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An aryl hydrocarbon receptor induces VEGF expression through ATF4 under glucose deprivation in HepG2

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Abstract

Background: Aryl hydrocarbon receptor (AhR) not only regulates drug-metabolizing enzyme expression but also regulates cancer malignancy. The steps to the development of malignancy include angiogenesis that is induced by tumor microenvironments, hypoxia, and nutrient deprivation. Vascular endothelial growth factor (VEGF) plays a central role in the angiogenesis of cancer cells, and it is induced by activating transcription factor 4 (ATF4).

Results: Recently, we identified that glucose deprivation induces AhR translocation into the nucleus and increases *CYP1A1* and *1A2* expression in HepG2 cells. Here, we report that the AhR pathway induces VEGF expression in human hepatoblastoma HepG2 cells under glucose deprivation, which involves ATF4. *ATF4* knockdown suppressed VEGF expression under glucose deprivation. Moreover, *AhR* knockdown suppressed VEGF and ATF4 expression under glucose deprivation at genetic and protein levels.

Conclusions: The AhR-VEGF pathway through ATF4 is a novel pathway in glucose-deprived liver cancer cells that is related to the microenvironment within a cancer tissue affecting liver cancer malignancy.

Keywords: Aryl hydrocarbon receptor (AhR), Vascular endothelial growth factor (VEGF), Angiogenesis, Activating transcription factor 4 (ATF4), Human hepatocellular liver carcinoma cell

Background

The aryl hydrocarbon receptor (AhR) is a well-known transcription factor that is involved in the detoxification response to pollutants and intrinsic biological processes of multicellular organisms. AhR forms a complex with AhR nuclear translocator (ARNT) and regulates the expression of drug-metabolizing enzymes.

AhR has been shown to be activated by xenobiotics including benzo[a]pyrene and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, and it induces the expression of genes containing an XRE domain in their promoter region [1,2]. The genes, which are activated by AhR, include loci encoding the cytochrome P450 family, which is responsible for the phase I detoxification response, enzymes metabolizing endobiotics and xenobiotics, and other molecules that function in cell differentiation [3-7]. In addition,

AhR is one of the stress response molecules in mouse hepa1c1c7 cells; crosstalk between AhR and nuclear factor erythroid-derived 2-related factor 2 (Nrf2) mediates the oxidative stress response through NAD(P)H dehydrogenase quinone 1 (NQO1) [8]. Miao et al. demonstrated that Nrf2 transcription is directly modulated by an activated AhR [9]. AhR has been studied not only as a mediator of chemical toxicity but also as a regulator of vascular development or angiogenesis [10]. Vascular endothelial growth factor (VEGF) is a potent angiogenic factor that plays a central role in angiogenesis, and it is a recognized gene marker for angiogenesis [11]. VEGF expression is induced by various environmental stresses, nutrient deprivation [12], and hypoxia [13]. Glucose deprivation, one of the nutrient deprivations of MCF-7/ADR cells, induces the expression of cellular homologs of oncogenes and angiogenic factors [14,15]. These results support the hypothesis that glycolytic metabolism is associated with cancer malignancy [16]. In human hepatoblastoma HepG2 cells, glucose deprivation induces VEGF expression [17]. We reported that the nuclear localization of AhR was

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induced in HepG2 cells in low glucose conditions [18]. The AhR localization induced not only *cytochrome P450 family 1* member *A1* (*CYP1A1*) and *IA2* expression but also *Nrf2* expression. In addition, AhR mediates TCDD induced VEGF expression [19] and relates to angiogenesis in mouse [20]. In this study, we report a novel pathway that induces VEGF expression in HepG2 cells in response to glucose deprivation. The response to glucose deprivation that is mediated by AhR induces VEGF expression through activating transcription factor 4 (ATF4) expression in HepG2 cells.

Results

Glucose deprivation induces VEGF expression through AhR in HepG2 cells

In the HepG2 human liver carcinoma cell line, glucose deprivation, or hypoglycemia, enhances *VEGF* mRNA expression [17]. When the medium was exchanged from high glucose medium (4.5 g/L D-glucose) to no glucose medium (0 g/L), *VEGF* mRNA expression was increased in HepG2 cells at 12 and 24 h after the medium exchange (Figure 1A). Correspondingly, the expression level of protein was increased under glucose deprivation at 12 and 24 h after the medium exchange and secreted VEGF was increased under glucose deprivation at 24 h after medium exchange (Figure 1B and C).

VEGF is a potent angiogenic factor that plays a central role in angiogenesis [11], and angiogenesis is a significant step in the pre-malignancy and malignancy of cancer [21]. We expected that AhR was related to the process of cancer cell malignancy. Tryptophan-2,3-dioxygenase (TDO)-derived kynurenine promotes tumor cell survival through AhR with the progression of cell malignancy [22]. AhR is translocated to the nucleus by low glucose conditions in HepG2 cells. AhR that is translocated to the nucleus activates *CYP1A1*, *IA2*, *IB1*, and *Nrf2* expression in HepG2 cells in low glucose conditions [18]. We expected that the nucleus-translocated AhR was related to VEGF expression that was induced by glucose deprivation. When *AhR* expression was knocked down by RNAi, *VEGF* expression did not change in normal glucose conditions (D-glucose = 4.5 g/L). Under glucose deprivation (D-glucose = 0 g/L), *VEGF* expression was suppressed by RNAi against AhR (Figure 1D). The expression and secretion of VEGF protein were clearly suppressed by RNAi against *AhR* under glucose deprivation (Figure 1E and F).

AhR and VEGF pathway analysis *in silico*

Figure 1D, E and F show that AhR is required for increased expression of VEGF in HepG2 cells in glucose deprived-conditions. However, there are no AhR binding regions on the transcriptional regulatory domain of *VEGF*. Therefore, we expected that AhR did not regulate VEGF expression directly. We tried to extract candidate *VEGF* transcriptional

regulators that could be induced by glucose deprivation through *in silico* analyses using Ingenuity Pathway Analysis (IPA). We extracted the molecules that were classified as “regulation of expression” and could possibly regulate *VEGF* expression directly (Figure 2A). Moreover, the candidate molecules of interest were those molecules that could be integrated into the pathway where AhR was a starting molecule and *VEGF* was at the end of the pathway. As a result, ATF4, estrogen receptor 1 (ESR1), and endothelial PAS domain protein 1 (EPAS1) were integrated into the AhR to VEGF pathway (Figure 2B). In low glucose conditions, AhR induced *Nrf2* expression [18]. Similarly, in no glucose conditions, AhR induced *Nrf2* expression (data not shown). In nuclei, phosphorylated Nrf2 protein induced the expression of ATF4 protein [23] and bound to ATF4 protein [24,25]. We expected that ATF4 has the high possibility for interacting with AhR through Nrf2 and inducing VEGF expression. Therefore, we focused on the ATF4 interaction with Nrf2.

AhR regulates ATF 4 expression under glucose deprivation in HepG2 cells

The *ATF4* transcript level was higher after 12 and 24 h of culture in glucose-deprived conditions than in high-glucose conditions (Figure 3A). The protein levels differed between glucose deprivation and high glucose after 24 h of cell culture, but they did not differ much after 12 h of culture (Figure 3B). There are no reports that AhR regulates *ATF4* expression directly. According to our current *in silico* analysis, AhR possibly regulates *ATF4* expression through Nrf2, FOS, CCAAT enhancer-binding protein alpha (CEBPA), MAE, and p53. In HepG2 cells, *AhR* knock-down suppressed *ATF4* expression in glucose-deprived conditions (Figure 3C), and the expression level of ATF4 protein was suppressed by *AhR* knockdown (Figure 3D). These results indicate that ATF4 is included in the AhR pathway and that AhR regulates ATF4 expression.

ATF4 but not Nrf2 mediates the induction of VEGF in glucose deprivation

ATF4 was shown to induce the stress-induced expression of VEGF [26]. In addition, ATF4 regulates VEGF expression in stress-induced angiogenesis, which interacts with Nrf2 [27]. In HepG2 cells, the AhR pathway regulates the stress-induced expression of *Nrf2* [18]. Therefore, we raise the following 3 hypotheses: A) Nrf2 induces VEGF expression by activating ATF4 expression; B) protein interactions between Nrf2 and ATF4 induce VEGF expression; and C) ATF4 activates VEGF expression independent of Nrf2 in stress-induced angiogenesis (Figure 4).

Glucose deprivation-induced expression of VEGF was examined using RNAi against ATF4 or Nrf2 at the mRNA and protein levels (Figure 5). When HepG2 cells were cultured under high glucose conditions, RNAi against

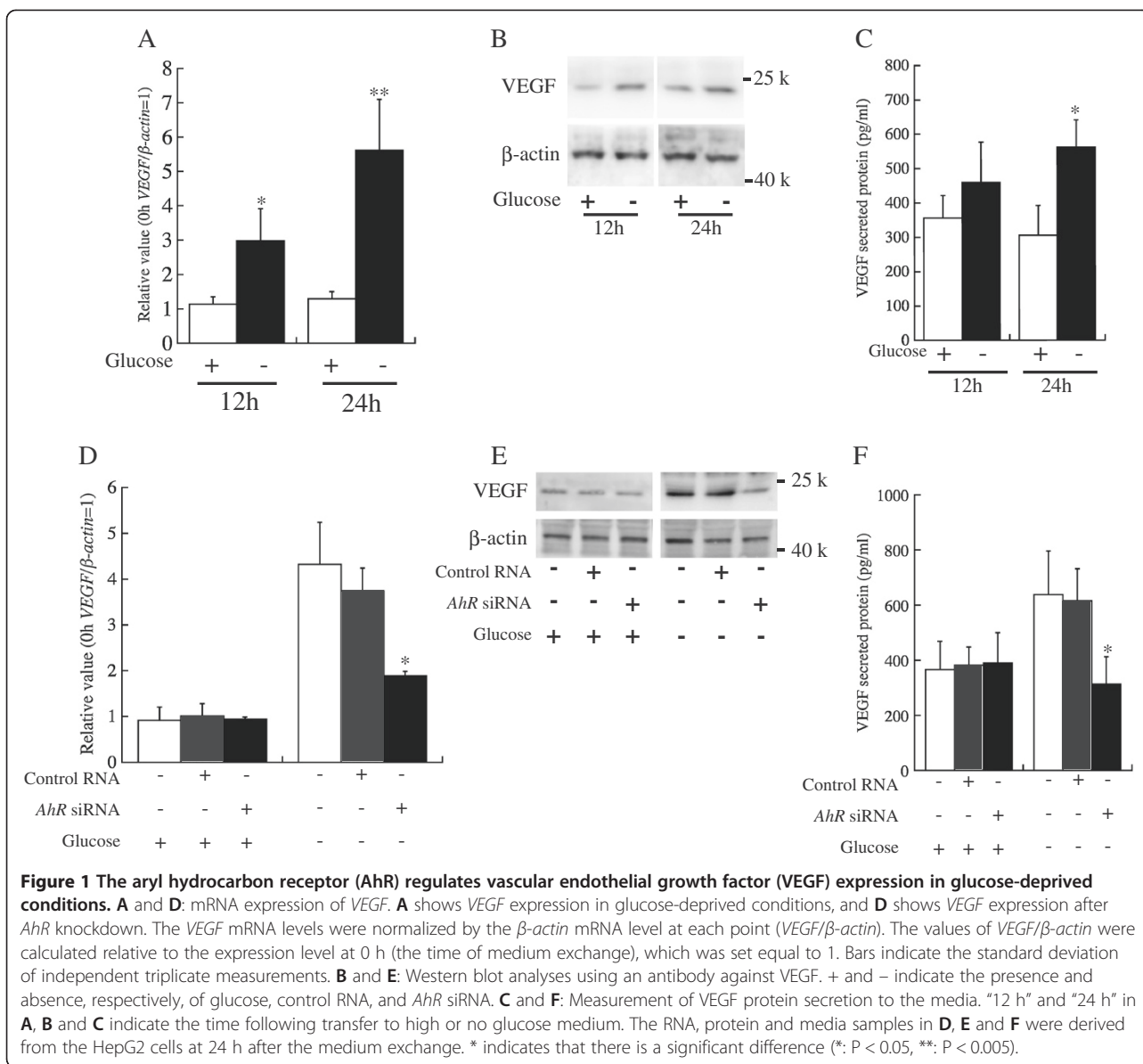


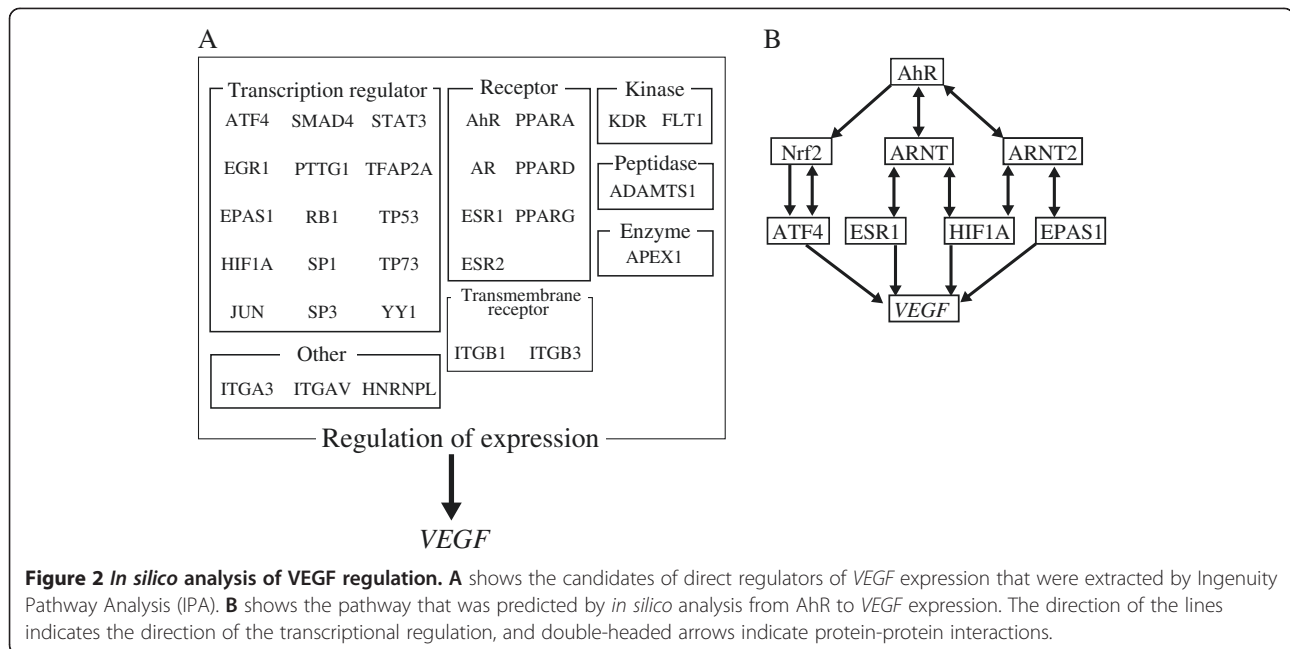
Figure 1 The aryl hydrocarbon receptor (AhR) regulates vascular endothelial growth factor (VEGF) expression in glucose-deprived conditions. **A** and **D**: mRNA expression of *VEGF*. **A** shows *VEGF* expression in glucose-deprived conditions, and **D** shows *VEGF* expression after *AhR* knockdown. The *VEGF* mRNA levels were normalized by the β -actin mRNA level at each point ($VEGF/\beta$ -actin). The values of $VEGF/\beta$ -actin were calculated relative to the expression level at 0 h (the time of medium exchange), which was set equal to 1. Bars indicate the standard deviation of independent triplicate measurements. **B** and **E**: Western blot analyses using an antibody against VEGF. + and - indicate the presence and absence, respectively, of glucose, control RNA, and *AhR* siRNA. **C** and **F**: Measurement of VEGF protein secretion to the media. *12 h" and "24 h" in **A**, **B** and **C** indicate the time following transfer to high or no glucose medium. The RNA, protein and media samples in **D**, **E** and **F** were derived from the HepG2 cells at 24 h after the medium exchange. * indicates that there is a significant difference (*: $P < 0.05$, **: $P < 0.005$).

ATF4 did not affect VEGF expression. However, when HepG2 cells were cultured under glucose-deprived conditions, *ATF4* knockdown suppressed VEGF expression and secretion (Figure 5A,B and C). In contrast, *Nrf2* knockdown did not affect VEGF expression at the mRNA and protein levels and secretion of the VEGF protein (Figure 5D,E and F). No appreciable change in *ATF4* expression at the mRNA and protein levels occurred by *Nrf2* knockdown regardless of the glucose concentration (Figure 6A and B).

Discussion

Lines of evidence suggest that AhR has important roles in cancer pathogenesis, promotion, and malignancy [28,29]. The activation of AhR by a typical agonist,

2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), has been shown to promote tumor formation in both liver and skin [30]. TCDD-mediated liver tumor promotion in multistage rat hepatocarcinogenesis has been studied in detail because of its relevance in xenobiotic-induced toxicity [31]. The expression of mutant AhR molecules by mice and rats suggested that AhR plays a key role in liver tumor promotion [32,33]. In addition, kynurenine acts as an endogenous ligand of the human AhR, and the TDO-AhR pathway is associated with the malignant progression of human brain tumors [22]. Takeuchi et al. and Rhoman et al. have shown that AhR has a role for VEGF expression and angiogenesis in mouse [19,20]. Our results indicate that AhR regulates VEGF expression through *ATF4* in glucose deprived-conditions in HepG2



cells (Figures. 2, 3, and 5). VEGF is an important molecule in angiogenesis for tumor malignancy during carcinogenesis [11]. Angiogenesis is well known as one of the malignancy stages of cancer progression. Shweiki et al. raised the possibility of inducing VEGF expression by hypoxia and glucose deprivation in C6 cells, a clonal glial cell line that is derived from a rat glial tumor [34]. Several studies have demonstrated that glucose deprivation also induces VEGF expression in different types of cells [35-37]. Yun et al. reported that glucose transporter 1 (GLUT1) and 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 (PFKFB3) are important molecules for VEGF expression under glucose deprived-conditions in DU145 cells, a human prostate carcinoma cell line [37]. GLUT1 and PFKFB3 are upregulated by hypoxia-inducible factor 1 (HIF1) [38,39]. Therefore, the VEGF pathway in glucose deprivation is possibly linked to a hypoxia response pathway.

Cancer cells in poorly vascularized solid tumors are constantly or intermittently exposed to glucose deprivation as well as hypoxia [40]. Glucose deprivation activates the unfolded protein response (UPR), a stress-signaling pathway in tumor cells [41]. The UPR induces the expression of the transcription factor ATF4 through PERK [42]. Glucose deficiency or deprivation induces AhR translocation into the nucleus, and the nuclear translocation of AhR activates not only CYP1A1 and CYP1A2 expression [18] but also ATF4 expression (Figure 3C). However, there are no binding elements for AhR in the ATF4 transcriptional regulatory domains, suggesting that other molecules mediate AhR-ATF4 regulation. AhR forms a dimer with ARNT, and the AhR-ARNT complex can bind to dioxin-responsive

elements to activate the transcription of various genes, including *CYP1A1* and *IA2* [43,44]. Tumor cells in the microenvironment of a solid tumor are exposed to hypoxia and/or nutrient deprivation. In the microenvironments within a solid tumor, angiogenesis is triggered by the expression of various molecules including VEGF.

To understand the response of liver cancer cells to nutrient deprivation, we propose 3 hypotheses for the AhR-regulated pathway that induces VEGF in glucose-deprived HepG2 cells: A) Nrf2 induces VEGF expression by activating ATF4 expression; B) protein interactions between Nrf2 and ATF4 induce VEGF expression; and C) ATF4 activates VEGF expression independent of Nrf2 (Figure 4).

Glucose deprivation-induced expression of VEGF was examined at the mRNA and protein levels using RNAi against *ATF4* or *Nrf2* (Figure 5). When HepG2 cells were cultured under glucose deprivation, *ATF4* knockdown suppressed VEGF expression (Figure 5A). In contrast, *Nrf2* knockdown did not affect VEGF expression at the mRNA and protein levels and secretion of the VEGF protein (Figure 5D,E and F). No appreciable change in ATF4 expression at the mRNA and protein levels resulted from Nrf2 knockdown regardless of the glucose concentration (Figure 6). These results clearly indicated that ATF4 activated VEGF expression independent of Nrf2 in the glucose-deprived HepG2 cells. We adopt hypothesis C as illustrated in Figure 4.

Conclusions

The AhR is well known as a ligand-activated transcription factor and transcriptional regulator for drug-metabolizing

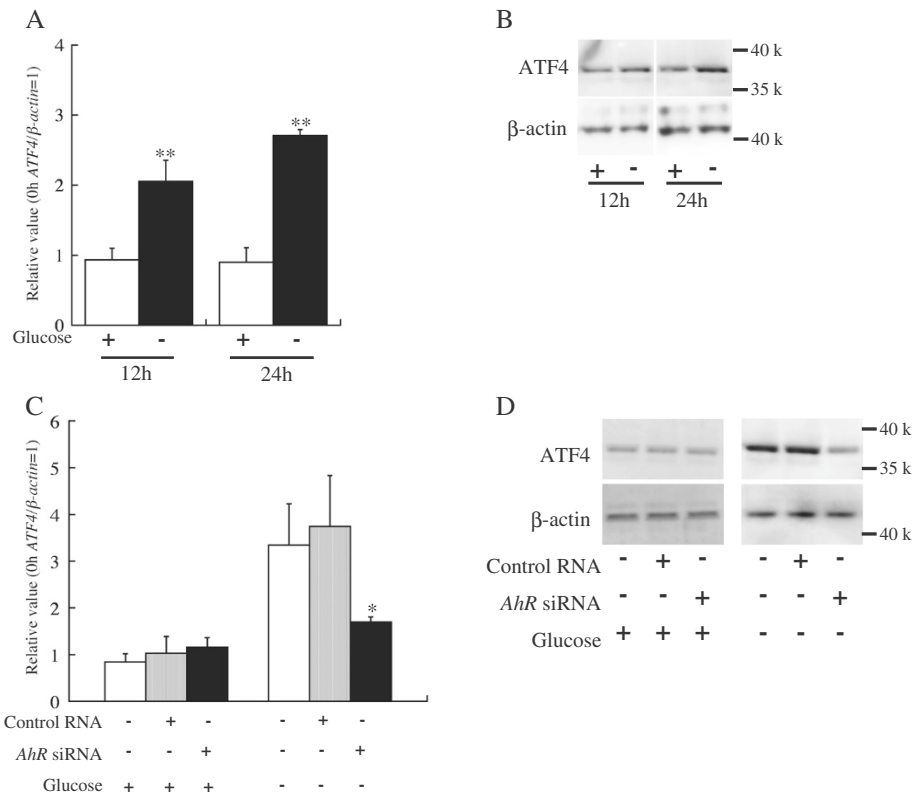


Figure 3 AhR regulates activating transcription factor 4 (ATF4) expression in glucose-deprived conditions. **A** and **C**: mRNA expression of *ATF4*. **A** shows *ATF4* expression in glucose-deprived conditions, and **C** shows *ATF4* expression after *AhR* knockdown. The *ATF4* mRNA levels were normalized by the β -actin mRNA level at each point ($ATF4/\beta$ -actin). The $ATF4/\beta$ -actin values were calculated relative to the expression level at 0 h (**A**: the time of medium exchange, **C**: the time of knockdown), which was set equal to 1. Bars indicate the standard deviation of independent triplicate measurements. * indicates that there is a significant difference (*: $P < 0.05$, **: $P < 0.005$). **B** and **D**: Western blot analyses using an antibody against ATF4. + and - indicate presence and absence, respectively, of glucose, control RNA, and *AhR* siRNA. "12 h" and "24 h" in **A** and **B** indicate the time following transfer to high or no glucose medium. The RNA and protein samples in **C** and **D** were derived from the HepG2 cells at 24 h after the medium exchange.

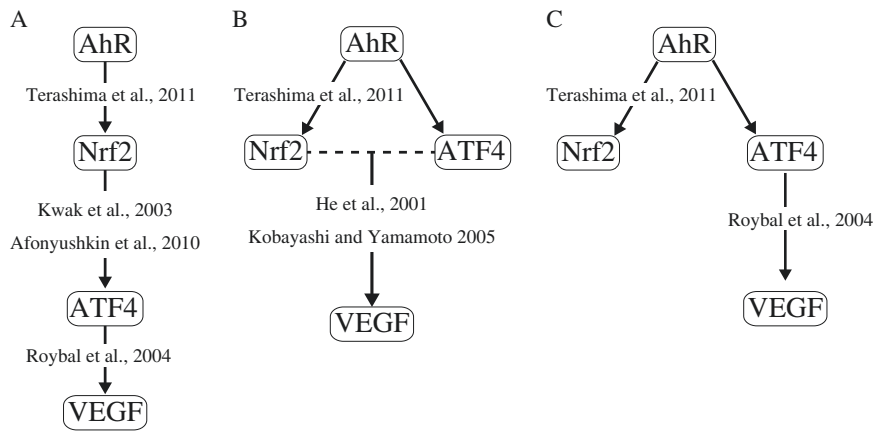


Figure 4 Hypotheses of the relationships between AhR, nuclear factor erythroid-derived 2-related factor 2 (Nrf2), ATF4, and VEGF. The hypotheses by IPA and past reports are raised (**A**, **B** and **C**, see the text for detail). Solid arrows indicate the activation of gene expression, and the dotted line indicates a protein-protein interaction.

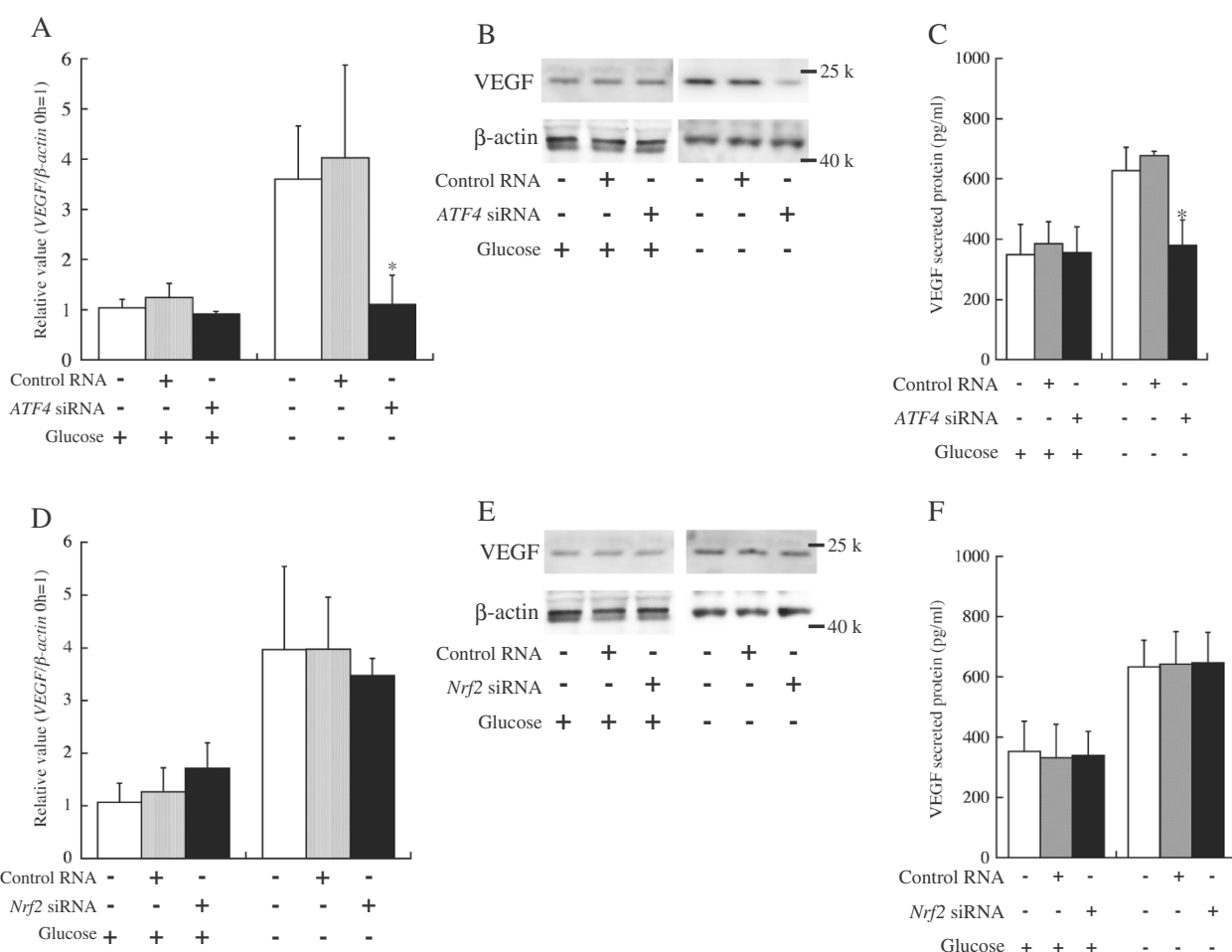


Figure 5 VEGF expression under glucose deprivation is induced by ATF4 but not Nrf2. **A** and **D**: mRNA expression of *VEGF*. **A** shows *VEGF* expression after *ATF4* knockdown, and **D** shows *VEGF* expression after *Nrf2* knockdown. The *VEGF* mRNA levels were normalized by the β -actin mRNA level at each point (*VEGF*/ β -actin). The *VEGF*/ β -actin values were calculated relative to the expression level at 0 h (the time of knockdown), which was set equal to 1. Bars indicate the standard deviation of independent triplicate measurements. **B** and **E**: Western blot analyses using an antibody against VEGF. + and - indicate the presence and absence, respectively, of glucose, control RNA, and *ATF4* or *Nrf2* siRNA. **C** and **F**: Measurement of VEGF protein secretion to the media. All RNA, protein and media amples were derived from HepG2 cells at 24 h after knockdown. * indicates that there is a significant difference (*: $P < 0.05$, **: $P < 0.005$).

enzyme, *CYP1* family genes. AhR translocates into the nucleus following ligand binding and forms a complex with ARNT. The AhR-ARNT complex binds to the XRE domain to regulate target gene expression. In conclusion, the AhR pathway, which involves ATF4, induces VEGF expression in glucose-deprived human hepatoblastoma HepG2 cells. AhR translocates into the nucleus in response glucose deprivation in HepG2 and regulates gene expression. However, there are no binding domains of AhR on the transcriptional regulatory domain of *ATF4*. We expect that there are some mediators between the AhR translocation into the nucleus and *ATF4* expression. The pathway from AhR to VEGF through ATF4 is a novel pathway in glucose-deprived liver cancer cells, which is related to the microenvironment within cancer tissue that affects liver cancer malignancy.

Methods

Cell culture

HepG2 (a human hepatocellular carcinoma, ATCC No.: HB-8065) cells (1.0×10^5 cells/mL) were cultured in Dulbecco's modified Eagle's medium (DMEM; high glucose: 4.5 g/L D-glucose; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Hyclone, South Logan, VT, USA, Lot FRC25965) and antibiotics (Gibco) at 37°C in a CO₂ incubator for 48 h as a pre-culture. After the pre-culture, the media were changed to high or low glucose (1.0 g/L D-glucose, Gibco) DMEM.

RNA extraction and quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted from 3 independent HepG2 cell cultures using the RNeasy Mini Kit following the

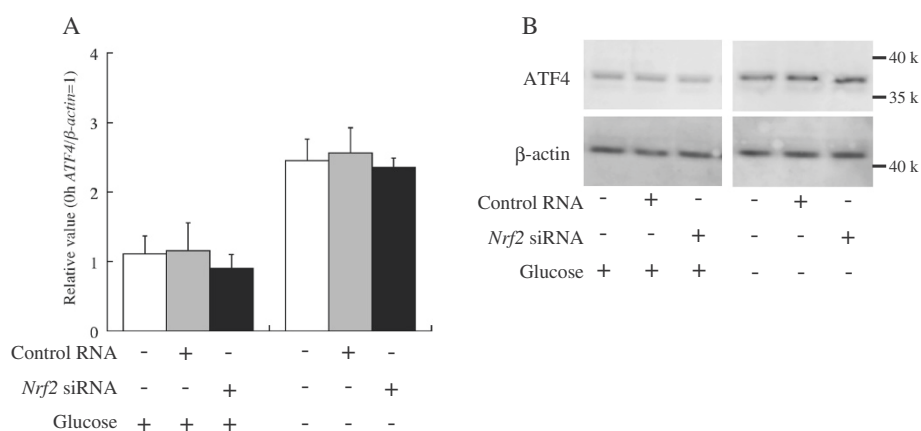


Figure 6 Nrf2 is not related to ATF4 expression. **A** shows ATF4 expression after Nrf2 knockdown. The ATF4 mRNA levels were normalized by the β -actin mRNA level at each point (ATF4/ β -actin). The ATF4/ β -actin values were calculated relative to the expression level at 0 h (the time of knockdown), which was set equal to 1. Bars indicate the standard deviation of independent triplicate measurements. * indicates that there is a significant difference (*: $P < 0.05$, **: $P < 0.005$). **B** shows western blot analyses using an antibody against ATF4. + and - indicate the presence and absence, respectively, of glucose, control RNA, and Nrf2 siRNA. All RNA or protein samples were derived from HepG2 cells at 24 h after knockdown.

manufacturer's instructions (Qiagen, Dusseldorf, Germany). Total RNAs (1 μ g) were used for cDNA synthesis with a cDNA synthesis kit (Roche, Basel, Switzerland). Using the first-strand cDNA, real-time PCR was performed with a 7500 Real-Time PCR System (Applied Biosystems, Tokyo, Japan).

RNA interference (RNAi) assay

The small interfering RNAs (siRNAs) against *Ahr*, *Nrf2*, and *ATF4* and control siRNAs were supplied as Stealth RNAs by Invitrogen (Carlsbad, CA, USA). The siRNA sequences targeting human *Ahr*, *Nrf2*, and *ATF4* mRNAs were designed. In addition, negative control siRNAs were designed by scrambling the nucleotide sequence (scramble sequence) of the siRNAs for human *Ahr*, *ATF4*, and *Nrf2*. The negative control siRNAs were designed without homology to any other gene.

HepG2 cells were individually transfected with siRNAs with lipofectamine (Invitrogen) for 48 h according to the manufacturer's instructions. All siRNA transfections were performed by treating the cells with 10 nM siRNA. The negative control group was treated with the same concentration of the negative control siRNA. All alternative control groups were treated with lipofectamine in the absence of siRNA. The HepG2 cells were cultured for 48 h in high glucose conditions as a pre-culture with siRNA or control siRNA. The suppression efficiency of gene expressions show in Additional file 1: Figure S1.

Western blotting

HepG2 cells were lysed and separated into the cytosol and nuclear fractions using the NER-PER Nuclear and Cytoplasmic Extraction Reagent (Thermo Scientific,

Rockford, IL, USA). The protein samples were subjected to sodium dodecyl sulfate 10% polyacrylamide gel electrophoresis (10 μ g protein) and transferred to a nitrocellulose membrane. The blots were blocked with Chemiluminescent Blocker (Millipore, Billerica, MA, USA) at room temperature for 1 h and rinsed with tris-buffered saline containing 0.1% Tween 20 (TBS-T). The blots were incubated overnight with antibodies against VEGF (1:1000, SC-7269 Santa Cruz), ATF4 (1:1000, SC-200, Santa Cruz), or β -actin (1:1000, SC-130656, Santa Cruz) at 4°C, followed by washing with TBS-T. The blot was subsequently incubated for 1.5 h with a mouse-IgG (1:1000, ZYMED) secondary antibody in Chemiluminescent Blocker at room temperature followed by washes with TBS-T. After a chemiluminescent reaction using Luminate Classico Western HRP Substrate (Millipore), the bands were visualized with a LAS-3000 (Fujifilm, Tokyo, Japan).

Quantitation of VEGF protein secretion

Media were collected from 300,000 cells in 60 mm dishes and centrifuged at 2000 r.p.m. for 10 min at 4°C. VEGF in the medium was measured by using the Quantikine human VEGF ELISA kit from R&D Systems (Minneapolis, MN, USA) according to manufacturer's instruction.

Analysis of the AhR-VEGF pathway by ingenuity pathway analysis

We used an Ingenuity Pathway Analysis tool to search the biological pathway from AhR to VEGF including the molecules involved in the pathway. We used this tool to identify candidate molecules that were glucose deprivation-induced VEGF transcriptional regulators in conjunction with biological pathways that are activated by AhR.

Additional file

Additional file 1: Figure S1. Suppression efficiency of gene expressions by RNAi. The graphs show *AhR*, *ATF4* and *Nrf2* expressions under addition of siRNA for *AhR*, *ATF4* and *Nrf2* respectively. Each mRNA levels were normalized by the β -actin mRNA level at each point (*AhR*, *ATF4* or *Nrf2*/ β -actin). The values of *AhR*, *ATF4* or *Nrf2*/ β -actin were calculated relative to the expression level at 0 h (the time of medium exchange), which was set equal to 1. Bars indicate the standard deviation of independent triplicate measurements. * indicates that there is a significant difference (*: $P < 0.05$, **: $P < 0.005$).

Abbreviations

AhR: Aryl hydrocarbon receptor; VEGF: Vascular endothelial growth factor; ATF4: Activating transcription factor 4; ARNT: AhR nuclear translocator; Nrf2: Nuclear factor erythroid-derived 2-related factor 2; CYP: Cytochrome P450.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

JT, HW, KK and SO conceived and designed the experiments and analyzed the data. JT, CT, and SO wrote the manuscript: JT and CT performed the experiments. All authors read and approved the final manuscript.

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