Basic-helix-loop-helix (bHLH) transcription factor DEC2 negatively regulates vascular endothelial growth factor expression

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DEC1 (BHLHB2/Sharp2/Stra13) and DEC2 (BHLHB3/Sharp1) are basic-helix-loop-helix (bHLH) transcription factors, involved in cellular differentiation, responses to hypoxia and circadian rhythms. We recently showed that the expression of DEC1 and DEC2 was up-regulated by hypoxia; however, the functions of these two factors under hypoxic conditions have not been elucidated in detail. It is well established that the expression of vascular endothelial growth factor (VEGF) is up-regulated by hypoxia, and the expression of VEGF in response to hypoxia depends on transcriptional activation by a heterodimer comprising hypoxia-inducible factor 1α (HIF-1α) and arylhydrocarbon receptor nuclear translocator 1 (ARNT1). In the present study, we showed that DEC2, but not DEC1, suppressed VEGF gene expression under hypoxic conditions. DEC2 protein was co-immunoprecipitated with HIF-1α but not with ARNT1. The binding of HIF-1α to the hypoxia response element (HRE) in the VEGF promoter was decreased by DEC2 over-expression, and increased by DEC2 knockdown. We also showed that the circadian expression of VEGF showed a reciprocal pattern to that of DEC2 in cartilage. DEC2 had a circadian oscillation in implanted Sarcoma 180 cells. We conclude that DEC2 negatively regulates VEGF expression and plays an important role in the pathological conditions in which VEGF is involved.

Introduction

Angiogenesis is an important biological reaction for tumor cell growth, and its inhibition is a potential strategy for treating tumors (Kim *et al*. 1993; Takeshita *et al*. 1994; Kini *et al*. 2001). Vascular endothelial growth factor (VEGF) is a major cytokine involved in angiogenesis. It is important to clarify the precise mechanisms by which VEGF expression is regulated. Expression of VEGF is regulated by oxygen concentrations, inflammation, microbial infections, cytokines and circadian rhythms (Shweiki *et al*. 1992; Hlatky *et al*. 1994; Ben-Av *et al*. 1995; Li *et al*. 1995; Koyanagi *et al*. 2003; Sato *et al*. 2007).

Hypoxia is a physiological stress, which induces the expression of VEGF. Hypoxia-inducible factor 1 (HIF-1) is a transcription factor, which plays a central role in the gene expression induced by hypoxia, and also in the development of cancer (Forsythe *et al*. 1996). HIF-1 is a heterodimer of HIF-1α and aryl hydrocarbon receptor nuclear translocator 1 (ARNT1; also known as HIF-1β). The expression of HIF-1α is regulated by oxygen concentrations while ARNT1 is constitutively expressed.

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There is a hypoxia response element (HRE) in the promoter region of the VEGF gene (Forsythe *et al.* 1996), and the binding of HIF-1α/ARNT1 to the HRE initiates the transcription of VEGF mRNA. Mutation of the HRE abrogates the gene transcription induced by hypoxia (Kimura *et al.* 2000).

It is reported that expression of the VEGF gene was regulated by circadian rhythms (Koyanagi *et al.* 2003). Mammalian circadian rhythms are regulated by molecular clockwork systems based on a negative feedback loop in normal and tumor cells. *Clock* and *Brain-muscle-arnt-like-protein 1/2 (Bmal 1/2)* are well known clock genes that positively regulate the expression of target genes. *Period 1/2/3 (Per 1/2/3)*, *Cryptochromes 1/2/3 (Cry 1/2/3)* and *Differentiated embryo-chondrocyte 1 (Dec1;* also named as *Bhlhb2/Sharp2/Stra13)* and *Dec2 (Bhlhb3/Sharp1)* are also designated as clock genes, but negatively regulate the expression of their targets. Circadian rhythms are tightly regulated by these clock genes. The heterodimer comprising CLOCK and BMAL 1/2 (CLOCK/BMAL 1/2) enhances transcription of *Dec*, *Per* and *Cry* via CACGTG E-boxes, and the products of these genes, DEC, PER and CRY, suppress the transactivation by CLOCK/BMAL 1/2 (Sangoram *et al.* 1998; Hamaguchi *et al.* 2004). It was also reported that PER, but not CRY, regulated the expression of the VEGF gene induced by HIF-1α/ARNT1 (Koyanagi *et al.* 2003). However, there have been no reports about the regulation of VEGF by other clock genes.

The basic-helix-loop-helix (bHLH) transcription factors are involved in the expression of various genes. We reported that DEC1 and DEC2 are bHLH transcription factors, and play an important role in the circadian rhythm of the suprachiasmatic nucleus (SCN) (Honma *et al.* 2002) or peripheral tissue (Hamaguchi *et al.* 2004; Noshiro *et al.* 2004; Kondo *et al.* 2006) as a negative regulator. These two DECs were shown to regulate the differentiation of chondrocytes, skeletal muscles and nervous systems (Boudjelal *et al.* 1997; Shen *et al.* 1997; Fujimoto *et al.* 2007). Recently, we reported that the expression of DEC1 and DEC2 is induced by hypoxia (Miyazaki *et al.* 2002). This suggests that DEC1 and DEC2 are involved in the expression of other hypoxia-inducible genes. In the present study, we investigated the role of DEC1 and DEC2 in hypoxia-induced VEGF expression. Our results showed that DEC2 regulated the expression of the VEGF gene induced by HIF-1α/ARNT1 transactivation by interacting with HIF-1α and interfered with the binding of HIF-1α to the VEGF promoter under hypoxic conditions, and that DEC2 showed the opposite phase to VEGF in circadian rhythm.

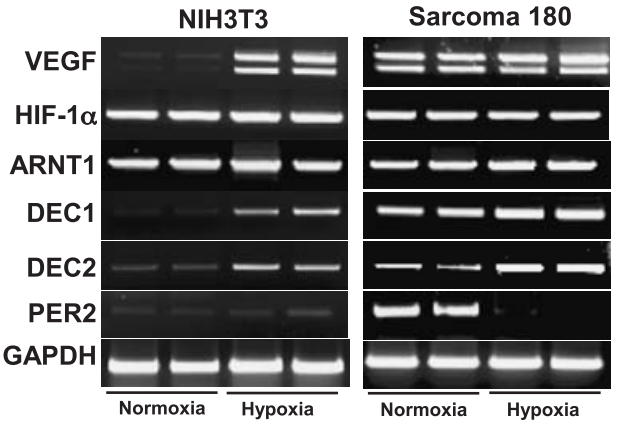

Figure 1 Effect of hypoxia on the expression of mRNAs for VEGF, HIF-1α, ARNT1, DEC1, DEC2, and PER2 in NIH3T3 and Sarcoma 180 cells. Cells were exposed to normoxia (21% O_2) or hypoxia (1% O_2 for NIH3T3 cells or 3% O_2 for Sarcoma 180 cells) for 24 h, and total RNA was isolated from the cells. Single stranded cDNA was synthesized from RNA and RT-PCRs for VEGF, HIF-1α, ARNT1, DEC1, DEC2, PER2 and GAPDH were performed. The results of duplicate experiments are shown.

Results

Effects of hypoxia on the expression of mRNAs for VEGF, HIF-1α, ARNT1, DEC1, DEC2, and PER2 in NIH3T3 and Sarcoma 180 cells

We previously showed that the mRNA expression of DEC1 and DEC2 was induced by hypoxia in 293 T and HeLa cells (Miyazaki *et al.* 2002). In the present study, we first examined the effect of hypoxia on the expression of mRNAs for VEGF, HIF-1α, ARNT1, DEC1, DEC2, and PER2 in NIH3T3 (mouse fibroblast) and Sarcoma 180 (mouse Sarcoma) cells. Cells were incubated under normoxic or hypoxic conditions for 24 h, and mRNA was prepared. The mRNA was subjected to semi-quantitative RT-PCR analyses. The expression of mRNA for VEGF, DEC1 and DEC2 was faint under normoxic conditions in NIH3T3 cells, but was significantly up-regulated by hypoxia (Fig. 1, left panel). The level of PER2 mRNA was low during normoxia and not changed by hypoxia in NIH3T3 cells, whereas the mRNA of HIF-1α and ARNT1 was abundantly expressed under normoxic conditions and was not changed by hypoxia. In Sarcoma 180 cells, substantial amounts of mRNAs for VEGF, HIF-1α, ARNT1, DEC1 and PER2 were detected under normoxic conditions (Fig. 1, right panel). Hypoxia did not induce a significant change in the expression of mRNA for VEGF, HIF-1α, ARNT1 and DEC1, but inhibited the expression

of PER2 mRNA. On the other hand, the expression of DEC2 mRNA was induced by hypoxia also in this cell type. These results are consistent with previous finding in other tumor cell lines (Ishibashi *et al.* 2001; Ivanova *et al.* 2001; Qin *et al.* 2001; Li *et al.* 2003).

Effects of DEC1 and DEC2 on the promoter activity of *Vegf*

We next examined if DEC1 or DEC2 affects the promoter activity of *Vegf*. Cells were transiently transfected with a full-length *Vegf* promoter containing a HRE (Fig. 2A1) together with the expression vector for DEC1 or DEC2. The promoter activity of *Vegf* was increased by hypoxia about 100-fold and the increase was not affected by DEC1 in NIH3T3 cells (Fig. 2B, upper panel). In Sarcoma 180 cells, the promoter activity of *Vegf* was strong even under normoxic conditions, and was not altered by DEC1 (Fig. 2B, lower panel). The promoter activity of *Vegf* under hypoxia was not suppressed by DEC1. On the other hand, DEC2 suppressed, in a dose-dependent manner, the promoter activity of *Vegf* induced by hypoxia in NIH3T3 cells (Fig. 2C, upper panel) or the activity in Sarcoma 180 cells under both normoxia and hypoxia (Fig. 2C, lower panel). We confirmed, by Western blot analysis, that over-expression of DEC1 or DEC2 does not alter the endogenous levels of HIF-1α and ARNT1 proteins in cells.

Effect of DEC2 on the VEGF promoter activity induced by HIF-1α/ARNT1 transactivation

The HIF-1α/ARNT1 heterodimer plays a critical role in the transcription of *Vegf* induced by hypoxia (Forsythe *et al.* 1996). We next examined if DEC2 affects the promoter activity of *Vegf* induced by HIF-1α/ARNT1 transactivation. When the constructs for HIF-1α and ARNT1 were co-transfected into NIH3T3 or Sarcoma 180 cells, the promoter activity of *Vegf* increased about 100-fold even under normoxic conditions (Fig. 3A upper and lower panel), and the increase was suppressed by DEC2 in a dose-dependent manner.

We also examined if DEC2 affects the promoter activity of truncated constructs containing only HRE (pVEGF-HRE-TK-luc) or HREm (pVEGF-HREm-TK-luc) (Fig. 2A2 and A3). When the constructs for HIF-1α and

Figure 2 Effect of DEC1 or DEC2 on the promoter activity of the VEGF gene. (A) The diagram shows the structure of luciferase reporter constructs containing the hypoxia response element (HRE) or HRE mutant (HREm) of the 5′-flanking regions of *Vegf* and *Dec1*. 1. pVEGF-luc; full-length mouse *Vegf* containing 1289 bp upstream of the gene. 2. pVEGF-HRE-TK-luc; HRE with 29 bp of flanking sequence in *Vegf* was subcloned into pGL3-TK. 3. pVEGF-HREm-TK-luc; HREm with 29 bp of flanking sequence in *Vegf* was subcloned into pGL3-TK. 4. pDEC1-HRE-TK-luc; HRE with 30 bp of flanking sequence in *Dec1* was subcloned into pGL3-TK. 5. pDEC1-HREm-TK-luc; HREm with 30 bp of flanking sequence in *Dec1* was subcloned into pGL3-TK. (B) The reporter construct pVEGF-luc was co-transfected with the expression vector for DEC1 into NIH3T3 or Sarcoma 180 cells. After 16 h of transfection, the cells were incubated under normoxic or hypoxic conditions for an additional 24 h. Each value represents the mean ± SE (*bars*) for three independent experiments. The expression of DEC1, HIF-1α, ARNT1 and actin protein was confirmed by Western blot analysis. (C) The reporter construct pVEGF-luc was co-transfected with the expression vector for DEC2 into NIH3T3 or Sarcoma 180 cells. The cells were cultured under normoxic or hypoxic conditions and subjected to a luciferase assay or Western blot analysis as in (B). Each value represents the mean ± SE (*bars*) for three independent experiments. $^*P < 0.05$, by *t*-test. The expression of DEC2, HIF-1α, ARNT1 and actin protein was confirmed by Western blotting.

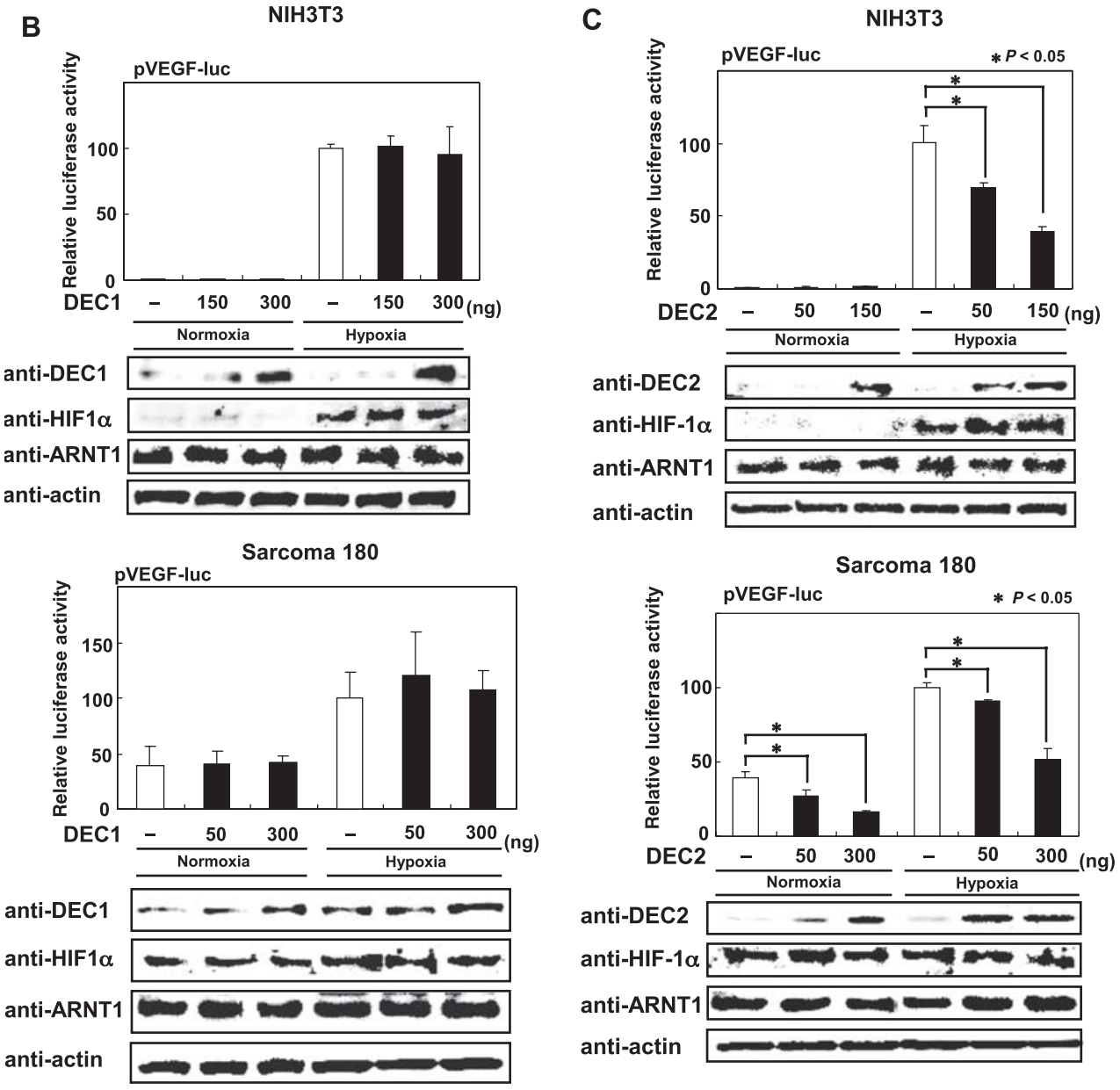

Figure 2 *Continued*

ARNT1 were co-transfected into NIH3T3 cells, the promoter activity of VEGF-HRE increased about fivefold under normoxic conditions (Fig. 3B, upper panel). Co-transfection of the PER2 construct slightly suppressed the promoter activity of VEGF-HRE as previously described (Koyanagi *et al.* 2003). When the DEC2 construct was co-transfected, the promoter activity of VEGF-HRE was suppressed in a dose-dependent manner (Fig. 3B, upper panel). The inhibitory effect of DEC2 on the promoter activity was more significant than that of PER2. The effect of the DEC1 construct was weak. Co-expression of HIF-1α and ARNT1 did not induce the promoter activity of VEGF-HREm. The activity of VEGF-HREm was not changed by the co-transfection of DEC2, DEC1 or PER2.

Dec1 is a target of HIF-1α like *Vegf* (Miyazaki *et al.* 2002). We next examined whether DEC2 suppresses the activity of the DEC1-HRE promoter (Fig. 2A4 and A5) induced by HIF-1α/ARNT1 transactivation. The promoter activity of DEC1-HRE induced by the transactivation

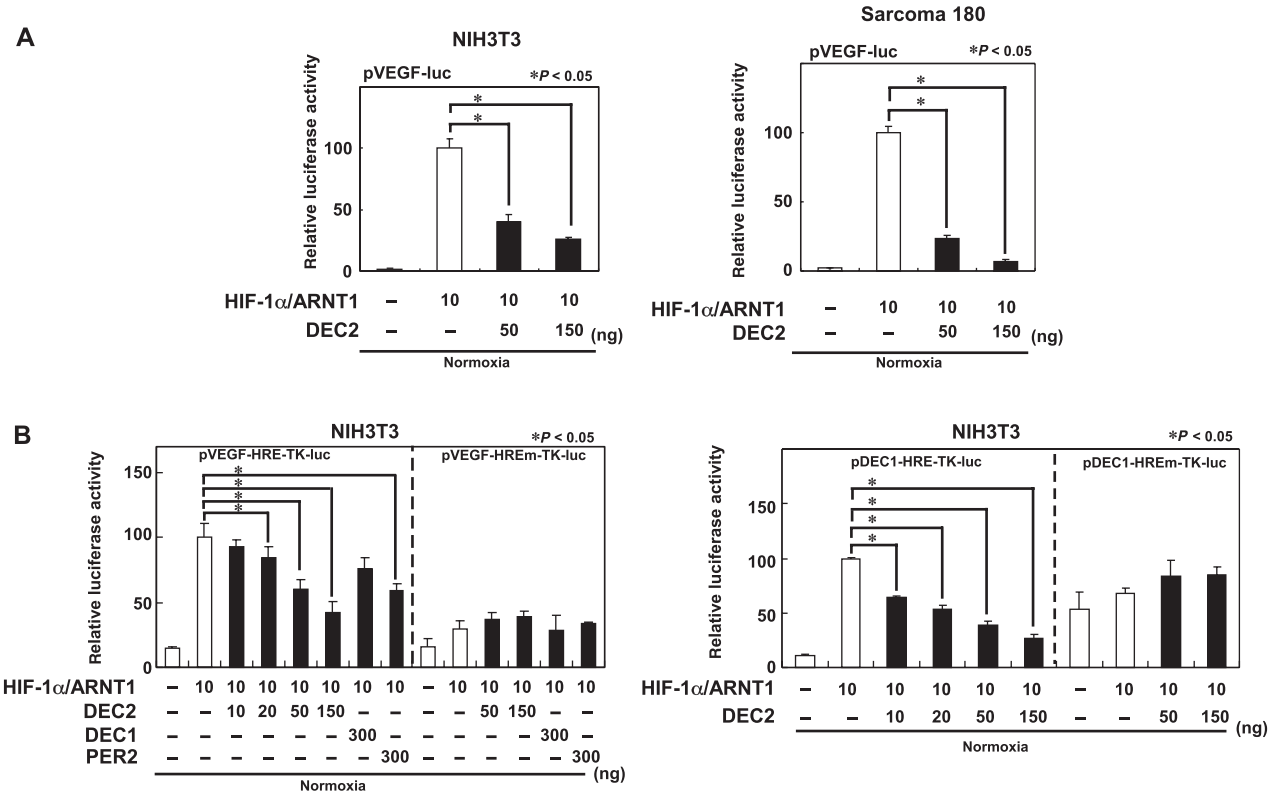

Figure 3 DEC2 suppressed the VEGF promoter activity induced by HIF-1α/ARNT1 transactivation in NIH3T3 and Sarcoma 180 cells. (A) The reporter construct pVEGF-luc was co-transfected with the expression vectors for HIF-1α and ARNT1 together with DEC2 into NIH3T3 or Sarcoma 180 cells. After incubation under normoxic conditions for 40 h, a Luciferase assay was performed. Each value represents the mean ± SE (*bars*) for three independent experiments. $*P < 0.05$, by *t*-test. (B) The reporter construct pVEGF-HRE-TK-luc or pVEGF-HREm-TK-luc was co-transfected with expression vectors for HIF-1α and ARNT1 together with DEC2, DEC1 or PER2 into NIH3T3 cells (upper panel). The reporter construct pDEC1-HRE-TK-luc or pDEC1-HREm-TK-luc was co-transfected with expression vectors for HIF-1α and ARNT1 together with DEC2 into NIH3T3 cells (lower panel). After 40 h of incubation, the cells were lysed and subjected to a luciferase assay. Each value represents the mean ± SE (*bars*) for three independent experiments.

was also suppressed by the co-transfection of DEC2 (Fig. 3B, lower panel). On the other hand, the promoter activity of DEC1-HREm was not induced by HIF-1α/ARNT1 transactivation, similar to VEGF-HREm, and the activity of DEC1-HREm was not changed by co-transfection of DEC2.

DEC2 protein interacts with HIF-1α protein in cells

We performed an immunoprecipitation assay in order to examine the interaction between DEC2 and HIF-1α or ARNT1 (Fig. 4). COS-7 cells were transfected with expression vectors for ARNT1, FLAG, FLAG-HIF-1α, FLAG-DEC1 or FLAG-DEC2. After 40 h of transfection, the cells were lysed and the expression of ARNT1, DEC2 or DEC1 protein was confirmed by immunoblotting using aliquots of total cell lysate. FLAG-tagged proteins in the lysates were immunoprecipitated with anti-FLAG antibody, and immunoblotted with antibodies against ARNT1 (Fig. 4A), DEC2 or DEC1 (Fig. 4B). HIF-1α was co-immunoprecipitated with ARNT1 as previously described (Jiang *et al.* 1996). DEC2 was co-precipitated with HIF-1α but not with ARNT1. On the other hand, DEC1 was not bound to HIF-1α or ARNT1. These results demonstrated that DEC2 protein could physically bind to HIF-1α in cells.

DEC2 suppressed the endogenous expression of VEGF

We further examined if DEC2 suppresses the endogenous VEGF expression induced by hypoxia. NIH3T3 cells were transfected with the expression vector for

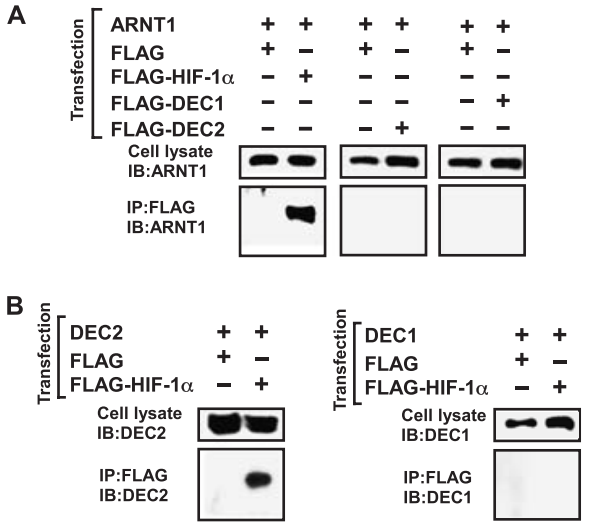

Figure 4 DEC2 interacts with HIF-1α. (A, B) COS-7 cells were transfected with the expression vector for ARNT1, FLAG, FLAG-HIF-1α, FLAG-DEC1 or FLAG-DEC2. After 40 h of transfection, cells were lysed and the lysates were immunoprecipitated (IP) with anti-FLAG antibody, and immunoblotted (IB) with antibodies against ARNT1 (A), DEC2 or DEC1 (B). To confirm the expression of ARNT1, DEC2 and DEC1 proteins, aliquots of total cell lysate were immunoblotted with each antibody.

DEC2. After 16 h of transfection, the cells were incubated for an additional 24 h under hypoxic conditions. The expression level of VEGF mRNA was examined by RT-PCR (Fig. 5A), and the amount of VEGF protein in the conditioned medium was measured by an ELISA (Fig. 5B). DEC2 suppressed, in a dose-dependent manner, the endogenous expression of VEGF mRNA and protein induced by hypoxia, but had no effect on the expression under normoxic conditions. In Sarcoma 180 cells, DEC2 suppressed the endogenous expression of VEGF during normoxia in a dose-dependent manner (Fig. 5C and D).

Effect of hypoxia on the expression of HIF-1α and DEC2 proteins in HSC-3 cells

NIH3T3 and Sarcoma 180 cells were useful for plasmid transfection studies, but the endogenous expression of DEC2 protein was weak in these cells. Thus, in order to observe the expression of endogenous DEC2 protein, we used another cell line, human squamous cell carcinoma (HSC-3) cells. We examined the effect of hypoxia on the expression of endogenous HIF-1α, DEC2, DEC1, PER2 or actin protein in HSC-3 cells (Fig. 6A). Under normoxic conditions, the expression of HIF-1α,

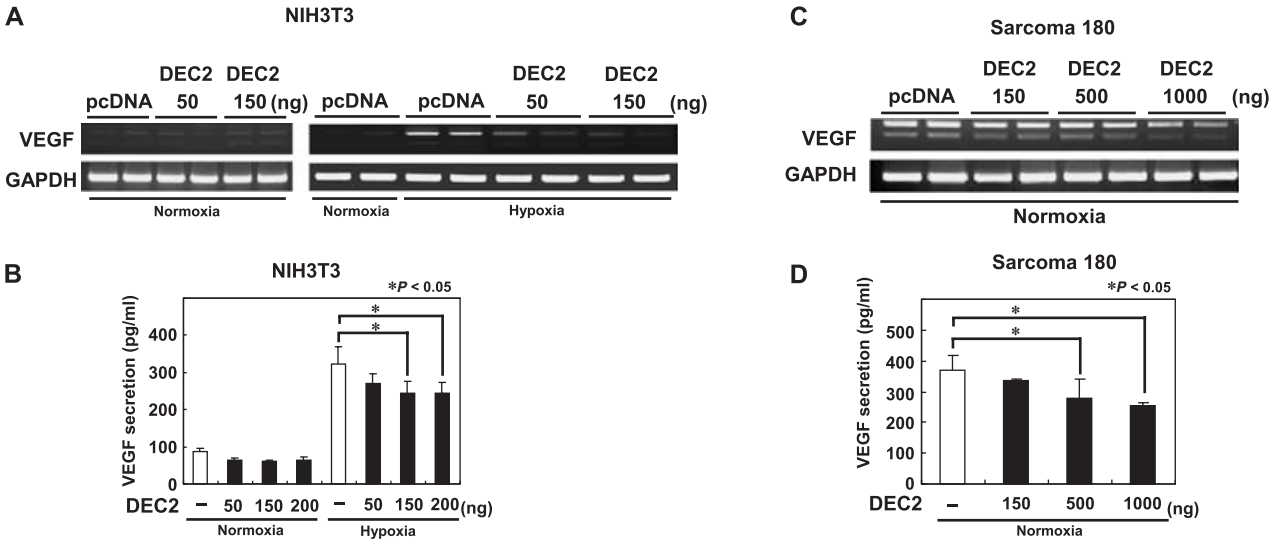

Figure 5 DEC2 suppressed the endogenous expression of VEGF. NIH3T3 cells were transfected with the expression vector for DEC2. After 16 h of transfection, the cells were exposed to hypoxia and incubated for an additional 24 h. (A) RT-PCR analysis for VEGF mRNA was performed as shown in Fig 1. (B) The conditioned medium was collected and the secretion of VEGF protein into the medium was measured by an ELISA. Each value represents the mean ± SE (*bars*) for three independent experiments. $^*P < 0.05$, by *t*-test. Sarcoma 180 cells were transfected with the expression vector for DEC2. After 40 h of transfection, RT-PCR (C) and ELISA (D) were performed as above. Each value represents the mean ± SE (*bars*) for three independent experiments. $^*P < 0.05$, by *t*-test.

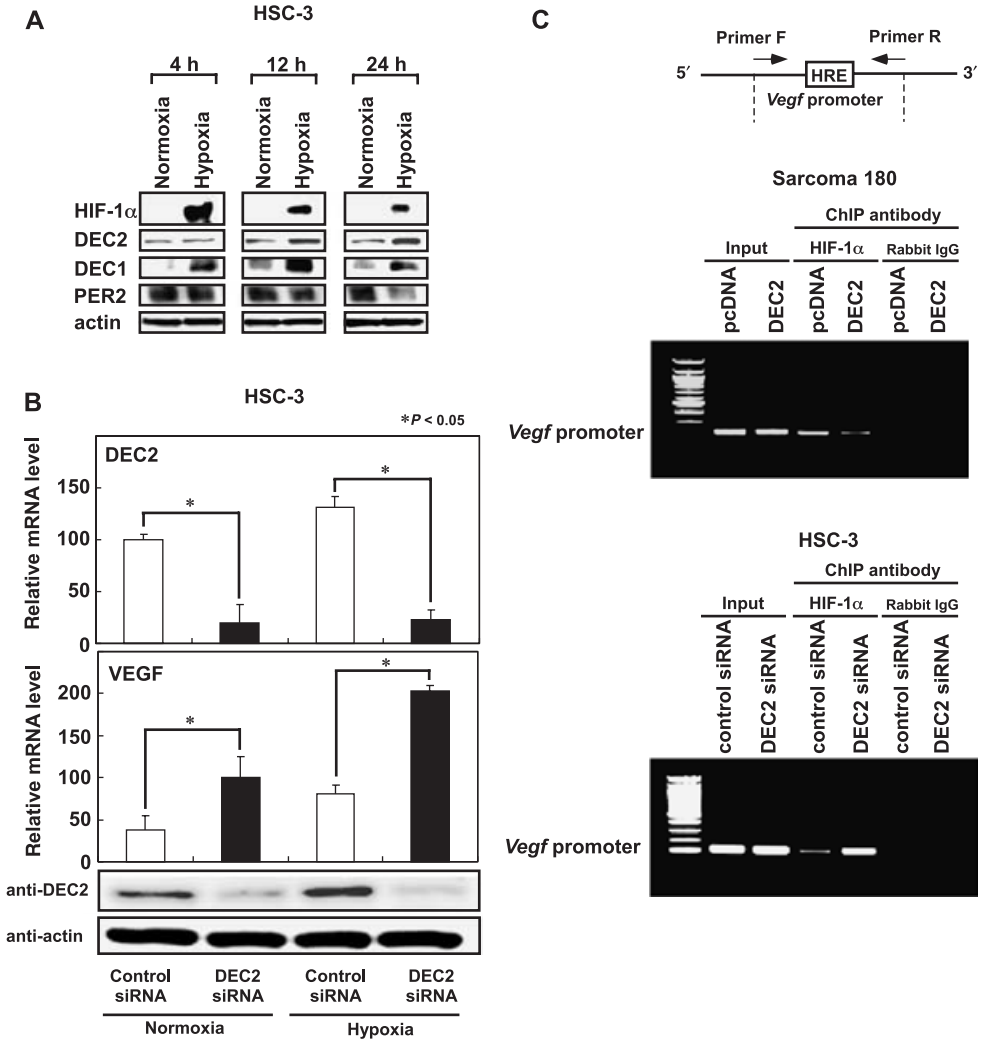

Figure 6 Effect of hypoxia on the expression of HIF-1α, DEC2, DEC1 or PER2 protein in HSC-3 cells. (A) HSC-3 cells were cultured under normoxic or hypoxic (1% O_2) conditions for 4, 12 or 24 h, and lysed. The lysates were subjected to a Western blot analysis for HIF-1α, DEC2, DEC1, PER2 or actin. (B) HSC-3 cells were transfected with specific siRNA against DEC2 (DEC2 siRNA) or scrambled sequence siRNA (control siRNA). After 50 h of transfection, the cells were exposed to normoxia or hypoxia (1% O_2) for an additional 12 h. RNA was prepared and subjected to real-time RT-PCR analysis. Each value represents the mean ± SE (*bars*) for three independent experiments. $*P < 0.05$, by *t*-test. A Western blot analysis for DEC2 siRNA was performed as above. (C) Chromatin immunoprecipitation assay. Sarcoma 180 cells were transfected with the expression vector for DEC2 or with control pcDNA. After 40 h of transfection, the cells were exposed to 1% hypoxia for an additional 4 h (upper panel). HSC-3 cells were transfected with specific siRNA against DEC2 or with control siRNA. After 50 h of transfection, the cells were incubated for an additional 12 h under hypoxic conditions (lower panel). Cells were fixed and lysed, and the DNA was sheared by sonication. Immunoprecipitation was carried out with an anti-HIF-1α antibody. The precipitated protein/DNA complexes were denatured, and DNA was recovered and purified. The fragment of the *Vegf* promoter which was co-immunoprecipitated with HIF-1α, was amplified by PCR. PCR products were analyzed by gel electrophoresis.

DEC2 and DEC1 protein was faint, but PER2 protein was abundantly expressed. The cells were exposed to hypoxia for 4, 12 or 24 h. The amount of HIF-1α protein was increased markedly when exposed to hypoxia for 4 h, and decreased thereafter. In contrast, the amount of DEC2 protein increased gradually and was highest after the expose of hypoxia for 24 h. The expression of DEC1 protein was also increased when exposed to

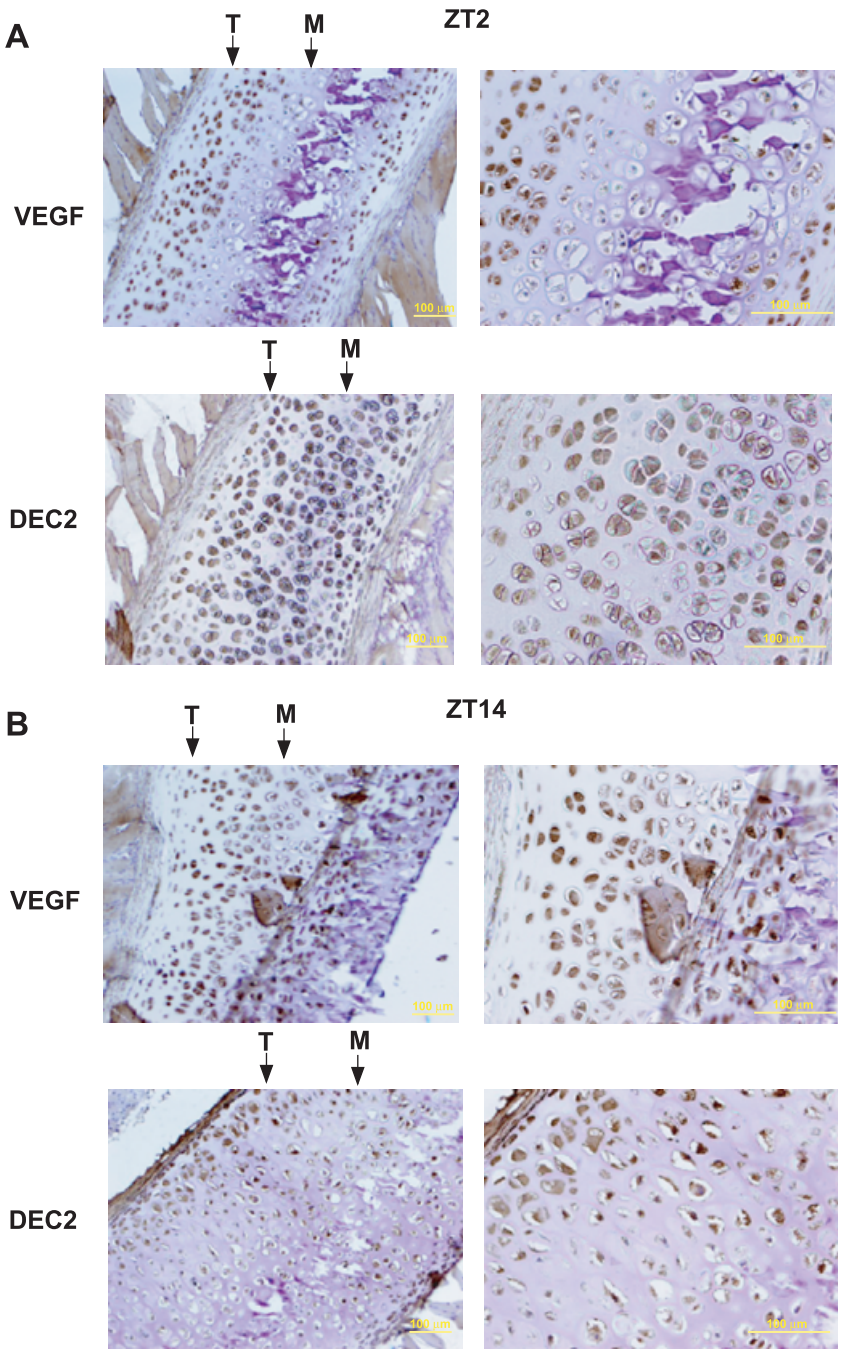

Figure 7 Circadian expression of VEGF and DEC2 protein in cartilage from mice. Mice were housed under 12 : 12-h LD conditions for 19 days. The animals were killed at ZT2 and ZT14, and rib cartilage was obtained. Immunohistochemical detection of VEGF, DEC2, HIF-1α and ARNT1 was performed using anti-VEGF, anti-DEC2, anti-HIF-1α and anti- ARNT1 antibody, respectively. The arrow shows the terminal zone (T) and middle zone (M). The results are representative of three mice.

hypoxia, and peaked at 12 h. The level of PER2 protein was little changed by hypoxia for up to 12 h, but was decreased markedly by hypoxia for 24 h. These results demonstrated that the expression of HIF-1α, DEC1, DEC2 and PER2 is differentially regulated by hypoxia. We further examined the effect of the knockdown of endogenous DEC2 by siRNA in HSC-3 cells. Inhibition of DEC2 expression by siRNA resulted in the increased expression of VEGF mRNA (Fig. 6B). We next performed a chromatin immunoprecipitation (ChIP) assay to examine if DEC2 interferes with the binding of HIF-1α to the VEGF-promoter in Sarcoma 180 and HSC-3 cells. As shown in Fig. 6C, DEC2 over-expression decreased the binding of HIF-1α to

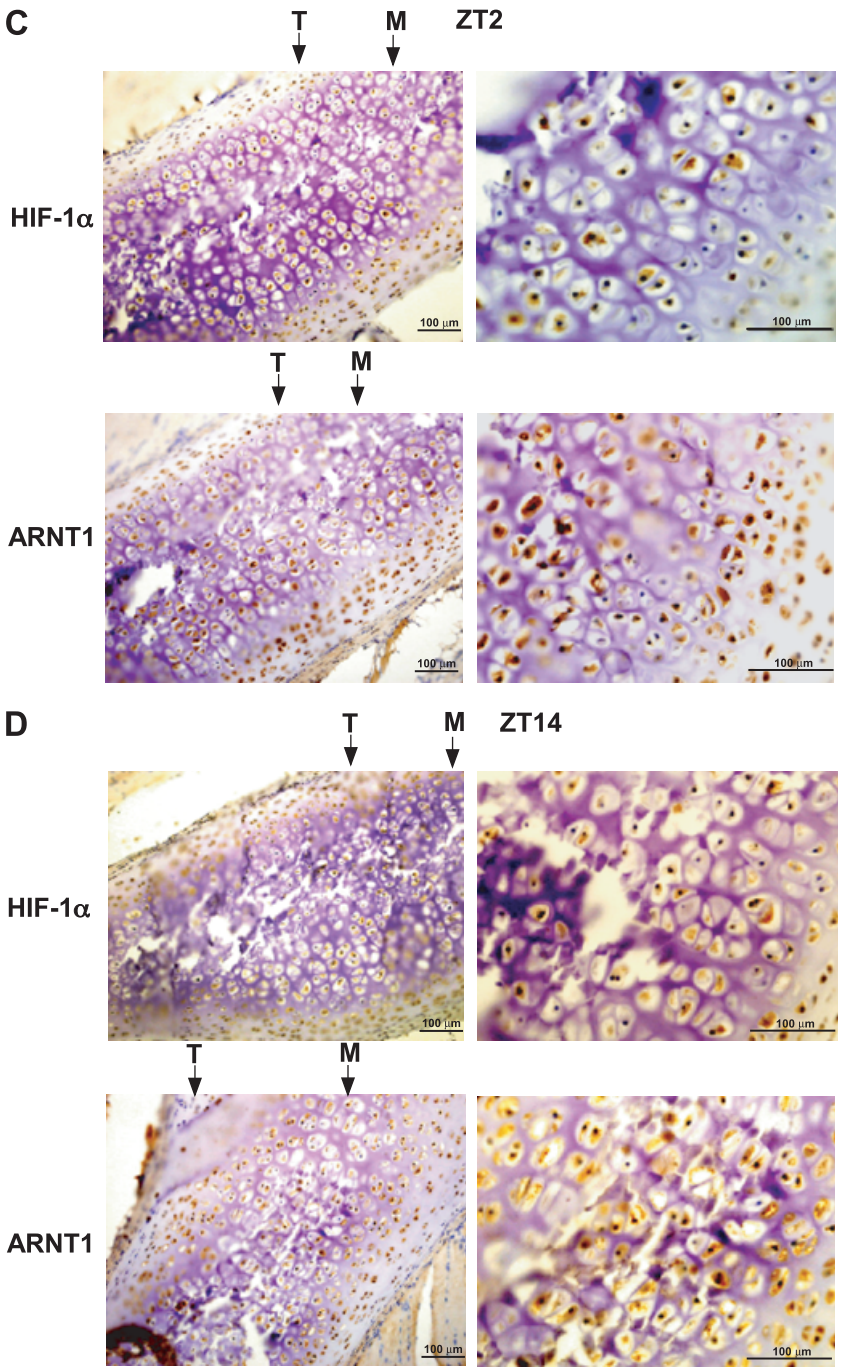

Figure 7 *Continued*

the VEGF promoter in Sarcoma 180 cells exposed to hypoxia. In addition, siRNA knockdown of DEC2 markedly increased the binding of HIF-1α to the VEGF promoter in HSC-3 cells under hypoxic conditions. These results demonstrated that DEC2 expression regulates the binding of HIF-1α to the VEGF promoter during hypoxia.

Day-night difference of VEGF and DEC2 protein in cartilage

We next examined the circadian rhythm of VEGF and DEC2 protein expression *in vivo*. Samples of mouse rib cartilage were prepared at ZT 2 (10 : 00) and at ZT 14 (22 : 00), light-dark (LD) conditions, and we performed an immunohistochemical analysis of VEGF, DEC2,

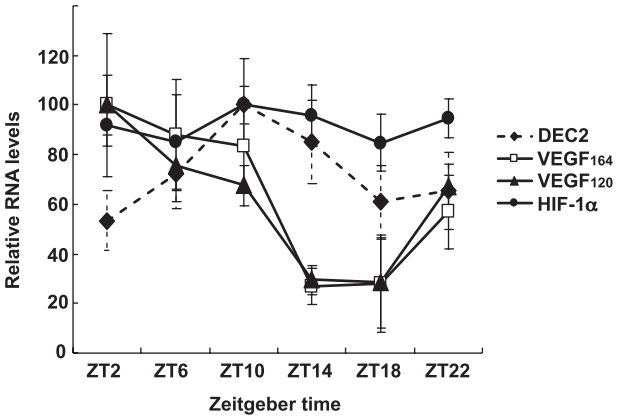

Figure 8 Circadian expression of VEGF and DEC2 mRNA in implanted tumor cells. Temporal profiles of mRNA expression of HIF-1α, DEC2, $VEGF_{164}$ and $VEGF_{120}$ in tumor masses. For plots of RNA, the mean peak values for HIF-1α, DEC2, $VEGF_{164}$, and $VEGF_{120}$, are set at 100. Each point represents the mean ± SE (bars; $n = 4–6$). The mRNA levels for DEC2, $VEGF_{164}$ and $VEGF_{120}$ exhibit significant circadian variations ($P < 0.01$, respectively; ANOVA).

HIF-1α and ARNT1 in cartilage. At ZT2, VEGF protein was observed in the terminal zone (T), but not in the middle zone (M) (Fig. 7A). On the other hand, DEC2 was stained intensely in the middle zone, but weakly in the terminal zone. At ZT14, VEGF was strongly expressed in the middle zone, but DEC2 was dominant in the terminal zone (Fig. 7B). These results indicate that the expression of VEGF shows a reciprocal pattern to that of DEC2 in circadian rhythm. HIF-1α and ARNT1 were positively stained in both the terminal and middle zones (Fig. 7C and D).

Day-night difference of VEGF and DEC2 mRNA in implanted tumor cells

As shown in Fig. 8, mice were housed under a LD cycle and a 50 μL volume containing 1.5×10^6 viable Sarcoma 180 cells was injected into the right hind footpad of each mouse. The tumor volume was estimated as previously described (Koyanagi *et al.* 2003). The mRNA levels for DEC2 and VEGF in implanted Sarcoma 180 cells exhibited an opposite circadian phase at ZT2 and ZT14 ($P < 0.01$), but HIF-1α did not show a circadian rhythm.

Discussion

The hypoxia-inducible factor HIF-1α is the key regulator in cellular adaptation to hypoxia. Hypoxia stabilizes HIF-1α allowing it to form a heterodimer with ARNT1 (Forsythe *et al.* 1996): The heterodimer HIF-1α/ARNT1 binds to the HRE, and causes an increase in the expression of target genes such as the VEGF and erythropoietin genes (Grimm *et al.* 2002). In the present study, we demonstrated that DEC2 interacts with HIF-1α, and interferes with the binding of HIF-1α to the HRE, resulting in down-regulation of the expression of HIF-1α-inducible genes such as the VEGF gene during hypoxia. Recent studies have shown that both DEC1 and DEC2 were induced to express by hypoxic conditions (Miyazaki *et al.* 2002; Yun *et al.* 2002; Chakrabarti *et al.* 2004; Ivanov *et al.* 2007). Here we showed that the expression of DEC1 and DEC2 was also induced by hypoxia in mouse fibroblasts, Sarcoma 180 cells, and human squamous cell carcinoma cells. Although HIF-1α was activated by hypoxia in these cells, the induction of DEC2 expression by hypoxia was slower than that of HIF-1α and DEC1 expression.

This is a new molecular mechanism in which the DEC2-mediated feedback loop participates in the regulation of gene expression induced by hypoxia. We also found that DEC1 did not bind to HIF-1α, and had little effect on the promoter activity of the VEGF gene. This finding indicates that DEC1 and DEC2, which differ in structure of the C-terminal, have distinct roles in adaptation to hypoxia: DEC2 has the alanine and glycine-rich region in its C-terminal half, but DEC1 does not.

In contrast to DEC1 and DEC2 expression, PER2 expression was down-regulated by hypoxia in mouse Sarcoma 180 and human squamous cell carcinoma cells. It was reported that PER2 also inactivates HIF-1α in a similar manner to DEC2 (Koyanagi *et al.* 2003): PER2 inhibited HIF-1α/ARNT1-induced VEGF promoter activity in Sarcoma 180 cells by interacting with HIF-1α protein. The different expression profiles of DEC2 and PER2 under hypoxic conditions suggest distinct functions in the transcriptional regulation of target genes of HIF-1α.

Both DEC1 and DEC2 show a circadian expression in many tissues (Honma *et al.* 2002; Noshiro *et al.* 2004; Furukawa *et al.* 2005). We also showed, in the present study, that DEC2 and VEGF protein expression had a circadian rhythm in cartilage. The expression of VEGF showed a reciprocal pattern to that of DEC2 at ZT2 and ZT14. Furthermore, both DEC2 and VEGF had circadian oscillations in implanted Sarcoma 180 cells, and the expression of VEGF mRNA showed the opposite circadian phase to that of DEC2 mRNA. These findings indicate that DEC2 regulates VEGF expression in both normal and tumorous tissues.

Several recent reports showed that clock genes play important roles in cancer biology: (i) The mRNA expression of DEC1 was suppressed by the over-expression of VHL in renal cell carcinoma (Ivanova *et al.* 2001),

(ii) Knockdown of BMAL2 enhanced cell proliferation in hepatocellular carcinoma (Yeh *et al.* 2003), and (iii) PER1 plays an important role in cell growth and DNA damage control in human cancer cells (Gery *et al.* 2006). DEC2 may contribute to the regulatory mechanism in tumor cell biology.

In summary, we revealed that DEC2 negatively regulates VEGF expression by interacting with HIF-1α, and interferes with the binding of HIF-1α to the HRE in the VEGF promoter under hypoxic conditions. Thus, DEC2 may regulate the other genes targeted by HIF-1α. In addition, DEC2 regulates the transcription of its targets by interacting with E-box in a circadian fashion (Fujimoto *et al.* 2007). DEC2 may be a key transcription factor, which plays an important role in the cross-talk between the hypoxia-response system and circadian regulation.

Experimental procedures

Cell culture

NIH3T3, Sarcoma 180 and COS-7 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). HSC-3 cells were obtained from the Japanese Cancer Research Resources Bank. Cells were cultured in Dulbecco's Modified Eagle's Medium-high glucose (Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum. The cells were cultured at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 (20% O_2). Hypoxic exposure was performed in a hypoxic chamber (1% O_2 for NIH3T3 or HSC-3 cells, and 1% O_2 for 4 h or 3% O_2 for 24 h for Sarcoma 180 cells; Sarcoma 180 cells died in the presence of 1% O_2 for 24 h).

Construction of reporter and expression plasmids

The luciferase reporter plasmid, *Vegf*-luc, containing a 1289-bp fragment of the mouse *Vegf gene* in pGL3-basic (Fig. 2A1), was previously described (Koyanagi *et al.* 2003). A 34-bp fragment containing the hypoxia-response element (HRE) of *Vegf* with the flanking sequence was made by annealing the oligonucleotides 5′-CTAGTGTGCATACGTGGGTTTCCACAGGTCGTCT-3′ and 5′-TCGAAGACGACCTGTGGAAACCCACGTATGCA-CA-3′. Another 34-bp fragment which has a mutation in the HREm of *Vegf* was made in a similar manner using 5′-CTAGTGTGCAT*GATAT*GGTTTCCACAGGTCGTCT-3′ and 5′-TCGAAGACGACCTGTGGAAACC*ATATC*AT-GCACA-3′. These fragments were ligated into the *Nhe*I and *Xho*I sites of pTK-Luc upstream of the TK promoter (Fig. 2A; pVEGF-HRE-TK-Luc and pVEGF-HREm-TK-Luc). The promoter constructs of HRE and HREm of DEC1 (Fig. 2A; pDEC1-HRE-TK-Luc and pDEC1-HREm-TK-Luc), and the expression plasmids for FLAG-HIF-1α, FLAG-DEC2, FLAG-DEC1, DEC2 pcDNA, DEC1 pcDNA and PER2 pcDNA were previously described (Honma *et al.* 2002; Miyazaki *et al.* 2002; Sato *et al.* 2004; Fujimoto *et al.* 2007). Expression constructs encoding full-length mouse ARNT1 were obtained by subcloning PCR-generated fragments into the vector pcDNA3.1.

Transient transfection and luciferase assay

NIH3T3 or Sarcoma 180 cells were seeded at 1×10^4 cells per 16-mm well 24 h before transfection. The transfection of plasmid DNA was performed using Lipofectamine 2000 Reagent (Invitrogen Corp., Carlsbad, CA) as previously described (Kondo *et al.* 2006). After the transfection, the cells were incubated under normoxic conditions for 16 h. Then, these cells were incubated for an additional 24 h under hypoxic or normoxic conditions. The cells were harvested and the luciferase activity in the cell lysate was determined using a Dual Luciferase Reporter Assay System (Promega, Madison, WI). Luciferase activity was normalized using pTK-luc activity as a control. Values are mean ± SD for three wells.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated using an RNEasy RNA Isolation Kit (Qiagen, Hilden, Germany). First-strand cDNA was synthesized from 1 μg of total RNA, using ReverTra Ace (Toyobo, Osaka, Japan). PCR was performed using an aliquot of first-strand cDNA as a template under standard conditions with *ExTaq* DNA polymerase (Takara, Siga, Japan). The cDNA for mouse VEGF, ARNT1, DEC1, DEC2, PER2 and GAPDH were amplified up to 30 cycles. The cDNA for mouse HIF-1α was amplified 32 cycles. The primers used were as follows: VEGF-F: 5′-ATGAACTTTCT-GCTCTCTTGG-3′, VEGF-R: 5′-TCACCGCCTTGGCTT-GTCACATC-3′, HIF-1α-F: 5′-AGCCCTAGATGGCTTT-GTGA-3′, HIF-1α-R: 5′-TATCGAGGCTGTGTCGACTG-3′, ARNT1-F: 5′-GCTATAATCATTCCCAGGTTTCT-3′, ARNT1-R: 5′-CATTGTTGTAGGTGTTGCTTTGG-3′, DEC1-F: 5′-GTGAGTCACTCTCCAGTTT-3′, DEC1-R: 5′-ATCCGTGTCTAGCTGTGCAAT-3′, DEC2-F: 5′-ATGC-TCGACAGGCTTAGGACA-3′, DEC2-R: 5′-TGTGTGAGCT-GAGACATGAAAC-3′, PER2-F: 5′-CAGACTCATGATGAC-AGAGG-3′, PER2-R: 5′-AGGGCTGGCTCTCACTGGACA-3′, GAPDH-F: 5′-ACATTCGAGGCTCCAGTGAATTCGG-3′ and GAPDH-R: 5′-ACGACATACTCAGCACCGGCCTCAC-3′. As a result of alternative splicing, there are three transcripts encoding VEGF containing 120, 164 and 188 amino acid residues ($VEGF_{120}$, $VEGF_{164}$ and $VEGF_{188}$). The predicted size of the amplified product was 440 bp for $VEGF_{120}$ and 572 bp for $VEGF_{164}$. The band corresponding to $VEGF_{188}$ was not amplified. The products for HIF-1α, ARNT1, DEC1, DEC2, PER2 and GAPDH were 466, 768, 534, 612, 253 and 612 bp, respectively. The PCR products were separated on 1.5% (w/v) agarose gels.

SiRNA and real-time RT-PCR

Short interference RNA (siRNA) against DEC2 was synthesized by Qiagen. The sequences for the sense and anti-sense DEC2 siRNAs were 5′-r (CGUUGCAACCUAUUCUGAA) d (TT)-3′ and

5′-r (UUCAGAAUAGGUUGCAACG) d (TG)-3′. The negative control siRNAs were 5′-r (UUCUCCGAACGUGUCACGU) d (TT)-3′ and 5′-r (ACGUGACACGUUCGGAGAA) d (TT). The siRNA was transfected into cells using Lipofectamine 2000 reagent. After 50 h under normoxic conditions, the cells were incubated for an additional 12 h under hypoxic or normoxic conditions. RNA was prepared and subjected to a real-time quantitative-PCR analysis. The real-time PCR was performed using the SYBR Green Master Mix (Applied Bio-Science) and specific primers. The primer sets are as follows: DEC2-qF: 5′-CGCCCATTCAGTCCGACTT-3′, DEC2-qR: 5′-CGGGAGAGGTATTGCAAGACTT-3′, VEGF-qF: 5′-TACCTCCACCATGCCAAGTG-3′, VEGF-qR: 5′-ATGATTCTGCCCTCCTCCTTCT-3′, GAPDH-qF: 5′-GCACCGTCAAGGCTGAGAAC-3′, GAPDH-qR: 5′-ATGGTGGTGAAGACGCCAGT-3′.

Enzyme-linked immunosorbent assay (ELISA)

The concentration of mouse VEGF in the conditioned medium was measured using an ELISA kit (R&D systems, Minneapolis, MN). This assay recognizes the soluble forms of VEGF (mouse $VEGF_{120}$ and $VEGF_{164}$). The values were represented as the mean ± SD for the three wells.

Western blot analysis

The cells were seeded at 1×10^5 cells per 35-mm well and lysed using M-PER lysis buffer (Pierce, Rockford, IL). Protein concentrations were determined by BCA assay. The lysates (30 μg protein) were subjected to SDS/PAGE, and proteins were transferred to PVDF membranes. The membranes were incubated with antibodies specific for DEC2 (1 : 2000) (Kondo *et al.* 2006), DEC1 (1 : 1000) (Sato *et al.* 2004), HIF-1α (1 : 1000, Abcam, Cambridge, England), ARNT1 (1 : 3000, Abcam), PER2 (1 : 500, Trans Genic Inc, Kobe, Japan) or actin (1 : 30 000) followed by a horseradish peroxidase-conjugated secondary antibody (1 : 5000). An ECL Advance Western Blotting Detection kit (Amersham, Uppsala, Sweden) was used for detection.

Immunoprecipitation

COS-7 cells were seeded at 1×10^6 cells per 100-mm well 24 h before transfection, and transfected with expression vectors using Lipofectamine 2000 reagent. After 40 h, cells were lysed in lysis buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA and 1% Triton X-100. The lysates were incubated with anti-FLAG M2 agarose affinity gel (Sigma) overnight at 4 °C. Immunoprecipitates were washed 3 times with buffer containing 0.5 M Tris–HCl (pH7.4) and 1.5 M NaCl, and then were eluted in SDS sample buffer. The immunoprecipitated proteins were analyzed by Western blotting.

ChIP

ChIP was done using a kit from Upstate (Lake Placid, NY). Briefly, Sarcoma 180 and HSC-3 cells growing in 100-mm dishes under normoxic or 1% hypoxic conditions (4 h for Sarcoma 180 cells or 12 h for HSC-3 cells) were cross-linked using 1% formaldehyde at 37 °C for 7 min. After two washes with cold-PBS, cells were resuspended in 300 μL of lysis buffer (50 mM Tris–HCl (pH 8.1), 10 mM EDTA, 1% SDS and protease inhibitor cocktail mixture) and incubated on ice for 15 min. Cell lysate was sonicated to give DNA ranging in size from 200 to 900 bp. Samples were centrifuged for 10 min at 4 °C. The supernatants were diluted tenfold with dilution buffer (167 mM NaCl, 16.7 mM Tris–HCl (pH 8.1), 1.2 mM EDTA, 1.1% Triton X-100 and 0.01% SDS), pre-cleared with 60 μL of Protein G agarose for 1 h at 4 °C, and then incubated with either anti-HIF-1α antibody or normal rabbit IgG antibody over-night at 4 °C. The beads were washed with low-salt, high-salt, LiCl and TE buffers. Immunocomplexes were extracted from the beads with 1% SDS and 0.1 M $NaHCO_3$. Cross-linking was reversed by heating the eluates at 65 °C for 10 h. DNA was purified by ethanol precipitation, and the fragment of the mouse or human *Vegf* promoter containing the HRE, was amplified by PCR. The primers were designed to amplify the DNA fragment containing the HRE as shown in Fig. 6C. The sequences of the primers were mouse *Vegf*-promoter-F: 5′-CAGTTCCCTGGCAACATCTCTG-3′, mouse *Vegf*-promoter-R: 5′-CCAAATTTGTGGCACTGA-3′, human *Vegf*-promoter-F: 5′-CCTTTGGGTTTTGCCAGA-3′ and human *Vegf*-promoter-R′: 5′-CCAAGTTTGTGGAGCTGA-3′.

The sizes of products were 208 and 119 bp, respectively.

Animals and immunohistochemistry

Six-week-old male C57/BL6 mice (Crea Japan, Tokyo, Japan) were housed under 12 : 12-h LD conditions for 19 days, and killed at the indicated zeitgeber (ZT-an environmental agent or event that provides the setting or resetting of the biological clock of an organism: ZT-0 corresponds to 8:00 am lights on) time on day 20, and mouse rib cartilage samples were placed within phosphate-buffered saline (PBS) and stored in a deep freezer. Mice were housed under LD conditions. Sarcoma 180 cells were implanted into the mice and RNA was prepared as previously described (Koyanagi *et al.* 2003). The cDNA of HIF-1α, DEC2, VEGF and GAPDH was amplified by RT-PCR. To evaluate the quantitative reliability of RT-PCR, we performed a kinetic analysis of amplified products to ensure that signals were derived only from the exponential phase of amplification. The density of each band was analyzed with the use of NIH image software.

Serial paraffin sections of cartilage in rib were immunostained using DAKO ENVISION Kit/HRP (DAB) (Dako Cytomation, Kyoto, Japan). Endogenous peroxidase was blocked with 0.03% hydrogen peroxidase containing sodium azide for 10 min. This was followed by incubation for 1 h at room temperature with rabbit polyclonal anti-DEC2 antibody (1 : 50), VEGF (1 : 100; Santa Cruz, CA), HIF-1α (1 : 200) and ARNT1 (1 : 200) diluted in antibody diluent. The sections were then sequentially incubated with the Labeled Polymer, HRP for 30 min, and with a ready-to-use DAB + substrate-chromogen solution (1–3 min). Finally, the sections were counterstained with Mayer's hematoxylin. Specificity was confirmed by applying PBS instead of the

primary antibodies, or by replacing the primary antibodies with non-immune serum.

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