused the appearance of the obesity phenotype ($p < 0.01$; Figure 1). This increase also occurred in the *siah-1* mutant ($p < 0.01$) and *skn-1* mutant ($p < 0.01$) but did not occur in *hif-1* mutant ($p > 0.05$).

Total Lipid
The results of total body lipid examination in wild-type N2 strains showed that hypoxic conditions induced an increase in total lipid levels, which indicated that hypoxia leads to

the emergence of one obesity phenotype namely an increase in fat depots ($p < 0.01$; Figure 2). This increase also occurred in the *siah-1* mutant ($p < 0.01$) and *skn-1* mutant ($p < 0.01$) but did not occur in *hif-1* mutant ($p > 0.05$). This supports the results of an examination of body width/length ratios where induction of obesity phenotype occurs in wild-type N2, *siah-1*, and *skn-1* mutant, but did not occur in the *hif-1* mutant.

SBP-1 Protein Levels
The results of the examination showed the induction of hypoxia in wild-type N2 strains induced an increase in levels of active SBP-1 protein ($p < 0.01$; Figure 3). This increase also occurs in the *siah-1* mutant ($p < 0.05$) and *skn-1* mutant ($p < 0.01$), but it did not occur in *hif-1* mutant ($p > 0.05$).

***fat-6* mRNA Levels**
The *fat-6* genes is a downstream putative of the SBP-1 transcription factor. The results of the examination showed the induction of hypoxia in wild-type N2, *siah-1* mutant and *skn-1* mutant strains induced an increase in *fat-6* mRNA levels ($p < 0.05$ for all) but did not occur in the *hif-1* mutant ($p > 0.05$; Figure 4).

Discussion

The results of this study indicate that hypoxia causes obesity phenotypes including elevation of body width/length ratio and increasing total fat in *C. elegans*. The results of this study are in line with previous studies showing in C57BL6/J mice that short-term hypoxia causes hypertriglyceridemia
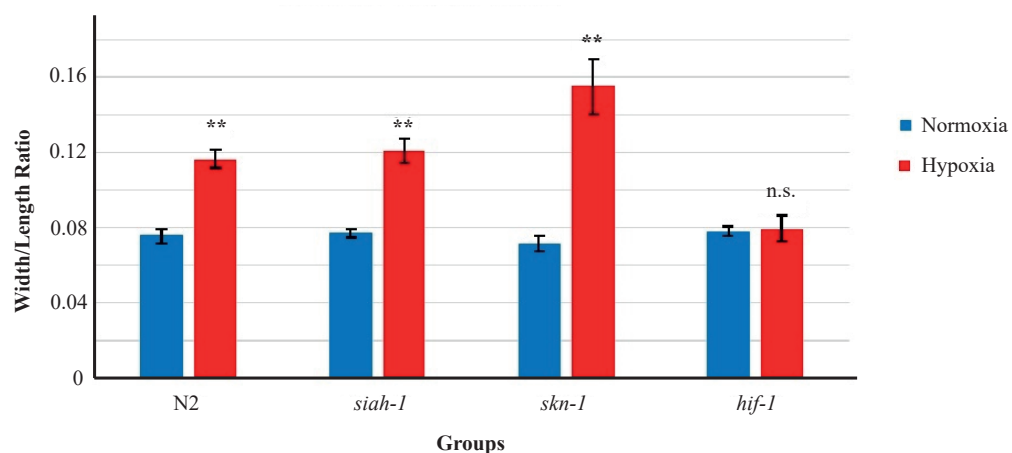
Figure 1. Quantitative results of width/length ratio of *C. elegans* body under normoxia and hypoxia conditions from wild-type N2, *siah1* mutant, *skn-1* mutant, and *hif-1* mutant. **significance $p < 0.01$; n.s.: not significant compared to normoxia treatment in the same group.
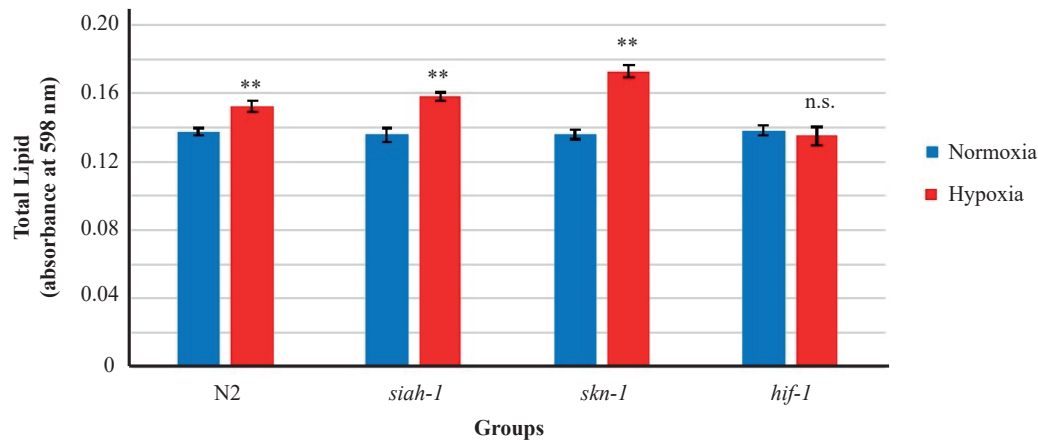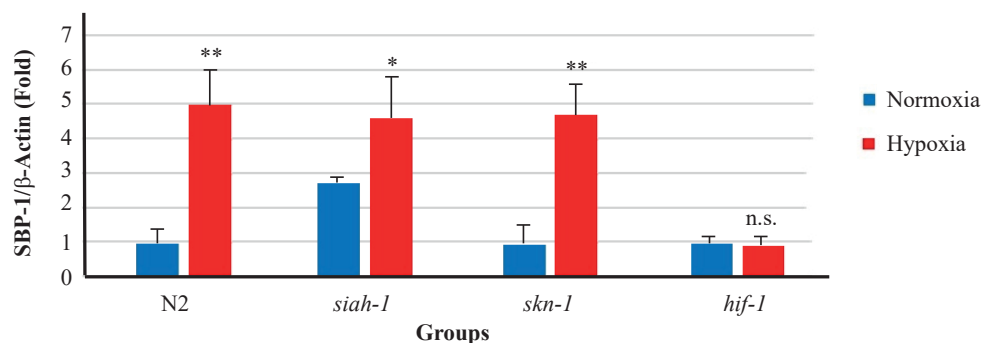
Figure 2. Total lipid levels of *C. elegans* in normoxia and hypoxia conditions from wild-type N2, *siah1* mutant, *skn-1* mutant, and *hif-1* mutant. **significance $p<0.01$, n.s.: not significant compared to normoxia treatment in the same group.

and hypercholesterolemia, which are the common features of adiposity-related metabolic diseases.(5) This theory is supported by the fact that people who suffer from hypoxic related diseases are at high risk for fat buildup and obesity. (9) Study also supports that populations living in the high altitude that is vulnerable to low oxygen concentrations have a higher risk of obesity.(10)
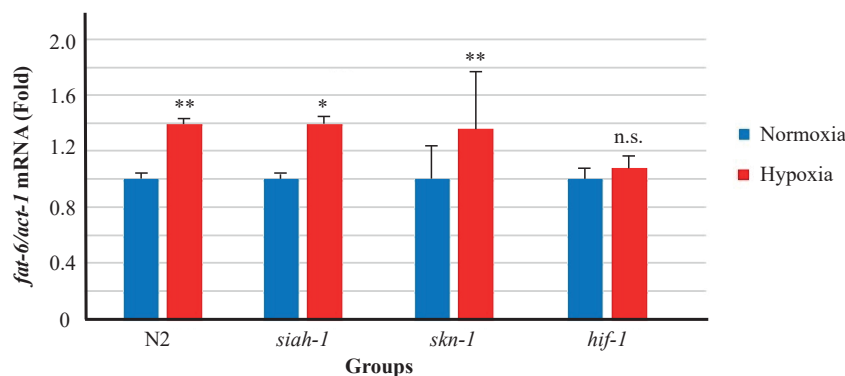
SBP-1 has an important role in lipid homeostasis in *C. elegans* and has previously been shown to regulate fat accumulation.(18) The study discussed the role of SBP-1 in hypoxic-induced lipid accumulation which found a strong causal relationship between activation of SBP-1 by anoxia and increased body size and shape.(7) It is also interesting to note that people living in high altitude areas have shorter, less muscular and fatter bodies than peers with similar genetic backgrounds that live at lower altitudes.(19) Whether the mechanism that depends on sterol regulatory element-binding protein (SREBP)-1 involved in this phenomenon

remains to be studied. Because several recent studies have been conducted on both human animal models and rodents that have shown activation of SREBPs in the pathogenesis of obesity and obesity-related disorders, activation of SREBP-1 can also function as an oxygen sensor for lipid metabolism and can play an important role in accumulated pathogenesis lipids and obesity in humans who lack chronic oxygen.

Hypoxia increases the biosynthesis of triglyceride (TG) in the liver by increasing SREBP -1, an important transcription factor in lipid biosynthesis (20), and its downstream, including important enzymes from TG biosynthesis, stearoyl-CoA desaturase (SCD)-1 (21). The hypoxic mechanism for increasing SREBP-1 has not been previously known. Hypoxia induces the transcription activity of HIF-1 (22), shows that HIF-1 can regulate the SREBP-1 pathway that augments lipid biosynthesis. HIF-1 is the main regulator of oxygen homeostasis and controls

Figure 3. Quantitative SBP-1 protein levels of the *C. elegans* in normoxia and hypoxia conditions from wild-type N2, *siah1* mutant, *skn-1* mutant, and *hif-1* mutant. *significance $p<0.05$; **significance $p<0.01$; n.s.: not significant compared to normoxia treatment in the same group.

DOI: 10.18585/inabj.v12i1.1007

The Role of Oxygen-Sensitive Factors in Hypoxia-Induced Adiposity (Widhiantara IG, *et al.*)
Indones Biomed J. 2020; 12(1): 51-6

Figure 4. The examination of *fat-6* mRNA levels isolated from wild-type N2, *siah1* mutant, *skn-1* mutant, and *hif-1* mutant under both normoxia and hypoxia conditions. *significance $p<0.05$; **significance $p<0.01$; n.s.: not significant compared to normoxia treatment in the same group.

various physiological responses to hypoxia, including erythropoiesis, angiogenesis, and glucose metabolism.(17) But the effects of HIF-1 on lipid metabolism have never been studied before.

This study produced several new findings. Partial HIF-1 deficiency prevents an increase in body width/length ratio, total lipids, SBP-1 and fat-1 caused by hypoxia. Previously, studies in mice showed that hypoxia had a clearer effect on SREBP-1 mRNA levels in het mice than in wild-type mice, indicating that HIF-1 does not regulate SREBP-1 expression at the transcription level. However, a partial deficiency of HIF-1 prevents an increase caused by hypoxia in SREBP, which indicates that HIF-1 affects SREBP-1 at the posttranscriptional level. HIF-1 can be set on SREBP-1 through SREBP cleavage-activating protein (SCAP). SCAP facilitates the transport of SREBPs from the endoplasmic reticulum to the Golgi apparatus, where active SREBP isoforms are cleaved by specific proteases.(23-25). Specifically, the SCAP gene promoter contains a binding site for HIF-1, namely 5-ACGTG-3.(26) Het mice show impaired SCAP induction in response to hypoxia. Thus HIF-1 can increase lipid biosynthesis in the liver during hypoxia by stimulating SREBP-1 activity through SCAP. (5) However, further research is still needed to examine the nature of regulation of SBP-1/SREBP-1 which depends on HIF-1.

The Siah1 and SKN-1 apparently do not contribute to the hypoxia-induced adiposity in *C. elegans*. These results are in contrast with previously reported role of mammalian Siah2 in lipid synthesis of hypoxic tumor growth which found that knockdown of Siah2 attenuates the hypoxic depletion of αKGDH activity, triggers glutamine oxidation, and decreases glutamine-dependent lipid synthesis.(27) Additionally, hypoxia decreases basal levels of SKN-1 (16) and the mammalian Nrf2 (SKN-1 homolog) is revealed to suppress both lipid biosynthesis and desaturation genes including (nicotinamide adenine dinucleotide phosphate) NADPH-utilizing enzymes fatty acid synthase (FASN) and stearoyl-CoA desaturase 1 (SCD1).(28) However, this study does not confirm that SKN-1 is important for hypoxia-induced adiposity. For further suggestion, it is necessary to conduct research using immunoprecipitation analysis to determine whether there is physical contact between HIF-1 and SBP-1 or SCAP.

Conclusion

In general, the results of this study provide evidence of the involvement of the transcription factor HIF-1, and not Siah-1 and SKN-1, in inducing adiposity under hypoxic conditions. Further research is needed by observing SCAP levels or observing the hypoxic effect of SBP-1/SREBP-1 regulation on SCAP mutants.

Acknowledgements

References

1. Hruby A, Hu FB. The epidemiology of obesity: a big picture. Pharmacoeconomics. 2015; 33: 673–89.
2. Agha M, Agha R. The rising prevalence of obesity. Int J Surg Oncol 2017; 2: e17. doi:10.1097/IJ9.0000000000000017.
3. Kuehn B. Obesity rates increasing. JAMA 2018; 320: 1632. doi: 10.1001/jama.2018.15094.

4.   Pi-Sunyer X. The medical risks of obesity. Postgrad Med 2009; 121: 21-33.

5.   Li J, Bosch-Marce M, Nanayakkara A, Savransky V, Fried SK, Semenza GL, *et al.* Altered metabolic responses to intermittent hypoxia in mice with partial deficiency of hypoxia-inducible factor-1α. Physiol Genomics. 2006; 25: 450-7.

6.   Jun J, Reinke C, Bedja D, Berkowitz D, Bevans-Fonti S, Li J, *et al.* Effect of intermittent hypoxia on atherosclerosis in apolipoprotein E-deficient mice. Atherosclerosis. 2010; 209: 381-6.

7.   Taghibiglou C, Martin HGS, Rose JK, Ivanova N, Lin CHC, Lau HL, *et al.* Essential role of SBP-1 activation in oxygen deprivation induced lipid accumulation and increase in body width/length ratio in Caenorhabditis elegans. FEBS Lett. 2009; 583: 831-4.

8.   Siques P, Brito J, Naveas N, Pulido R, De la Cruz JJ, Mamani M, *et al.* Plasma and liver lipid profiles in rats exposed to chronic hypobaric hypoxia: changes in metabolic pathways. High Alt Med Biol. 2014; 15: 388-95.

9.   McClean KM, Kee F, Young IS, Elborn JS. Obesity and the lung: 1 - Epidemiology. Thorax. 2008; 63: 649-54.

10. Mohanna S, Baracco R, Seclén S. Lipid profile, waist circumference, and body mass index in a high altitude population. High Alt Med Biol. 2006; 7: 245-55.

11. Zheng J, Greenway FL. Caenorhabditis elegans as a model for obesity research. Int J Obes. 2012; 36: 186-94.

12. Nomura T, Horikawa M, Shimamura S, Hashimoto T, Sakamoto K. Fat accumulation in Caenorhabditis elegans is mediated by SREBP homolog SBP-1. Genes Nutr. 2010; 5: 17-27.

13. Kartiko BH, Siswanto FM. Overtraining elevates serum protease level, increases renal p16INK4α gene expression and induces apoptosis in rat kidney. Sport Sci Health. 2018; 14: 1-7. doi: 10.1007/s11332-018-0433-6.

14. Siswanto FM, Jawi IM, Kartiko BH. The role of E3 ubiquitin ligase seven in absentia homolog in the innate immune system: An overview. Vet World. 2018; 11: 1551-7.

15. Sutphin GL, Kaeberlein M. Measuring Caenorhabditis elegans life span on solid media. J Vis Exp. 2009; 27: 1152. doi:10.3791/1152.

16. Siswanto FM. The mechanism behind the elongation of lifespan by SKN-1 (mammalian Nrf2 homolog) and the regulation of SKN-1 in Caenorhabditis elegans. Nishinomiya: Kwansei Gakuin University; 2018.

17. Siswanto FM, Oguro A, Imaoka S. Chlorogenic acid modulates hypoxia response of Hep3B cells. Pers Med Universe. 2017; 6: 12-6.

18. McKay RM, McKay JP, Avery L, Graff JM. C. elegans. Dev Cell. 2003; 4: 131-42.

19. Tripathy V, Gupta R. Growth among Tibetans at high and low altitudes in India. Am J Hum Biol. 2007; 19: 789-800.

20. Ferré P, Foufelle F. SREBP-1c Transcription factor and lipid homeostasis: clinical perspective. Horm Res Paediatr. 2007; 68: 72-82.

21. Li J. Intermittent hypoxia induces hyperlipidemia in lean mice. Circ Res. 2005; 97: 698-706.

22. Yuan G, Nanduri J, Bhasker CR, Semenza GL, Prabhakar NR. Ca2+/calmodulin kinase-dependent activation of hypoxia inducible factor 1 transcriptional activity in cells subjected to intermittent hypoxia. J Biol Chem. 2005; 280: 4321-8.

23. Horton JD. Sterol regulatory element-binding proteins: transcriptional activators of lipid synthesis. Biochem Soc Trans. 2002; 30: 1091-5.

24. Matsuda M, Korn BS, Hammer RE, Moon YA, Komuro R, Horton JD, *et al.* SREBP cleavage-activating protein (SCAP) is required for increased lipid synthesis in liver induced by cholesterol deprivation and insulin elevation. Genes Dev. 2001; 15: 1206-16.

25. Shimano H. SREBPs: physiology and pathophysiology of the SREBP family. FEBS J. 2009; 276: 616-21.

26. Nakajima T, Hamakubo T, Kodama T, Inazawa J, Emi M. Genomic structure and chromosomal mapping of the human sterol regulatory element binding protein (SREBP) cleavage-activating protein (SCAP) gene. J Hum Genet. 1999; 44: 402-7.

27. Sun RC, Denko NC. Hypoxic Regulation of Glutamine Metabolism through HIF1 and SIAH2 Supports Lipid Synthesis that Is Necessary for Tumor Growth. Cell Metab 2014; 19: 285–92. doi: 10.1016/j.cmet.2013.11.022.

28. Wu KC, Cui JY, Klaassen CD. Beneficial role of Nrf2 in regulating NADPH generation and consumption. Toxicol Sci 2011; 123: 590–600. doi: 10.1093/toxsci/kfr183.