

Involvement of c-Myc in the proliferation of MCF-7 human breast cancer cells induced by bHLH transcription factor DEC2

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Abstract. Differentiated embryonic chondrocyte expressed gene 1 (DEC1; BHLHE40/STRA13/SHARP2) and differentiated embryonic chondrocyte expressed gene 2 (DEC2; BHLHE41/SHARP1) are basic helix-loop-helix (bHLH) transcriptional factors that are involved in the regulation of cell differentiation, circadian rhythms, response to hypoxia and carcinogenesis. Previous studies have demonstrated that the expression of DEC2 is induced under hypoxic conditions in various normal and cancer cell lines. In the present study, using RT-qPCR and western blot analysis, we demonstrated that hypoxia induced the expression of DEC1 and DEC2 in MCF-7 human breast cancer cells; their expression levels reached a peak at different time points. In particular, we found that the expression pattern of the hypoxia-inducible factor (HIF)-1 α protein was similar to DEC1, and that of the HIF-2 α protein was identical to that of DEC2. The knockdown of HIF-2 α using siRNA suppressed the phosphorylation of Akt, as well as the expression of DEC2 and c-Myc. Hypoxia failed to affect the expression of DEC2 and c-Myc when the PI3K/Akt signaling pathway was blocked. In addition, the overexpression of DEC1 and DEC2 was induced by transfecting the cells with a pcDNA vector. The overexpression of DEC2, but not that of DEC1, increased the proliferation of the MCF-7 cells under both normoxic and hypoxic conditions. Concomitantly, the expression of c-Myc was upregulated by

exposure to hypoxia and by the overexpression of DEC2. In conclusion, DEC2 participates in hypoxia-induced cell proliferation by functioning as a target gene of the PI3K/Akt signaling pathway and regulating the expression of c-Myc.

Introduction

Breast cancer is the most common type of cancer in women worldwide. It has become the second most common cause of cancer-related mortality in women according to the statistical data supplied by Siegel *et al* in 2013 (1). Although the mortality rate due to breast cancer has declined by approximately 30% over the past 20 years, metastatic breast cancer remains incurable (1). A low oxygen status (hypoxia or anoxia) is a characteristic of most solid tumors and is associated with malignant progression in several types of cancer, including breast cancer (2). It also severely affects the efficacy of radiotherapy or chemotherapy, as hypoxia usually protects tumor cells from being damaged through several pathways (3-6): i) severe structural and functional abnormalities of tumor microvessels (perfusion-limited O₂ delivery), ii) deterioration of diffusion geometry (diffusion-limited O₂ delivery) and iii) tumor-associated and/or therapy-induced anemia leading to a reduced O₂ transport capacity of the blood (anemic hypoxia) (7). The hypoxia-inducible factor (HIF) transcription factors mediate the primary transcriptional responses to hypoxic stress in normal and transformed cells. An O₂-labile α -subunit (HIF-1 α , HIF-2 α and HIF-3 α) and a stable β -subunit (Arnt1, Arnt2 and Arnt3) constitute the heterodimeric HIF proteins. The difference between HIF-1 α and HIF-2 α is mainly observed in the N-terminal transactivation domain (N-TAD), which contributes to target gene specificity, while the C-terminal transactivation domain (C-TAD) is homologous between the isoforms and promotes the expression of their common target genes (8). Recent studies have reported interesting data on distinguishing the roles of HIF-1 α and HIF-2 α . Although the two isoforms share some similar properties, they exhibit unique and even opposite characteristics when expressed in the same cell type (9,10). These effects are partly mediated through the regulation of distinct targets, as well as through direct and indirect interactions with complexes that contain important oncoproteins and tumor suppressors.

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Abbreviations: DEC1, differentiated embryonic chondrocyte expressed gene 1; DEC2, differentiated embryonic chondrocyte expressed gene 2

Key words: differentiated embryonic chondrocyte expressed gene 1, differentiated embryonic chondrocyte expressed gene 2, hypoxia, MCF-7

As one of the significant oncoproteins, c-Myc has been reported to participate in multiple aspects of cellular function, such as replication, growth, metabolism, differentiation, apoptosis and carcinogenesis (11-16). It activates and represses the transcription of target genes by binding to the E-box (CACGTG) motif after forming a heterodimer with another basic helix-loop-helix/leucine zipper (bHLH/Zip) protein MAX. The aberrant expression of c-Myc is closely related to various types of cancer.

Human differentiated embryonic chondrocyte expressed genes (DECs), mouse stimulated by retinoic acid (STRA), and rat split- and hairy-related protein (SHARP) constitute a structurally distinct class of bHLH proteins. DEC2 is an important factor in the regulation of apoptosis, cell differentiation, tumor progression, circadian rhythms and the response to hypoxia. However, the roles of DEC2 under hypoxic conditions have not yet been clarified. The present study demonstrates that DEC2 promotes the proliferation of MCF-7 breast cancer cells by regulating the expression of the c-Myc oncoprotein under hypoxic conditions and through the activation of the PI3K/Akt signaling pathway.

Materials and methods

Cell culture and treatment. MCF-7 human breast cancer cells were cultured as previously described (17). Hypoxia (3% O₂) was induced by the culture of cells for various periods of time (2, 8 and 24 h) inside an air-tight chamber with inflow and outflow valves that was infused with a mixture of 3% O₂, 5% CO₂, 92% N₂ (BNP-110; ESPEC Corp., Osaka, Japan). In order to block PI3K, the cells were incubated with the PI3K/Akt inhibitor, LY294002 (Calbiochem, San Diego, CA, USA) at 10 μM for 1 h, followed by incubation at 3% O₂ for 24 h.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Three independent RNA samples were prepared from the above cells for RT-qPCR. Total RNA was isolated using an RNeasy RNA Isolation kit (Qiagen GmbH, Hilden, Germany). First-strand cDNA was synthesized from 1 μg of total RNA using ReverTra Ace (Toyobo Co., Ltd., Osaka, Japan). Quantitative (real-time) PCR was performed using SYBR-Green Master Mix (Life Technologies, Carlsbad, CA, USA). The sequences and product sizes of the DEC1 (BHLHE40/STRA13/SHARP2) and DEC2 (BHLHE41/STRA13/SHARP1) primer sets were described in a previous study (18).

DEC1 and DEC2 overexpression. Human DEC1 and DEC2 cDNA were subcloned into pcDNA/zeo as previously described (18). MCF-7 cells were seeded at 5x10⁴ cells/35-mm well. DEC1 or DEC2 pcDNA was transfected into the cells using the lipofectamine LTX (Invitrogen, Carlsbad, CA, USA) 24 h later. Following transfection, the cells were incubated for an additional 24 h and subjected to western blot analysis.

Short interference RNA (siRNA). siRNA oligos against human HIF-2α were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). For the siRNA transfection experiments, the cells were seeded at 5x10⁴ cells/35-mm well. The siRNA was transfected into the cells 24 h later using the lipofectamine RNAiMAX reagent (Invitrogen) according to the

manufacturer's instructions. Following transfection, the cells were incubated under normoxic (O₂ 21%) or hypoxic (O₂ 3%) conditions for a further 24 h and subjected to western blot analysis.

Western blot analysis. The cells were lysed using M-PER lysis buffer (Thermo Fisher Scientific, Rockford, IL, USA) and the protein concentration was determined using the bicinchoninic acid (BCA) assay. Cell lysates were subjected to SDS-PAGE, and the proteins were transferred onto PVDF membranes (Immobilion P; Millipore, Tokyo, Japan), which were then incubated with antibodies. Bound antibodies were visualized by chemiluminescence using the ECL or ECL Prime Western Blotting Detection system (Amersham Biosciences, Uppsala, Sweden). The experiment was repeated 3 times.

Antibodies. The membranes for western blot analysis were incubated with antibodies specific to DEC1 (1:10,000; Novus Biologicals, Inc. Littleton, CO, USA), DEC2 (1:20,000; H-72X; Santa Cruz Biotechnology, Inc.), HIF-1α (1:3,000; H-206X; Santa Cruz Biotechnology, Inc.), HIF-2α (1:3,000; Santa Cruz Biotechnology, Inc.), c-Myc (1:3,000; Epitomics, Inc., Burlingame, CA, USA), phospho-Akt (1:6,000; Epitomics Inc.), Akt (1:10,000; Epitomics Inc.) and actin (1:20,000; Sigma, St. Louis, MO, USA), followed by horseradish peroxidase-conjugated secondary antibody (IBL, Fujioka, Gunma, Japan). Can Get Signal Immunoreactions Enhancer Solution (Toyobo Co., Ltd.) or Immunoshot Immunoreaction Enhancer Solution (Cosmobio Co., Ltd., Tokyo, Japan) was used to dilute the primary antibodies.

Cell proliferation assay. Cell proliferation was measured by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay. The MCF-7 cells were seeded in 96-well plates. The cells were transfected with an empty plasmid (pcDNA) or the DEC1 or DEC2 expression plasmid. After 18 h of transfection, the cells were cultured under hypoxic conditions for a further 24 h. Subsequently, the cells were added to each well along with the CellTiter 96® AQueous One Solution Reagent (Promega Corp., Madison, WI, USA) and were incubated at 37°C for an additional 1 h. Absorbance (OD₄₉₀ nm) was measured using a 96-well plate reader.

Statistical analysis. The results are presented as the means ± standard error of the mean (SEM) of the number of experiments indicated in the figure legends. Statistical analysis was performed using the Student's t-test. The level of statistically significant differences was set at P<0.05, and the level of highly significant differences at P<0.001.

Results

Effects of exposure to hypoxia on the expression of DEC1 and DEC2 in MCF-7 cells. Firstly, we analyzed the effects of exposure to hypoxia on gene expression in MCF-7 human breast cancer cells. As shown in Fig. 1, exposure of the cells to O₂ (3%) for 2, 8 and 24 h induced the protein expression of HIF-1α and HIF-2α when compared with the control cells exposed to normal oxygen conditions. In addition, HIF-1α expression

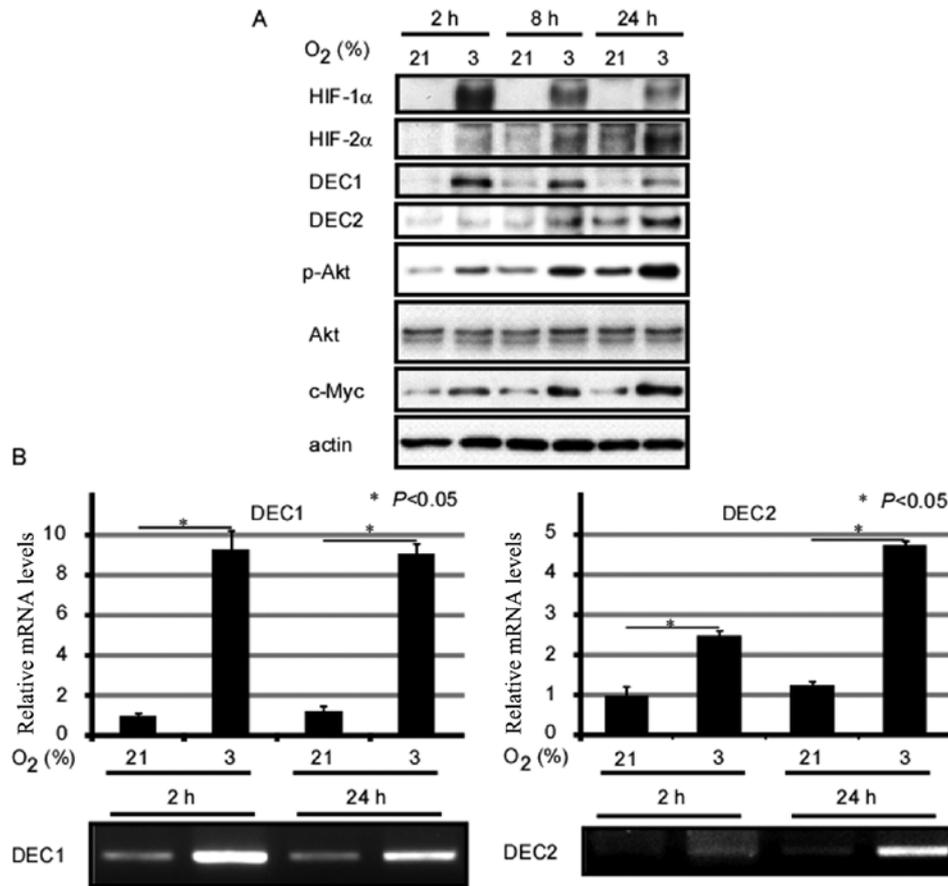


Figure 1. Exposure to hypoxia upregulates the expression of differentiated embryonic chondrocyte expressed gene (DEC)1 and DEC2 in MCF-7 breast cancer cells. (A) MCF-7 cells were cultured under hypoxic conditions for 2, 8 and 24 h. Cell lysates were prepared and subjected to western blot analysis for the expression of hypoxia-inducible factor (HIF)-1α, HIF-2α, DEC1, DEC2, phosphorylated (p-)Akt, Akt, c-Myc and actin. Representative data of 3 independent experiments with similar results are shown. (B) MCF-7 cells were exposed to hypoxia (O₂ 3%) or normoxia (O₂ 21%) for 2 and 24 h. Total RNA was prepared and subjected to RT-qPCR for the mRNA expression of DEC1 and DEC2. Each value represents the mean ± SEM (bars) of 3 independent experiments (*P<0.05, according to the t-test).

levels reached a peak at 2 h, whereas HIF-2α expression levels reached a peak at 24 h. Although the endogenous expression of DEC1 and DEC2 was weak in the MCF-7 cells, their mRNA and protein expression levels markedly increased under hypoxic conditions. Furthermore, the DEC1 protein level reached its peak when the cells were incubated under hypoxic conditions for 2 h, whereas the DEC2 protein expression level reached its peak following the exposure of the cells to hypoxia for 24 h. However, the relative mRNA expression of DEC1 showed no significant change between the 2- and 24-h time points following exposure to hypoxia. Moreover, exposure to hypoxia induced the expression of c-Myc and the phosphorylation of Akt in the MCF-7 cells (Fig. 1).

Exposure to hypoxia for 24 h enhances the proliferation of MCF-7 cells. Both Akt phosphorylation and c-Myc upregulation are closely associated with the proliferation of various types of cancer cells (14,19). Thus, we examined whether exposure to hypoxia affects the proliferation of MCF-7 human breast cancer cells by MTS assay (Fig. 2A). Exposure to hypoxia for 24 h induced the proliferation of the MCF-7 cells, and the DEC2-overexpressing MCF-7 cells showed an increased proliferation rate when compared with either the

pcDNA-transfected- or the DEC1 pcDNA-transfected cells. Moreover, DEC2 overexpression enhanced the proliferation of the MCF-7 cells under both normoxic and hypoxic conditions.

Overexpression of DEC2 induces c-Myc expression in MCF-7 cells. To clarify the biological functions of DEC1 and DEC2 in breast cancer cells, we transiently transfected the expression vectors for DEC1 or DEC2 into the MCF-7 cells and analyzed whether DEC1 or DEC2 affects the expression of c-Myc. Under normoxic conditions, c-Myc expression was slightly induced by the overexpression of DEC2; however, the c-Myc protein level was sharply elevated when DEC2 was overexpressed under hypoxic conditions. By contrast, DEC1 overexpression under both normoxic and hypoxic conditions did not affect the expression levels of c-Myc when compared with the empty vector-transfected cells (Fig. 2B). The above data suggest that DEC2, but not DEC1, regulates the expression of c-Myc in MCF-7 cells.

Knockdown of HIF-2α inhibits Akt phosphorylation, as well as the expression of DEC2 and c-Myc. It has been reported that HIF-2α induces the proliferation of renal cell carcinoma cells (20). We thus hypothesized that HIF-2α may contribute

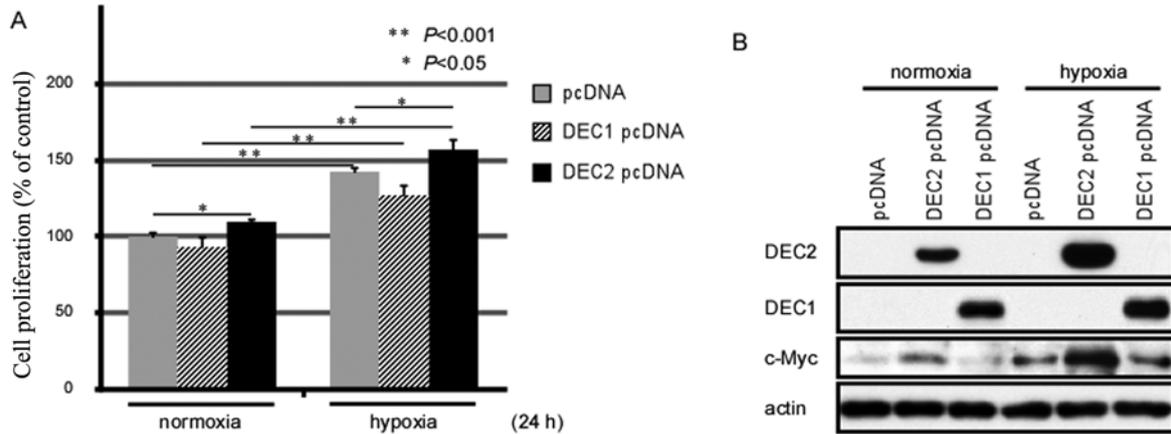


Figure 2. Differentiated embryonic chondrocyte expressed gene (DEC)2 overexpression induces cell proliferation through the upregulation of c-Myc. (A) The enforced expression of DEC2 increased MCF-7 cell proliferation. MCF-7 cells were transfected with pcDNA, DEC1 pcDNA or DEC2 pcDNA and then exposed to hypoxia or normoxia for 24 h, and cell proliferation was measured by MTS assay. The values are shown as a percentage of the control. Each value represents the mean \pm SEM (bars) of 3 independent experiments ($^{*}P<0.05$ or $^{**}P<0.001$, compared with the control pcDNA). (B) DEC2 overexpression upregulated the expression of the oncoprotein c-Myc. MCF-7 cells were treated as described above, and cell lysates were prepared from the cells and subjected to western blot analysis for the expression of DEC1, DEC2, c-Myc and actin. One representative of at least 3 independent experiments with similar results is shown.

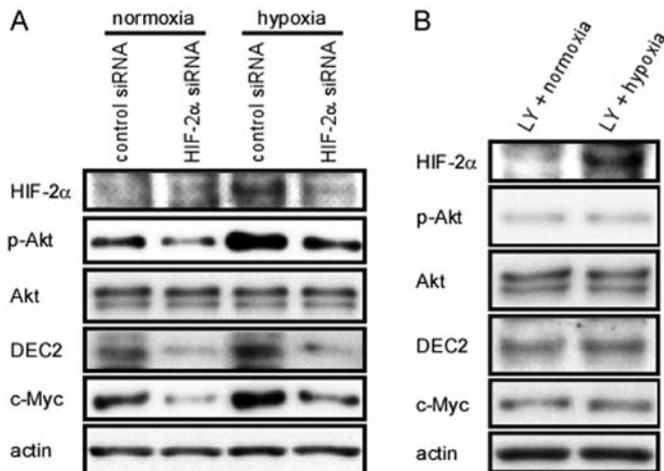


Figure 3. Hypoxia-inducible factor (HIF)-2 α regulates differentiated embryonic chondrocyte expressed gene (DEC)2 and c-Myc expression by activating Akt. (A) MCF-7 cells were transfected with HIF-2 α short interference RNA (siRNA) and incubated for 24 h. Subsequently, the cells were incubated under hypoxic or normoxic conditions for an additional 24 h, before being lysed. The lysates were subjected to western blot analysis for the expression of HIF-2 α , pAkt, Akt, DEC2, c-Myc and actin. (B) MCF-7 cells were incubated with LY294002 (PI3K/Akt inhibitor; 10 μ M) for 1 h before being exposed to hypoxia for 24 h. The lysates were prepared and subjected to western blot analysis for the expression of HIF-2 α , pAkt, Akt, DEC2, c-Myc and actin.

to the increased proliferation of MCF-7 cells under hypoxic conditions. In order to characterize the function of HIF-2 α protein, the MCF-7 cells were transiently transfected with siRNA against HIF-2 α for 24 h, followed by another 24 h of exposure to hypoxia or normoxia; western blot analysis was then performed. The silencing of HIF-2 α suppressed Akt phosphorylation, as well as DEC2 and c-Myc protein expression, regardless of the oxygen concentration (Fig. 3A). However, the expression of DEC1 was not affected by HIF-2 α siRNA (data not shown).

Inhibition of Akt phosphorylation decreases the induction of DEC2 by exposure to hypoxia. DEC1 has been reported to be involved in the phosphorylation of Akt (21). Therefore, we examined the association between Akt and DEC2. Pre-treatment of the MCF-7 cells with LY294002, an inhibitor of the PI3K/Akt signaling pathway, abrogated the hypoxia-induced Akt phosphorylation, as well as DEC2 expression (Fig. 3B). In addition, exposure to hypoxia failed to upregulate the protein level of c-Myc when the PI3K/Akt signaling was blocked.

Discussion

In the present study, we demonstrate that DEC2 plays a role, at least in part, in the proliferation of MCF-7 breast cancer cells induced by HIF-2 α under low oxygen conditions. Silencing the expression of HIF-2 α inhibited the phosphorylation of Akt, as well as the expression of DEC2 and c-Myc. It seems that DEC2 functions as an effector of the PI3K/Akt signaling pathway, since the interruption of this pathway blocked the induction of DEC2 expression following exposure to hypoxia. In addition, our results demonstrated that DEC2, but not DEC1, upregulated the expression of c-Myc. DEC2 and DEC1 are both transcription factors and have been shown to transrepress their target genes by binding to the E-box elements (22-24). Thus, it is suggested that the regulation of c-Myc by DEC2 may not be mediated through binding to the E-boxes. Future studies are required to clarify the details of the mechanisms through which DEC2 regulates c-Myc expression, and the differences between DEC2 and DEC1 in regulating their target genes.

Although no significant changes in the mRNA level of DEC1 were observed between the cells exposed to hypoxia for 2 and those exposed for 24 h, the protein expression of DEC1 was significantly reduced in the cells cultured under hypoxic conditions for 24 h compared with that in the cells cultured under hypoxic conditions for 2 and 8 h. DEC1 has been reported as a marker of tumor hypoxia in the A549 lung adenocarcinoma cell line (25). In that study, the mRNA and

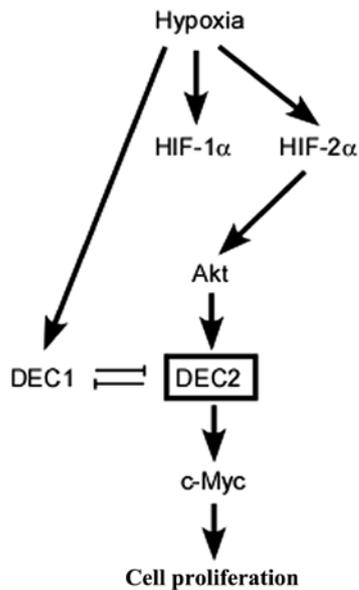


Figure 4. Role of differentiated embryonic chondrocyte expressed gene (DEC)2 in the hypoxia-induced proliferation of MCF-7 cells. Exposure to hypoxia upregulated the expression of hypoxia-inducible factor (HIF)-2 α , which activated the phosphorylation of Akt. DEC2 functioned as a target of the PI3K/Akt pathway and regulated the expression of c-Myc and contributed to the hypoxia-induced proliferation of human breast cancer cells.

protein expression levels of DEC1, as well as those of HIF-1 α , were upregulated by hypoxia in a time-dependent manner, and the expression levels reached their peak at the time point of 48 h (25). However, in our study, the protein expression of DEC1 decreased after the cells were cultured under hypoxic conditions for 24 h, when compared with those cultured for 2 h; however, the mRNA expression level of DEC1 remained stable at all time points. We hypothesized that the differences may arise from the specificity of the cell line and the different methods used for detecting the expression of the protein. In the other studies mentioned above, the expression of DEC1 and HIF-1 α was detected by immunocytochemical staining. Moreover, previous studies have demonstrated that DEC2 and DEC1 inhibit each other by binding the E-box on their promoters (23). Therefore, the reverse expression patterns observed under hypoxic conditions may be partly caused by the inhibitory effects exerted on each other.

DEC expression can be induced by a variety of other stimuli, such as transforming growth factor (TGF)- β , cytokines, retinoic acid, insulin and light (26-30). In the present study, we found that exposure of the cells to hypoxia increased the expression of DEC1 and DEC2 in a different manner. More specifically, DEC1 expression reached its peak at 2 h following exposure to hypoxia, whereas DEC2 expression reached its peak at 24 h following exposure to hypoxia. We also observed that DEC1 had an expression pattern similar to HIF-1 α protein, whereas DEC2 had an expression pattern similar to that of HIF-2 α protein. Although DEC1 and DEC2 exhibit a similar structure in their N-terminal, the C-terminal sequences are quite different. That is, the proline-rich region of DEC1 is replaced by the alanine/glycine-rich domain in DEC2 protein (31). Existing evidence suggests that DEC1 and DEC2 are expressed and function

in a different manner during physiological and pathological processes (31). In the present study, we proved that DEC2, but not DEC1, induced cell proliferation through the upregulation of c-Myc expression. Further studies are required to elucidate the function of the C-terminal of DEC1 and DEC2.

As an adverse prognostic factor, independent of standard prognostic factors, such as tumor stage and nodal status, hypoxia has been observed in a wide range of malignancies, including cancer of the breast, the uterine cervix, the rectum, the pancreas, the lungs, the brain, as well as soft tissue sarcomas, non-Hodgkin's lymphomas, malignant melanomas, metastatic liver tumors and renal cell cancer (7). The hypoxic microenvironment of tumors contributes to malignant progression, poor prognosis and resistance to chemotherapy and radiation. HIFs are crucial regulators of the response to low oxygen conditions (7). To date, HIF-1 α and HIF-2 α are the most extensively studied among the HIF isoforms. While the two isoforms share similar domain structures and overlapping target genes, it is now clear that they also have unique target genes. Moreover, HIF-1 α and HIF-2 α usually have divergent roles even in regulating the same genes (32). Recently, it has been reported that HIF-1 α is activated during the initial response to hypoxia, whereas HIF-2 α plays a major role during exposure to chronic hypoxia (33). The similar expression patterns of DEC2 and HIF-2 α demonstrated in our study suggests that DEC2 is an important mediator during exposure to chronic hypoxia.

The c-Myc gene, which was first described by Bishop *et al* in 1982, is a bHLH/Zip-type transcription factor (34). c-Myc forms a heterodimer with the bHLH/Zip protein MAX. Following dimerization, this complex binds to specific DNA sites, at CACGTG sequences known as the E-box motif, to activate and repress the transcription of target genes, as well as to modulate chromatin (35). As one of the major human oncogenes, c-Myc is frequently altered in many forms of cancer (35-38). It modulates the cell cycle and cell proliferation, increases cell metabolism and stimulates differentiation, among its many other biological functions. Studies have produced conflicting results as to how HIF-2 α affects c-Myc expression; some researchers have demonstrated that HIF-2 α inhibits c-Myc through direct interaction under physiological conditions (20,39,40). However, others have suggested that HIF-2 α enhances c-Myc activity by binding and stabilizing c-Myc/Max complexes (32). Our results are in agreement with the latter, as we demonstrated that DEC2 regulated c-Myc expression through a yet undetermined mechanism.

Fig. 4 summarizes the possible pathways responsible for the proliferation of MCF-7 breast cancer cells induced by exposure to hypoxia. Although the amino acid sequences of DEC1 and DEC2 share an approximately 40% homology, they have different effects on the cell proliferation induced by hypoxia and on apoptosis, as previously reported (17). In the present study, we found the c-Myc expression was increased by DEC2 overexpression in the MCF-7 cells. In general, DEC2 is thought to function as a transcriptional repressor by binding to the E-box in the promoters of its target genes (31). In our study, however, DEC2 was shown to function as a positive inducer by enhancing the expression of c-Myc. In our previous study, we demonstrated that siRNA against DEC2 decreased the mRNA level of c-Myc in MCF-7 cells (18). We deduced

that DEC2 regulated the expression of c-Myc, not only at the transcriptional level, but also at the post-transcriptional level. DEC1 and DEC2 have also been reported to play distinct roles in the epithelial-mesenchymal transition of pancreatic carcinoma cells (41). The differential protein structure and subcellular localization of DEC1 and DEC2 may contribute to the divergent roles in regulating target genes. Undoubtedly, further investigation is warranted to determine the detailed mechanisms through which DEC2 regulates c-Myc expression.

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