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Differential regulation of *DEC2* among hypoxia-inducible genes in endometrial carcinomas

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Abstract. In this study, we demonstrate an important role of activation of the hypoxia-inducible factor-1 (HIF-1) pathway in endometrial carcinogenesis and tumor phenotype development of endometrial carcinoma, and suggest a unique role of the HIF-1-target gene, differentiated embryo chondrocyte 2 (*DEC2)*, in carcinogenesis. Hypoxia caused an increase in HIF-1α protein expression in 4 endometrial carcinoma cell lines. The expressions of its 5 target genes - *DEC1*, *DEC2*, carbonic anhydrase-9 (*CA9*), vascular endothelial growth factor (*VEGF*), and solute carrier family 2, member 1 (*SLC2A1*) - also reactively increased in most of the cell lines, except for *DEC2* in the SNG-M cells. The expression levels of *DEC2*, *CA9*, and *SLC2A1* were significantly higher in the 4 atypical hyperplasia tissues and 82 endometrial carcinomas compared with those in the 21 normal endometria. Clinicopathological analyses of carcinoma patients revealed a significant correlation of the *VEGF* and *SLC2A1* expression with the status of lymph-vascular involvement and lymph node metastasis. The expression levels of *CA9* and *VEGF* were significantly higher in the tumors of post- as opposed to pre-menopausal patients. The *SLC2A1* expression was also related to the FIGO stage, but the *DEC2* expression was inversely related to the FIGO grade. The activation of the HIF-1 pathway could be related to endometrial carcinogenesis, and the component, *DEC2*, could have different expression-regulatory mechanisms and unique roles in carcinogenesis.

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Abbreviations: DEC, differentiated embryo chondrocyte; bHLH, basic helix-loop-helix; *CA9*, carbonic anhydrase-9; *VEGF*, vascular endothelial growth factor; *SLC2A1*, solute carrier family 2, member 1; HIF-1, hypoxia-inducible factor-1

Key words: endometrial carcinoma, differentiated embryo chondrocytes, hypoxia, gene expression

Introduction

Endometrial carcinoma is one of the most common gynecological malignancies in the world (1-3). Even though the overall 5-year survivals for all the stages are ~80%, many women die from the cancer each year, and existing screening and therapeutic modalities are unlikely to substantially decrease mortality. These unsatisfactory conditions have promoted research for novel biomarkers and targets for the prevention and/or therapy of this carcinoma, and the factors involved in cellular hypoxic reaction are increasingly highlighted as possible candidates.

Tissue hypoxia is now known to contribute to biologically aggressive tumor phenotypes and the emergence of therapeutic resistance (4-8). In solid tumors, including endometrial carcinomas, cancer cells are often exposed to low oxygen tension (hypoxia), low pH, and low nutrition due to inadequate vasculature, which cause a variety of biological changes through the alterations of various gene expressions (5-7). Hypoxia-inducible factor-1α (HIF-1α) is a key transcription factor regulating a variety of hypoxia-inducible genes: The protein rapidly degrades in the cells under normoxic conditions but is strikingly induced by hypoxia (4-7). Hypoxic stabilization of the HIF-1α protein leads to the multiple-step activation of the HIF-1α function involving its nuclear translocation and heterodimerization with HIF-1ß [also called aryl hydrocarbon receptor nuclear translocator (ARNT)] to form transcription factor HIF-1. HIF-1 then interacts with the cognate hypoxia-response elements (HRE) of target promoters, followed by the recruitment of transcriptional coactivators (4-7). To date, dozens of HIF-1-target genes have been identified, and their functional significances have been analyzed in a variety of cancers (5-7).

Previously, we found the functional HRE in the promoter region of the differentiated embryo chondrocyte 1 (*DEC1*)/*BHLHB2* and *DEC2*/*BHLHB3* genes which identified them

Table I. Clinicopathological characteristics of endometrial carcinomas.

Characteristics	n (%)
All	82 (100)
Menopausal status	
Pre	36 (43.9)
Post	46 (56.1)
Histological subtype	
Endometrioid	77 (93.9)
Adenocarcinoma	68 (82.9)
Adenocarcinoma with squamous differentiation	9 (11.0)
Serous	1 (1.2)
Clear cell	1 (1.2)
Mucinous	1 (1.2)
Mixed	2 (2.4)
FIGO[a] grade (endometrioid adenocarcinoma)	
1	47 (69.1)
2	14 (20.6)
3	7 (10.3)
MI[b]	
None	28 (34.1)
<50%	35 (42.7)
≥50%	19 (23.2)
LVI[c]	
Absent	37 (45.1)
Present	45 (54.9)
LN[d] status	
Negative	61 (74.4)
Positive	10 (12.2)
Undetermined	11 (13.4)
FIGO stage	
IA	12 (14.6)
IB	32 (39.0)
IC	10 (12.2)
IIA	5 (6.1)
IIB	4 (4.9)
III	17 (20.7)
IV	2 (2.4)
Recurrence status	
Negative	74 (90.2)
Positive	8 (9.8)
Survival	
Surviving	78 (95.1)
Deceased	4 (4.9)

[a]International Federation of Gynecology and Obstetrics, [b]depth of myometrial invasion, [c]lymphovascular invasion, [d]lymph node.

as HIF-1-target genes (9). The *DEC1* coding basic helix-loop-helix (bHLH) transcription factor was originally identified as the molecule expressed in cAMP-dependently differentiated embryo chondrocytes (10), and *DEC2* was cloned from the human DNA database of EST as a member of the DEC subfamily (11). Several reports have suggested that DEC subfamily proteins could play a role in tissue development and regulation of the circadian rhythm at the transcriptional level (9-15). Several hypoxia-inducible genes, including carbonic anhydrase-9 (*CA9*), vascular endothelial growth factor (*VEGF*), and solute carrier family 2, member 1 (*SLC2A1*), have been well-characterized in various cancer tissues. However, little is known about DECs in cancer tissues, and almost nothing about *DEC2* (16-23).

In this study, we evaluated the expression of *DEC1* and *DEC2*, along with the well-known hypoxia-inducible genes *CA9*, *VEGF*, and *SLC2A1* in endometrial carcinoma, complex atypical endometrial hyperplasia, and adjacent normal endometria, in order to clarify the clinical significance of DECs in carcinogenesis in anticipation of their being novel biomarkers or targets for the prevention and/or therapy of endometrial carcinomas.

Materials and methods

Chemicals. All chemicals were of analytical grade and were purchased from Wako Pure Chemicals (Osaka, Japan) and Sigma (St. Louis, MO, USA).

Cell culture. A cervical carcinoma cell line, HeLa, and three endometrial carcinoma cell lines, HEC-1, SNG-II, and SNG-M, were obtained from the Health Science Research Resources Bank (Osaka, Japan). The Ishikawa (3-H-12 No. 117) cell line was kindly provided by Dr M. Nishida (Tsukuba University, Japan) (24). The cell lines were maintained in Eagle's minimal essential medium (MEM; Sigma-Aldrich Japan, Tokyo, Japan) or Dulbecco's MEM (DMEM; Sigma) supplemented with 10% fetal bovine serum (FBS; BioWhittaker, Verviers, Belgium) plus penicillin (50 IU/ml) and streptomycin (50 μg/ml) with passage every 3 days. The cells were incubated for 12-24 h under normoxic (21% pO_2) or hypoxic (1% pO_2) conditions prior to analysis. The cells were collected after washing with PBS and the cell pellets were immediately frozen in liquid nitrogen and stored at -80˚C until use.

Tissue samples. Fresh specimens of 82 endometrial carcinomas, 21 adjacent normal endometria, and 4 complex atypical endometrial hyperplasia were surgically obtained from 91 patients, who had not received any treatment before tumor sampling, at the Department of Obstetrics and Gynecology in Hiroshima University Hospital between 1992 and 2004. The specimens were immediately frozen in liquid nitrogen and stored at -80˚C until use. Clinicopathological diagnosis was determined according to the classification of the International Federation of Obstetrics and Gynecology (FIGO) (Table I). Histological subtypes were classified according to the pathological criteria of the World Health Organization (WHO) (1994). Written informed consent was obtained from all the patients, and the study protocol was approved by the institutional ethics committees.

RNA preparation and real-time reverse transcription-polymerase chain reaction (real-time RT-PCR). Total RNAs

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pared from frozen cell pellets or tissue specimens RNeasy® mini kit (Qiagen) according to the manufacturer's instructions, and stored at -80˚C until use. All RNA samples were qualified using Agilent Technologies 2100 Bioanalyzer (Agilent, Palo Alto, CA, USA). Total RNA (2 μg) was reverse-transcribed using the High-Capacity cDNA Archive™ kit (Applied Biosystems, Foster City, CA, USA) with random primers according to the manufacturer's instructions. The two hundredth aliquot of the cDNA (equivalent to 10 ng total RNA) was subjected to real-time RT-PCR. Real-time PCR was performed using TaqMan Gene Expression Assay (Applied Biosystems). Each reaction was carried out in triplicate for all the cell lines and tissues, using the ABI Prism 7900HT sequence detection system (Applied Biosystems). These triplicate measurements were averaged, and the relative gene expression levels were calculated as the ratio to the expression level of *ACTB* (ß-actin). A cDNA mix, consisting of 17 various cell lines, was used to create a standard curve of gene expressions.

Immunoblot analysis. After incubation in a 10-cm diameter dish, the cells were collected by scraping, washed with cold-PBS, and the cell pellets were stored at -80˚C until use. Whole cell extracts were prepared by sonication with the lysis buffer (7 M Urea, 10 mM Tris-HCl pH 6.8, 10% Glycerol) supplemented with 1x Complete mini® protease inhibitor cocktail, 1 mM DTT, and 1 mM PMSF. Fifty micrograms of extracts were subjected to 7% SDS-PAGE and blotted onto nitrocellulose filters. Anti-HIF-1α (BD Biosciences, San Jones, CA, USA) or anti-ß-actin (Sigma) antibody was used as the primary antibody, diluted 1:500 or 1:5000 in TBS containing 0.1% Tween-20 (TBS-T) and 1% non-fat milk at room temperature for 1 h. After several washes, a 1:1000 dilution of anti-mouse IgG horseradish peroxidase conjugate (Amersham Life Science) was used as the secondary antibody and incubated for 1 h at room temperature. After extensive washing with TBS-T buffer, immunocomplexes were visualized using the enhanced chemiluminescence reagent, ECL Plus (NEN Life ScienceProduct, Inc., Boston, MA, USA).

Immunohistochemical staining. Immunohistochemical staining was performed on formalin-fixed, paraffin-embedded tissues. For HIF-1α staining, 1:500 diluted anti-HIF-1α mouse monoclonal antibodies (H1α67, Novus Biologicals, Littleton, CO, USA) and the CSA system (Dako, Carpinteria, CA, USA), were employed. The sections were initially immersed in Target Retrieval Solution (Dako) at 97˚C for 45 min, and subsequent steps were performed according to the manufacturer's instructions.

The expression of the HIF-1α protein was defined as positive if distinct nuclear and/or cytoplasmic staining was observed in normal and/or tumor cells in any optical field (Fig. 2). Stained intensity was compared between the normal and cancer cells.

Statistics. All statistical tests were performed using StatView® version 5.0 software (SAS Institute Inc., Cary, NC, USA), and the Student's *t*-test or Mann-Whitney *U* test were used to determine the *P*-value.

Results

Hypoxic induction of HIF-1α protein in vitro. In order to clarify whether a hypoxic signaling pathway was active in the endometrial carcinoma cells, the protein levels of HIF-1α were evaluated under normoxic or hypoxic conditions in 4 human endometrial carcinoma cell lines (Fig. 1A). After incubation under normoxic (21% pO_2) or hypoxic (1% pO_2) conditions for 12 h, the cells were harvested and whole cell extracts were prepared. HeLa, a cervical carcinoma cell line, was used as the positive control for HIF-1α induction (25). Immunoblot analysis showed that hypoxia caused an increase in HIF-1α protein expression in endometrial carcinoma cell lines, although the reaction to hypoxia significantly varied among the cell lines. Compared with the control HeLa cells, HIF-1α induction was greater in the Ishikawa cells, less in the SNG-M and HEC-1 cells, and least in the SNG-II cells. The expression of the HIF-1α protein was barely detected in the hypoxic SNG-II cells, although there was a slight increase compared to its expression in the normoxic cells.

Hypoxic inductions of DEC1, DEC2 and known hypoxia-inducible genes in endometrial carcinoma cell lines. The downstream activity of the HIF-1 signaling pathway after hypoxic treatment was investigated by expression analyses of the target genes, including *DEC1* and *DEC2*. The cells were incubated under the same hypoxic conditions used for the HIF-1α protein analyses, and then total RNAs were extracted from the cell pellets. Real-time RT-PCR analyses revealed that all of the 5 HIF-1 target genes responded to hypoxia in at least some of the cell lines (Fig. 1B). Hypoxic treatment significantly increased the expression of *DEC1*, *VEGF*, and *SLC2A1* in all endometrial carcinoma cell lines, as it did in HeLa ($P<0.001$), and with the exception of the SNG-M cells, the *CA9* expression was also increased in 4 of the 5 cell lines ($P<0.001$). However, the reaction of *DEC2* to hypoxia varied among the cell lines: Hypoxic treatment significantly increased the expression of *DEC2* in both the Ishikawa and HEC-1 cell lines ($P<0.001$), although it did not affect the expression in the SNG-II cells and further reduced it in the SNG-M cells ($P<0.001$).

Expression of HIF-1α protein in endometrial carcinoma tissues. In order to clarify whether the HIF-1α signaling pathway was active in the tumor tissues, we then performed immunohistochemical analyses for the HIF-1α protein using tissue sections obtained from 37 endometrial carcinoma patients: In 32 of the 37 tissues (85%), immunoreactivity for the HIF-1α protein was detected in both the normal and carcinoma cells. We found that there were certain differences in reactivity between the carcinoma and normal stromal cells, suggesting the specific role of HIF-1α in carcinoma cells (Table II). In 27 tissue sections, the staining intensity of the carcinoma cells was stronger than that of normal stromal cells, and the positive stain was observed in both the cytoplasm and nucleus of carcinoma cells (Fig. 2). However, in normal stromal cells, the protein was mainly stained in the nucleus.

Expressions of hypoxia-inducible genes in normal, complex atypical endometrial hyperplasia, and endometrial carcinomas.

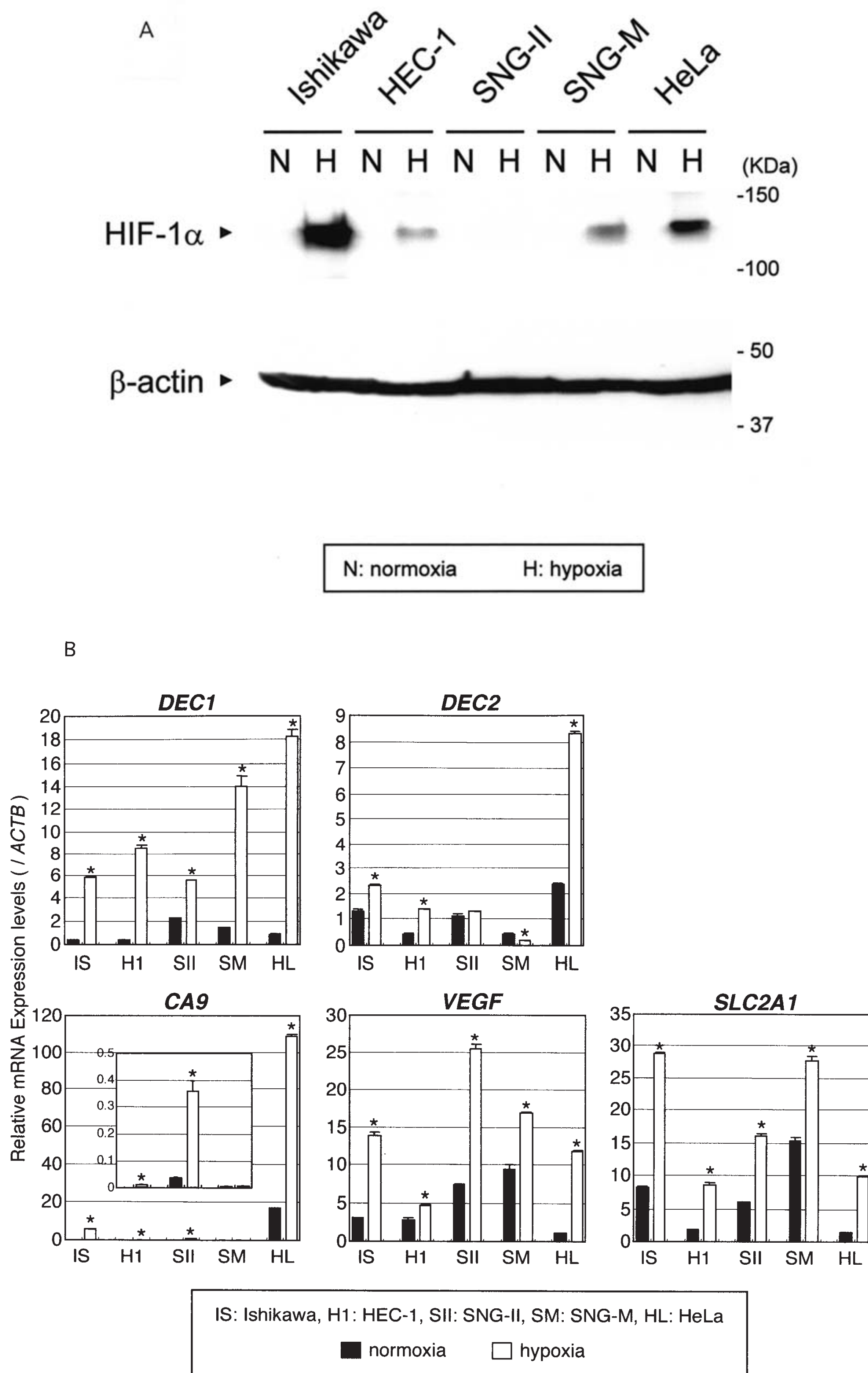

Figure 1. Activation of the HIF-1 pathway in the endometrial carcinoma cell lines. (A) The hypoxic induction of the HIF-1α protein. HIF-1α protein levels were evaluated under normoxic or hypoxic conditions in 4 human endometrial carcinoma cell lines by immunoblot analysis. After incubation under normoxic (21% pO_2) or hypoxic (1% pO_2) conditions for 12 h, the cells were harvested and whole cell extracts were prepared. HeLa, a cervical carcinoma cell line, was used as a positive control for the HIF-1α induction. (B) The hypoxic inductions of *DEC1* and *DEC2,* as well as known hypoxia-inducible gene expressions. The cancer cell lines were incubated under the same hypoxic conditions as above, and then total RNAs were extracted from the cell pellets. Real-time RT-PCR analyses of 5 HIF-1 target genes were performed, and the relative gene expression levels were calculated as the ratio to the expression level of *ACTB* (ß-actin). A cDNA mix, consisting of 17 various cell lines, was used to create standard curve of gene expression. Each value represents the mean ± SD for at least three independent experiments. The *P*-values were calculated using the Student's *t*-test. $^{*}P<0.001$.

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Summary of the immunohistochemical analysis of α expression.

All	HIF-1α expression status			
	Negative	Positive		
n (%)		N > T	N = T	N < T
37 (100)	5 (13.5)	0 (0)	5 (13.5)	27 (73)

N, normal; T, tumor.

We then analyzed the expressions of 5 HIF-1 target genes, *DEC1*, *DEC2*, *CA9*, *VEGF*, and *SLC2A1*, in endometrial carcinomas, atypical hyperplasias, and adjacent normal endometria, in order to clarify the significance of these genes in carcinogenesis (Fig. 3). The expression level of each gene was determined as the mean value of the triplicated real-time RT-PCR analyses for comparison in the 3 tissue groups: Eighty-two endometrial carcinomas, 21 adjacent normal endometria, and 4 complex atypical endometrial hyperplasias. Although there was no significant difference in the expression levels of *DEC1* and *VEGF* among the 3 groups, the expression levels of the other 3 genes, *DEC2*, *CA9*, and *SLC2A1*, were found to be higher in atypical hyperplasia (P=0.026, P<0.004 and P<0.005) and carcinoma tissues (P=0.0024, P<0.0001 and P<0.0001), compared with those in normal tissues. Although there was no significant difference in the expression levels in the atypical hyperplasia and carcinoma samples, increases in the expression of the 3 genes likely correlated with the carcinogenesis of endometrial tissues.

Clinicopathological analyses of hypoxia-inducible gene expression in endometrial carcinomas. Lastly, we investigated the association between the hypoxia-inducible gene expression and the clinicopathological features in 82 endometrial carcinomas (Table III). The association analysis revealed that all 4 genes, except *DEC1*, were related to some of the clinicopathological features of tumors: The *DEC1* expression did not relate to any of the clinicopathological features. However, the *DEC2* expression levels were likely associated with the FIGO grade (P=0.052); the high expression of *CA9* and *VEGF* significantly correlated with the menopausal status of patients; the expression of *VEGF* and *SLC2A1* was related to lymphatic involvement and lymph node metastasis; and the *SLC2A1* expression was also related to the FIGO stage. The mode of action of each HIF-1 target gene was discrete in the endometrial carcinomas, and the functions of *CA9*, *VEGF*, and *SLC2A1* in the carcinomas, overlapped in part.

Discussion

We have demonstrated here that hypoxia caused an increase in HIF-1α protein expression in 4 endometrial carcinoma cell lines, and that its 5 target genes - *DEC1*, *DEC2*, *CA9*, *VEGF*, and *SLC2A1* - also reactively increased in most of the cell

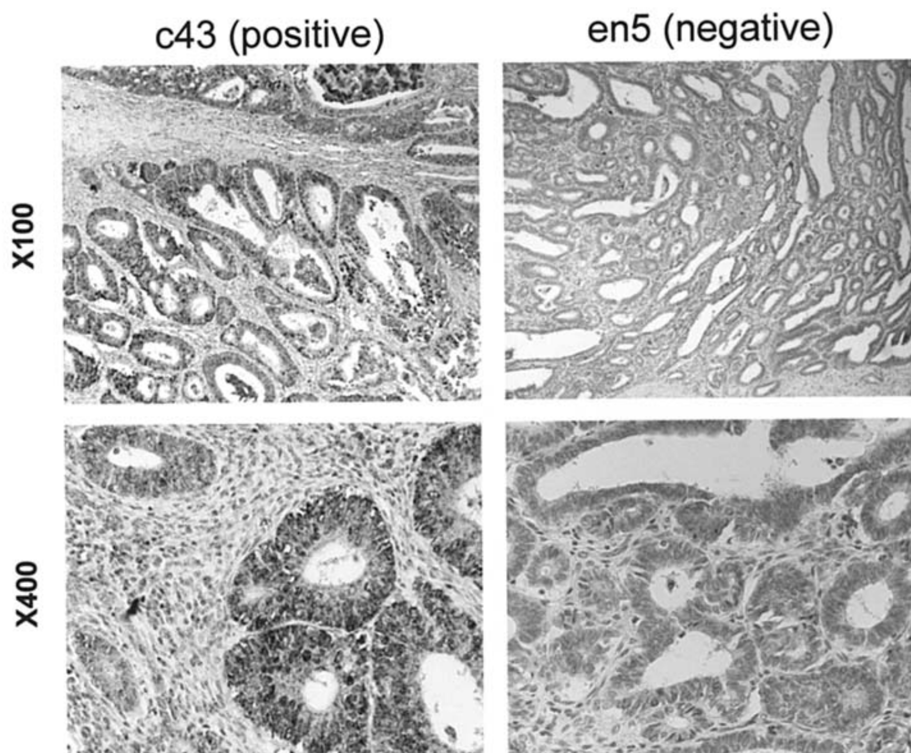

Figure 2. The expressions of the HIF-1α protein in endometrial carcinoma tissues. Representative examples of positive and negative staining for the HIF-1α protein were taken at x100 or x400 magnification. Immunoreactivity to HIF-1α was observed in the cytoplasm and nucleus of the cancer cells.

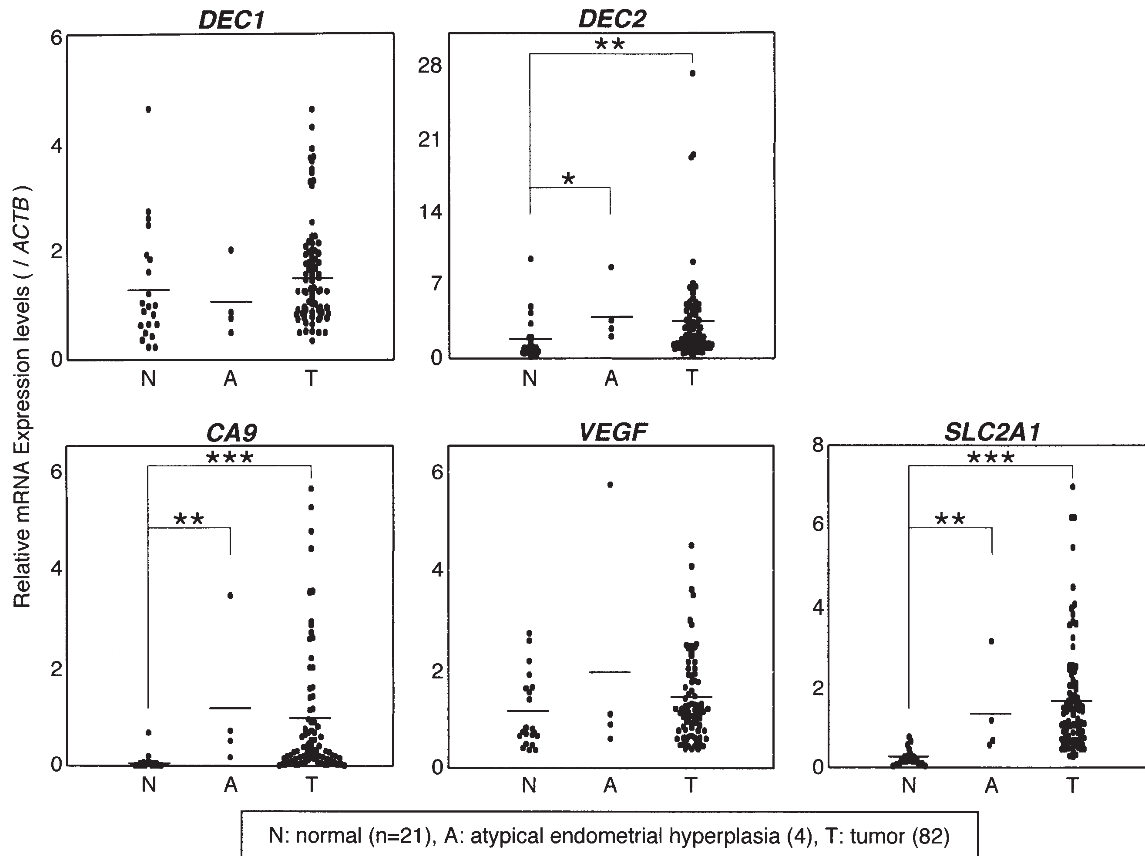

Figure 3. The expressions of hypoxia-inducible genes in normal, complex atypical endometrial hyperplasia, and endometrial carcinoma tissues. The expression levels of the 5 HIF-1 target genes were determined as the mean values of the triplicated real-time RT-PCR analyses for comparison in the 3 tissue groups: Eighty-two endometrial carcinomas, 21 adjacent normal endometria, and 4 complex atypical endometrial hyperplasias. The *P*-values were calculated using the Mann-Whitney *U* test. $^{*}P<0.05$, $^{**}P<0.005$, $^{***}P<0.0001$.

lines, except for *DEC2* in the SNG-M cells. We found that the expressions of *DEC2*, *CA9*, and *SLC2A1* were higher in complex atypical endometrial hyperplasia and endometrial carcinoma tissues compared with those in normal endometria. Furthermore, although the immunolabeling index of the HIF-1α protein in the carcinoma tissues did not correlate with any clinicopathological features of the tumors, the expression of 2 HIF-1 target genes correlated with aggressive clinicopathological features in 82 endometrial carcinomas: *VEGF* and/or *SLC2A1* correlated with lymphatic involvement, lymph-node metastasis, and the FIGO stage, and *CA9* and *VEGF*, correlated with the menopausal status, suggesting the existence of cross-talk between hypoxia- and estrogen-signaling. Since the expressions of several HIF-1-target genes increased in the process of endometrial carcinogenesis and were related to clinicopathological characteristics, it is likely that the activation of the HIF-1 pathway could play a key role in carcinogenesis and tumor phenotype development in endometrial carcinoma. However, only *DEC2* inversely correlated with the FIGO grade in its expression level.

DEC2 is involved in tissue development and regulation of the circadian rhythm as a transcriptional repressor (11,14,15), although its specific function in cancer remains to be clarified. Several reports have commented on the role of the other HIF-1 target genes including *DEC1* in various cancers (16-19), and GLUT1 (*SLC2A1* product) in endometrioid adenocarcinomas (23). However, ours is the first report to analyze the roles of *DEC2* in endometrial carcinoma. Our data suggest that *DEC2* can act on carcinogenesis and tumor phenotype development independently of the other HIF-1 target genes including, *DEC1*. It should be noted that, among the various genes, differences between *DEC1* and *DEC2* were noticeable: Contrary to the unchanged *DEC1* expression, *DEC2* increased its expression levels in atypical hyperplasia or endometrial carcinoma compared to normal endometria, suggesting an association with carcinogenesis. The *DEC1* protein expression analyses in lung or breast cancers (16-18), revealed an augmented expression in breast cancers. Although the *DEC1* protein expression was observed in 38% in a series of 115 non-small cell lung carcinomas, *DEC1* was persistently expressed in normal bronchial and alveolar tissues, suggesting that the loss of *DEC1* expression could be an early event in the development of lung cancer (19). Furthermore, it has been demonstrated that the *DEC1/2* distribution in the organs is different (11). Despite some inconsistent data, the overall reactivity of *DEC2* to hypoxia correlated with that of the HIF-1α protein *in vitro*, possibly suggesting its distinctive nature, i.e., HIF-1-dependent induction. Although *DEC2* promotes carcinogenesis, as opposed to *DEC1* in lung cancers, their functions in carcinomas could be origin- or cell type-specific and thereby discrete. The *DEC2* expression in cancer cell lines could be determined by certain *DEC2*-targeting mechanisms that occur during carcinogenesis. Despite an incomplete under-

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Statistical analysis of the clinicopathological characteristics relative to gene expression levels in endometrial carcinomas.

Variables (n)	Mean of relative expression/ACTB P-value														
	DEC1	*t*	*U*	*DEC2*	*t*	*U*	*CA9*	*t*	*U*	*VEGF*	*t*	*U*	*SLC2A1*	*t*	*U*
Menopausal status															
Pre (36)	1.507	0.470	0.266	3.669	0.632	0.550	0.677	0.167	**0.030**	1.137	**0.008**	**<0.001**	1.783	0.853	0.575
Post (46)	1.667			3.217			1.074			1.663			1.724		
Histological subtype															
Endometrioid (77)	1.632	0.349	0.410	3.208	0.079	0.337	0.928	0.437	0.568	1.391	0.106	0.094	1.770	0.620	0.720
Non-endometrioid (5)	1.195			6.610			0.463			2.065			1.444		
FIGO grade															
1 or 2 (61)	1.634	0.711	0.739	3.621	0.184	**0.052**	1.014	0.432	0.414	1.294	0.060	0.280	1.709	0.348	0.200
3 (7)	1.478			1.466			0.581			1.984			2.265		
MI															
<50% (63)	1.638	0.492	0.746	3.314	0.694	0.478	0.859	0.607	0.513	1.395	0.500	0.499	1.685	0.452	0.520
≥50% (19)	1.459			3.751			1.034			1.555			1.966		
LVI															
Absent (37)	1.453	0.233	0.137	3.069	0.503	0.140	0.971	0.650	0.246	1.226	0.060	**0.026**	1.270	**0.005**	**0.017**
Present (45)	1.715			3.699			0.841			1.602			2.145		
LN status															
Negative (61)	1.580	0.753	0.363	3.555	0.295	0.390	0.872	0.679	0.987	1.280	**0.011**	0.072	1.506	**0.001**	0.067
Positive (10)	1.690			2.101			0.687			2.038			3.057		
FIGO stage															
I or II (63)	1.629	0.592	0.987	3.686	0.290	0.359	0.947	0.549	0.672	1.363	0.210	0.145	1.557	**0.024**	0.178
III or IV (19)	1.490			2.517			0.743			1.661			2.389		
Recurrence status															
Negative (74)	1.623	0.462	0.815	3.293	0.428	0.827	0.947	0.311	0.553	1.387	0.175	0.195	1.730	0.703	0.719
Positive (8)	1.351			4.542			0.459			1.845			1.933		
Survival															
Surviving (78)	1.591	0.831	0.333	3.292	0.242	0.813	0.927	0.403	0.518	1.405	0.224	0.533	1.718	0.362	0.426
Deceased (4)	1.700			5.822			0.372			1.969			2.383		

t, Student's *t*-test; *U*, Mann-Whitney *U* test. Bold text, statistically significant.

standing of their functions as transcription repressors, findings that the expression of *DEC2* was stable in SNG-II, and further decreased in SNG-M under hypoxic conditions, could be explained by mutation(s) of the promoter region or the over-expression of transcriptional repressors such as *DEC1* (14).

We encountered unexpected data in our study: The activation of the HIF-1 downstream gene did not always correlate with the HIF-1α protein levels *in vitro*, and the immunolabeling index of the HIF-1α protein in carcinoma tissues did not correlate with any clinicopathological features of the tumors, contrary to the findings with the activated HIF-1 downstream genes *in vivo*. One hypothesis is that the HIF-1 pathway can be activated not only by hypoxia but also by the inactivation of several tumor suppressor genes such as *VHL*, *TP53* and *PTEN*, as reported elsewhere (5-7,26-28). Since mutations of *TP53* and *PTEN* were commonly observed in endometrial carcinomas, the inactivation of these tumor suppressor genes could be one of the mechanisms that activated the HIF-1 pathway in endometrial carcinogenesis (2,3). Our immnohistochemical analysis supported this hypothesis, since we found only nuclear staining in some adjacent stromal tissues, but both nuclear and cytoplasmic strong staining in the cancer cells, suggesting the aberrant activation of HIF-1α in cancer cells. The poor correlation in the activation between HIF-1α and its downstream genes could also be due to the involvement of the recruitment of coactivators and/or other transcription factors, including HIF-2α, among the mechanisms. Even so, the detailed mechanisms in the HIF-1 pathway activation and subsequently

in the carcinogenesis of endometrial carcinoma, as well as the functional roles of the HIF-1 downstream genes, remain unclear as with other cancers. Further investigation is required.

In conclusion, we demonstrated the increased expression of several hypoxia-inducible genes, including the previously unreported *DEC2* in endometrial carcinogenesis. The activation of the HIF-1 pathway played a part in carcinogenesis and tumor phenotype development in endometrial carcinoma. Among the HIF-1-target genes, the *DEC2* expression can be differentially regulated and plays a unique role in endometrial carcinogenesis.

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References

1. Amant F, Moerman P, Neven P, Timmerman D, Van Limbergen E and Vergote I: Endometrial cancer. Lancet 366: 491-505, 2005.
2. Prat J: Prognostic parameter of endometrial carcinoma. Hum Pathol 35: 649-662, 2004.
3. Shiozawa T and Konishi I: Early endometrial carcinoma: clinicopathology, hormonal aspects, molecular genetics, diagnosis, and treatment. Int J Clin Oncol 11: 13-21, 2006.
4. Vaupel P, Kallinowski F and Okunieff P: Blood flow, oxygen and nutrient supply, and metabolic microenvironment of human tumors (Review). Cancer Res 49: 6449-6465, 1989.
5. Semenza GL: Targeting HIF-1 for cancer therapy. Nature Rev 3: 721-732, 2003.
6. Kondoh-Kizaka S, Inoue M, Harada H and Hiraoka M: Tumor hypoxia: A target for selective cancer therapy. Cancer Sci 94: 1021-1028, 2003.
7. Poellinger L and Johnson RS: HIF-1 and hypoxic response: the plot thickens. Curr Opin Genet Dev 14: 81-85, 2004.
8. Fandrey J, Gorr TA and Gassmann M: Regulating cellular oxygen sensing by hydroxylation. Cardiovasc Res (in press).
9. Miyazaki K, Kawamoto T, Tanimoto K, Nishiyama M, Honda H and Kato Y: Identification of functional hypoxia response elements in the promoter region of the DEC1 and DEC2 genes. J Biol Chem 277: 47014-47021, 2002.
10. Shen M, Kawamoto T, Teramoto M, Makihira S, Fujimoto K, Yan W, Noshiro M and Kato Y: Induction of basic helix-loop-helix protein DEC1 (BHLHB2)/Stra13/Sharp2 in response to the cyclic adenosine monophosphate pathway. Eur J Cell Biol 80: 329-334, 2001.
11. Fujimoto K, Shen M, Noshiro M, Matsubara K, Shingu S, Honda K, Yoshida E, Suardita K, Matsuda Y and Kato Y: Molecular cloning and characterization of DEC2, a new member of basic helix-loop-helix proteins. Biochem Biophys Res Commun 280: 164-171, 2001.
12. Shen M, Yoshida E, Yan W, Kawamoto T, Suardita K, Koyano Y, Fujimoto K, Noshiro M and Kato Y: Basic Helix-loop-Helix protein DEC1 promotes Chondrocyte differenciation at the early and terminal stages. J Biochem Chem 51: 50112-50120, 2002.
13. Yun Z, Maecker HL, Johonson RS and Giaccia AJ: Inhibition of PPAR gamma 2 gene expression by the HIF-1-regulated gene DEC1/Stra13: a mechanism for regulation of adipogenesis by hypoxia. Dev Cell 3: 331-341, 2002.
14. Hamaguchi H, Fujimoto K, Kawamoto T, Noshiro M, Maemura K, Takeda N, Nagai R, Furukawa M, Honma S, Honma K, Kurihara H and Kato Y: Expression of the gene for Dec2, a basic helix-loop-helix transcription factor, is regulated by a molecular clock system. Biochem J 382: 43-50, 2004.
15. Honma S, Kawamoto T, Takagi Y, Fujimoto K, Sato F, Noshiro M, Kato Y and Honma K: Dec1 and Dec2 are regulators of the mammalian molecular clock. Nature 419: 841-844, 2002.
16. Chakrabarti J, Turley H, Campo L, Han C, Harris AL, Gatter KC and Fox SB: The transcription factor DEC1 (stra13, SHARP2) is associated with the hypoxic response and high tumour grade in human breast cancers. Br J Cancer 91: 954-958, 2004.
17. Currie MJ, Hanrahan V, Gunningham SP, Morrin HR, Frampton C, Han C, Robinson BA and Fox SB: Expression of vascular endothelial growth factor D is associated with hypoxia inducible factor (HIF-1α) and HIF-1alpha target gene DEC1, but not lymph node metastasis in primary human breast carcinomas. J Clin Pathol 57: 829-834, 2004.
18. Turley H, Wykoff CC, Troup S, Watson PH, Gatter KC and Harris AL: The hypoxia-regulated transcription factor DEC1 (Stra13, SHARP-2) and its expression in human tissues and tumors. J Pathol 203: 808-813, 2004.
19. Giatromanolaki A, Koukourakis MI, Sivridis E, Turley H, Wykoff CC, Gatter KC and Harris AL: DEC1 (STRA13) protein expression relates to hypoxia-inducible factor 1-alpha and carbonic anhydrase-9 overexpression in non-small cell lung cancer. J Pathol 200: 222-228, 2003.
20. Robertson N, Potter C and Harris AL: Role of carbonic anhydrase IX in human tumor cell growth, survival, and invasion: Cancer Res 64: 61610-6165, 2004.
21. Acs G, Zhang PJ, McGrath CM, Acs P, McBroom J, Mohyeldin A, Liu S, Lu H and Verma A: Hypoxia-inducible erythropoietin signaling in squamous dysplasia and squamous cell carcinoma of the uterine cervix and its potential role in cervical carcinogenesis and tumor progression. Am J Pathol 162: 1789-1806, 2003.
22. Airley RE, Loncaster J, Raleigh JA, Harris AL, Davidson SE, Hunter RD, West CM and Stratford IJ: GLUT-1 and CAIX as intrinsic markers of hypoxia in carcinoma of the cervix: relationship to pimonidazole binding. Int J Cancer 104: 85-91, 2003.
23. Wang BY, Kalir T, Sabo E, Sherman DE, Cohen C and Burstein DE: Immunohistchemical staining of GLUT1 in benign, hyperplastic, and malignant endometrial epithelia. Cancer 88: 2772-2781, 2000.
24. Nisahida M: The Ishikawa cells from birth to the present. Hum Cell 15: 104-117, 2002.
25. Kallio PJ, Pongratz I, Gradin K, McGuire J and Poellinger L: Activation of hypoxia-inducible factor-1α: posttranscriptional regulation and conformational change by recruitment of the Arnt transcription factor. Proc Natl Acad Sci USA 94: 5667-5672, 1997.
26. Tanimoto K, Makino Y, Pereira T and Poellinger L: Mechanism of regulation of the hypoxia-inducible factor-1α by the von Hippel-Lindau tumor suppressor protein. EMBO J 19: 4298-4309, 2000.
27. Ravi R, Mookerjee B, Bhujwalla ZM, Sutter CH, Artemov D, Zeng Q, Dillehay LE, Madan A, Semenza GL and Bedi A: Regulation of tumor angiogenesis by p53-induced degradation of hypoxia-inducible factor 1α. Genes Dev 14: 34-44, 2000.
28. Zundel W, Schindler C, Haas-Kogan D, Koong A, Kaper F, Chen E, Gottschalk AR, Ryan HE, Johnson RS, Jefferson AB, Stokoe D and Giaccia AJ: Loss of PTEN facilitates HIF-1-mediated gene expression. Genes Dev 14: 391-396, 2000.