

## **The basic helix-loop-helix (bHLH) transcription factor DEC2 negatively regulates**

### **Twist1 through an E-box element**

(塩基性ヘリックス・ループ・ヘリックス (bHLH) 転写因子 DEC2 は E-box 配列を介して抑制的に Twist1 を調整する)

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## **ABSTRACT**

Differentiated embryo chondrocyte 2 (DEC2/Sharp-1/Bhlhe41), a basic helix-loop-helix (bHLH) transcription factor, has been shown to regulate the transcription of target genes by binding to their E-box elements. We identified a possible DEC2-response element (consensus E-box: CACGTG) in the promoter region of Twist1. Forced expression of DEC2 significantly repressed Twist1 promoter activity under normoxia and under hypoxia as assessed by a luciferase reporter assay. In addition, over-expression of DEC2 repressed Twist1 mRNA expression assessed by quantitative real-time PCR. Site-directed mutagenesis studies showed that mutagenesis of the consensus E-box sequence eliminated the ability of DEC2 to reduce the Twist1 promoter activity. Chromatin immunoprecipitation (ChIP) assays confirmed that the DEC2-mediated repression is primarily achieved by binding to the E-box in the Twist1 promoter. Knockdown of DEC2 by siRNA significantly attenuated the repression of Twist1 expression. DEC2 and Twist1 exhibit inversed protein expression patterns during development of mouse embryo tongue tissue. Given the fact that DEC2 protein is emerging as an important regulator in a vast array of cellular events, including cell differentiation, maturation of lymphocytes and the molecular clock, our study elucidates an important mechanism by which DEC2 regulates cellular function by modulating the

expression of Twist1.

**Keywords:** DEC2, Twist1, E-box, hypoxia, tongue, growth and development

## **1. Introduction**

Basic helix-loop-helix (bHLH) transcription factors play important roles in the regulation of cellular differentiation of many distinct cell types. Differentiated embryonic chondrocyte (DEC) 1 and 2, bHLH transcription factors, were identified in the course of exploring novel genes involved in the proliferation and differentiation of human chondrocytes [1,2]. bHLH transcription factors bind to a common DNA sequence called the E-box (CANNTG) that is commonly found in the promoter and/or enhancer regions of numerous developmentally regulated genes [3] and they function as transcriptional activators or repressors. The molecular mechanism of gene regulation by DEC has been shown to be through E-box elements in the promoters of the target genes [4-6]. DEC2 binds to the E box as a homodimer and acts as a potent transcriptional repressor of MyoD. DEC2 is expressed in the late stage of neurogenic differentiation [7] and suppresses the transcription of M1 muscarinic acetylcholine receptor [8].

Twist1 is also a bHLH transcription factor involved in the negative regulation of cellular determination [9] and in the differentiation of several lineages, including myogenesis and osteogenesis [10,11]. In the mouse embryo, Twist1 protein is expressed in the branchial arches, head mesenchyme, sclerotome, dermatome, limb buds, somatic lateral plate, eyes, vibrissae and developing ears and teeth [12]. Beginning at day 8

post-conception, Twist1 is mainly expressed in undifferentiated cells of the mouse embryo that are committed to muscle and cartilage development [13]. In mammals, Twist1 expression during a precise time frame during embryogenesis allows for the migration and differentiation of several mesodermal and neural crest cell lineages [13].

Embryogenesis is regulated by a number of complex cell signaling pathways, which are critical for normal development. During embryogenesis, in a morphogenetic process known as epithelial to mesenchymal transition (EMT), normal epithelial cells transiently acquire the phenotype of mesenchymal cells, whereby they dislodge from their sites of origin and migrate to distant sites. Hypoxia (or induction of HIF-1 $\alpha$ ) is an important micro-environmental factor that induces the expression of EMT regulators, such as Snail and Zeb1, and coordinates the interaction between those EMT regulators [14]. Levels of vascular endothelial growth factor (VEGF) transcripts and of other HIF-1 $\alpha$  targets were found to decline in tumors displaying increasing levels of DEC2 [15]. DEC2 is required, and is sufficient, to limit the expression of HIF-1 $\alpha$ -target genes and is an endogenous buffer against the effects of hypoxia. HIF-1 $\alpha$  interacts with the hypoxia-response element (HRE) of target promoters, followed by the recruitment of transcriptional co-activators [16]. We previously demonstrated that DEC2 directly interacts with HIF-1 $\alpha$ , and interferes with the binding of HIF-1 $\alpha$  to the HRE, resulting

in down-regulated expression of the VEGF gene under hypoxia. On the contrary, elevated Twist1 expression leads to higher VEGF expression, promotes angiogenesis and correlates with chromosomal instability in breast cancer [17]. Mutant phenotypes of HIF-1 $\alpha$ - and Twist1-null mice show similarities, including defects in somites and abnormal formation of neural folds [18-20]. This suggests that the genes encoding HIF-1 $\alpha$  and Twist1 may be involved in the same pathway that regulates development.

Many of the phenotypes attributed to Twist1 occur as a result of its binding to E-boxes in the regulatory domains of target genes [12]. The DNA-binding and transcriptional properties of DEC2 and Twist1 within the bHLH regulatory network have not been characterized and their biological targets have not been defined. In this study, we focused on the mechanisms of regulation of DEC2 and Twist1, and we demonstrate for the first time that the hypoxia-inducible transcription repressor DEC2 participates in the transcriptional regulation through its binding to an E-box motif in the promoter region of Twist1. These findings contribute to a better understanding of the biological functions of differentiation and hypoxia. Identification of this additional molecular mechanism may aid in understanding the effects of DEC2 and Twist1 during development.

## **2. Materials and Methods**

### *2.1. Cell Culture*

The human oral cancer HSC-3 and embryonic kidney HEK293 cell lines 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, USA) supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>. Hypoxic exposure was performed in a hypoxic chamber ( 2% O<sub>2</sub>).

### *2.2. Luciferase Reporter Assays*

To determine the activity of the Twist1 promoter in the presence of ectopically over-expressed DEC2, luciferase assays were performed. The expression plasmid used for human DEC2 was described previously [21]. The Twist1 promoter was subcloned into PGL3-basic (Promega Corp, Madison, WI, USA) at the KpnI site. In brief, a luciferase reporter plasmid of the human Twist1 promoter (-1560 to +1) with a potential DEC2-responsive element, consensus E-box (CACGTG; -1535 to -1540), was made by subcloning the fragment representing nucleotides relative to the transcription start site of the Twist1 promoter into the vector. The QuikChange Site-Directed Mutagenesis Kit

(Stratagene, La Jolla, CA, USA) was used to introduce specific mutations into the E-box of the Twist1 promoter. Mutagenetic primers were designed for the E-box with a 6 bp mismatch (in bold):

forward, 5'- cgagtctcagcaggggaacagc**TCGAAT**gcctgctgcgcctcgcctgg - 3';

reverse, 5'- ccaggcgaggcgcaggcaggc**ATTCGA**gctgttcctgctgagactcg - 3'.

The PCR conditions were 18 cycles at 95°C for 30 s, at 55°C for 60 s and at 68°C for 10 min. A PGL3-basic vector containing the wild-type Twist1 promoter was used as template DNA. The sequence of the mutated plasmids was confirmed by DNA sequencing (ABI Prism 3130 Genetic Analyzer, Applied Biosystems, Tokyo, Japan). Transient transfections were performed using FuGENE6<sup>TM</sup> (Roche, Indianapolis, IN, USA) for HEK293 cells. Luciferase activity was measured using the Dual-luciferase® Reporter Assay System (Promega Corp) and a GloMax®-20/20 Single-Tube Luminometer (Promega Corp). All assays were performed in triplicate, and were repeated at least three times, and the most representative results are shown.

### *2.3. Chromatin Immunoprecipitation (ChIP) Assays*

ChIP assays were performed using a kit from Upstate (Charlottesville, VA, USA) as described previously [22]. Briefly, HSC-3 cells growing in 100-mm dishes under



normoxic or hypoxic conditions for 24 h, were cross-linked using 1% formaldehyde at 37°C for 7 min. After two washes with cold phosphate-buffered saline (PBS), cells were resuspended in 300 µL 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 lysates were sonicated to give DNA fragments ranging in size from 200 to 900 bp. Samples were centrifuged for 10 min at 4°C. The supernatants were diluted 10-fold 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 Protein G agarose for 1 h at 4°C, and then incubated with either an anti-DEC2 antibody or normal rabbit IgG antibody overnight at 4°C. The beads were then washed with low-salt, high-salt, LiCl and TE buffers. Immunocomplexes were extracted from the beads with 1% SDS and 0.1 M NaHCO<sub>3</sub>. 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 human Twist1 promoter containing the consensus E-box was amplified by PCR. The sequences of the primers were: human Twist1-F: 5'- CAATTTGTCCCTCCC ATGAA-3' and human Twist1-R: 5'- CTCAAGCTGAAGGCAAGAGC -3'. The PCR product size was 207 bp.

#### 2.4. Quantitative Real Time-PCR (QRT-PCR)

Total RNAs were isolated using an RNeasy Mini kit (Qiagen, Hilden, Germany) and a TURBO DNA-free™ Kit (Applied Biosystems) was used to remove contaminating DNA from the RNA preparations. First-strand cDNA was synthesized from 1 µg total RNA using High Capacity RNA-to-cDNA Master Mix (Applied Biosystems). Real-time PCR was carried out in 96-well plates using the LightCycler® 480 Real-Time PCR System (Roche Applied Science). All reactions were done in triplicate. All TaqMan probes (Hs00229146\_m1 for DEC2, Hs00361186\_m1 for Twist1, and Hs99999903\_m1 for beta-actin) were obtained from Applied Biosystems. An empty plasmid transfected cells without reverse transcriptase (RT) was used as a negative control.

#### 2.5. Short interference RNA (siRNA)

Short interference RNAs (siRNAs) against human DEC2 were synthesized by Qiagen. The sequences for the sense and antisense DEC2 siRNAs were 5'-r (CGACAGGCAUAAACAAGAA) d (TT)-3' and 5'-r (UUCUUGUUUAUGCCUGUC G) d (TG)-3', respectively. We also used another siRNA against DEC2 (DEC2 siRNA-2). The sequences for the sense and antisense DEC2 siRNA-2 were 5'-r (CGUUGCAACCUAUUCUGAA) d (TT)-3' and 5'-r (UUCAGAAUAGGUUGCAAC

G) d (TG)-3', respectively. The negative control (scrambled) siRNA sequences were 5'-r (UUCUCCGAACGUGUCACGU) d (TT)-3' and 5'-r (ACGUGACACGUUCGGA GAA) d (TT)-3'. For the siRNA transfection, HSC-3 cells were seeded at  $5 \times 10^4$  cells per 35-mm dishes. After 24 h, the siRNAs were transfected into the cells using the lipofectamine RNA iMAX reagent (Invitrogen, Carlsbad, CA, USA), and the cells were incubated for 48 h under normoxic or hypoxic conditions, and then subjected to various analyses.

## *2.6. Immunohistochemistry*

C57BL/6 mouse tongue embryo specimens were purchased from GenoStaff (Tokyo, Japan). An anti-DEC2 antibody (1:100; a kind gift from Yukio Kato, Hiroshima University, Japan) and an anti-Twist1 antibody (1:100, Abcam) were used as primary antibodies. After an overnight incubation at 4°C, the specimens were rinsed with PBS and incubated at room temperature for 30 min with a secondary antibody conjugated to peroxidase (Nichirei Biosciences, Tokyo, Japan). All specimens were color-developed with diaminobenzidine (DAB) solution (DAKO) and counterstained with hematoxylin. The immunostaining of all specimens was performed simultaneously to ensure the same antibody reaction and DAB exposure conditions.

### *2.7. Statistical analysis*

Student's two-tailed  $t$ -test was used to determine the  $p$  value.  $p < 0.05$  is considered to be statistically significant.

### **3. Results**

#### *3.1. DEC2 transrepresses the Twist1 promoter*

To determine whether DEC2 directly regulates the Twist1 promoter, we found a consensus E-box sequence motif that overlaps the HRE, representing a possible binding site for DEC2. A luciferase reporter assay was performed to examine whether DEC2 was able to transrepress the Twist1 promoter under normoxic and/or hypoxic conditions. The results indicated that DEC2 repressed the Twist1 promoter activity in a dose-dependent manner under normoxia and under hypoxia (Fig. 2A). Next, we constructed a mutant reporter in which several nucleotides were substituted in the consensus E-box. Transfection of the DEC2 expression plasmid had little effect on the E-box mutant activity under normoxia and under hypoxia (Fig. 2B). These results suggested that DEC2 represses the Twist1 promoter activity through the E-box. Furthermore, DEC2 over-expression repressed the Twist1 mRNA expression in a dose-dependent manner under normoxia and under hypoxia (Fig. 2C). Next, we performed knockdown assays for DEC2, to estimate how the DEC2 pathway contributes to Twist1 expression. Transfection of a specific siRNA for DEC2 in HSC-3 cells induced Twist1 expression compared to the nonspecific siRNA under both normoxic and hypoxic conditions, suggesting that knockdown of DEC2 reversed the

repression of Twist1 (Fig. 2D).

### *3.2. Direct binding of DEC2 to the E-box motif on the Twist1 promoter*

To demonstrate that DEC2 directly binds to the E-box in the Twist1 promoter, we performed chromatin immunoprecipitation (ChIP) assays. HSC-3 cells were cultured and transfected with pcDNA, DEC2 pcDNA, control siRNA and DEC2 siRNA. Our data clearly demonstrate that immunoprecipitation of the chromatin fragment containing the E-box in the Twist1 promoter increased in samples transfected with the DEC2 over-expression vector (Fig. 3A), whereas DEC2 siRNA decreased the amount of endogenous DEC2 bound to the Twist1 promoter (Fig. 3B).

### *3.3. Opposing functions of DEC2 and Twist1 in mouse tongue development*

Representative examples of immunohistochemical staining of mouse tongue at various stages of development are shown in Fig. 4. At embryonic E11.5 day, expression of Twist1 was present predominantly in the tongue cells. DEC2 expression was strong in newborn mice. Our data provide the first evidence that there is an inverse expression pattern between DEC2 and Twist1 protein during mouse tongue development.



#### 4. Discussion

We show for the first time that the bHLH transcription factor DEC2 negatively regulates Twist1 through an E-box in its promoter *in vitro and in vivo*. bHLH proteins are intimately associated with developmental events such as cell differentiation and lineage commitment. The HLH domain in the bHLH motif is responsible for dimerization, whereas the basic region mediates DNA binding to target genes [23]. We and others have suggested that DEC2 participates in circadian rhythm, adipogenesis, immune response and carcinogenesis through the transcriptional repression of several genes via an E-box in their promoter regions [4,24,25].

Our previous reports showed that DEC2 regulates the transcription of target genes by binding to an E-box element [26,27]. DEC2 negatively regulates Slug through its E-box [28]. The effects of DEC2 on Twist1 could be explained in part by a binding specificity to the element sequence identified as the binding site, which contains a canonical E-box motif (CACGTG). Several lines of evidence in this study support this notion. Our studies provide *in vitro* evidence that DEC2 can bind to the E-box in the Twist1 promoter, which is supported *in vivo* by the detection of endogenous DEC2 on the Twist1 promoter by ChIP assays. Since mutation of the E-box site in the Twist1 proximal promoter significantly abrogates DEC2-mediated repression, the repression of



Twist1 expression by DEC2 is entirely dependent on E-box binding. These findings provide direct evidence that DEC2 negatively regulates the expression of Twist1, which is largely achieved through direct DNA binding to the E-box in the Twist1 promoter. The repressive effects of DEC2 on the E box are similar to several negative bHLH factors, including Mist1 [29] and MyoR [30], which also utilize several overlapping mechanisms to regulate E box activity. Because DEC2 is expressed in a variety of tissues, it is likely that it not only forms a homodimer with the Twist1 protein but also with other positive factors expressed in a tissue-specific manner to regulate their functional activity.

The hypoxic response has been clearly shown to involve alterations in gene transcription [16], and HIF-1 $\alpha$  is well known as the pivotal factor that regulates cellular responses to hypoxia via transactivation of a variety of genes. We previously demonstrated that DEC2 is transcriptionally activated by HIF-1 $\alpha$ , suggesting that it plays a crucial role in the HIF-1 $\alpha$ -mediated cellular hypoxic reaction [31]. Hypoxia induces the up-regulation of HIF-1 $\alpha$  in tumor cells, activates numerous signal transduction pathways, induces EMT and the secretion of VEGF. VEGF is a Twist1-induced angiogenic factor [32]. We previously demonstrated that DEC2 interacts with HIF-1 $\alpha$ , and interferes with the binding of HIF-1 $\alpha$  to the HRE, resulting

in the down-regulated expression of the VEGF gene under hypoxia. Owing to the critical role of hypoxia in maintaining self-renewal [33], we hypothesized that bHLH transcription factors activated by hypoxia could modulate the expression of EMT regulators, resulting in the suppression of EMT. In our screening of possible suppression of the EMT regulator Twist1 by different bHLH transcription factors, we observed a tight correlation between DEC2 and Twist1. Co-transfection of a DEC2 expression vector and a Twist1 promoter that includes overlapping nucleotides between the consensus E-box and the HRE sequence significantly decreased the basal luciferase activity of Twist1 in hypoxia. Mutation of those sequences failed to repress Twist1 promoter activity. These findings suggest that the consensus E-box sequence of the Twist1 promoter is required for the regulation of transcription in the presence of hypoxia. DEC2-mediated repression, although profound, may not always dictate the expression of Twist1. For example, DEC2 and Twist1 are both up-regulated in response to hypoxia induction. Acute hypoxia is considered a severe cytotoxic stimulus, and rapid induction of both genes maximizes the cellular survival mechanism. Intriguingly, the effect of DEC2 is independent of oxygen levels, as down-regulation of HIF-1 $\alpha$  protein occurs highly efficiently in cells cultured both in normoxic and in hypoxic conditions [15].

As a negative regulator of differentiation, Twist1 might function through different mechanisms in different cell lineages. Twist1 silencing can up-regulate osteoblast differentiation of murine mesenchymal cells [34]. Twist1 can bind to the E1-box in the RUNX2 P2 promoter and down-regulates its activity in human mesenchymal cells [35]. Twist1 is also functionally linked during early embryogenesis. In early *Xenopus* embryos, Twist1 negatively regulates the expression of Cerberus, an inhibitor of Nodal, BMP and Wnt signaling [36]. During *Xenopus* embryonic vasculogenesis, knockdown of Twist1 provoked embryonic hemorrhage and expression of Twist1 could rescue the hemorrhage defects due to Myc knockdown [37]. These data show that Twist1 is highly conserved and functionally important during embryogenesis. We previously demonstrated that DEC2 is also involved in tissue development and regulation of the hypoxia response as a transcriptional repressor [2,4,26]. In this study, we demonstrated that DEC2 and Twist1 exhibit inversed expression patterns during mouse tongue development. The results of our immunohistochemical study suggest that the opposing expression of DEC2 and Twist1, i.e., Twist1-high/DEC2-low or Twist1-low/DEC2-high, might be a key index that determines development or differentiation during tongue embryogenesis. Based on the immunohistochemical analysis of mouse tongue embryo specimens, not all Twist1-positive staining is present in the nucleus. Instead, the vast

majority is located in the cytoplasm, although the underlying mechanism that regulates the cytoplasmic and nuclear localization of Twist1 is not clear at this time.

Although we focused on Twist1 in the present study, most bHLH promoters contain an E-box and/or an E-box-like motif. Thus, it is interesting to postulate that interactions between bHLH proteins and DEC2 may play a general role in controlling the expression of multiple bHLH genes during differentiation and phenotypic modulation. The demonstration of the DEC2-Twist1 axis provides a mechanistic explanation for the link between hypoxia and differentiation. Although the DEC2-Twist1 axis may not be the only pathway involved, the regulation of DEC2 and/or other bHLH transcription factors through different mechanisms requires further exploration and should be the subject of immediate attention.

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**Figure 1**

**Figure 1** Nucleotide sequence of the human Twist1 promoter. (+1) denoting the position of the transcription initiation site. The consensus E-box (CACGTG) that overlaps with the hypoxia response element (HRE: ACGTG) sequence is shown in bold.

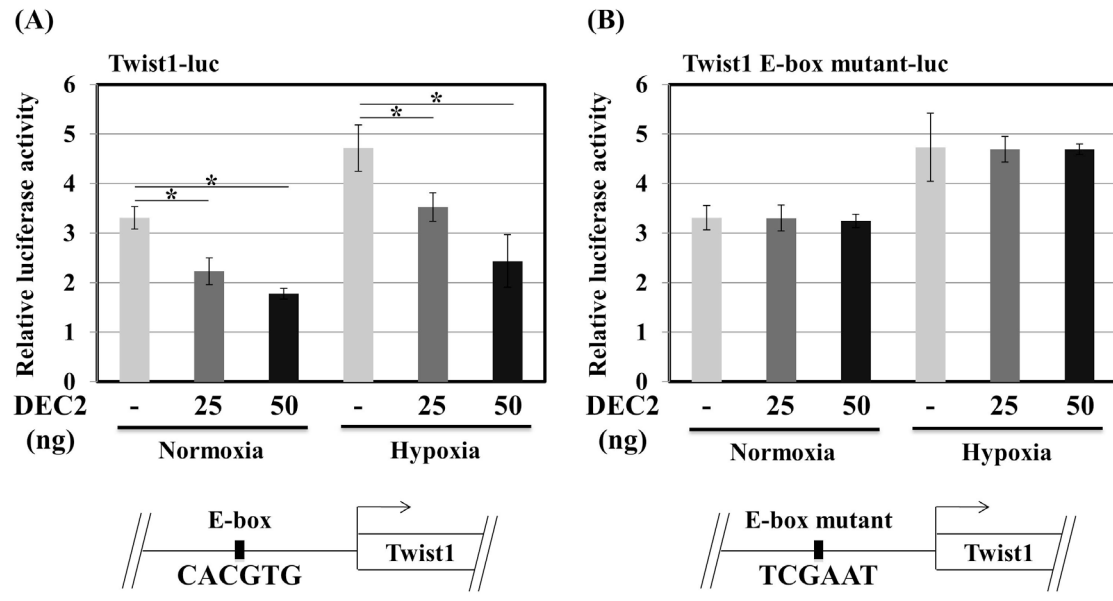


Figure 2

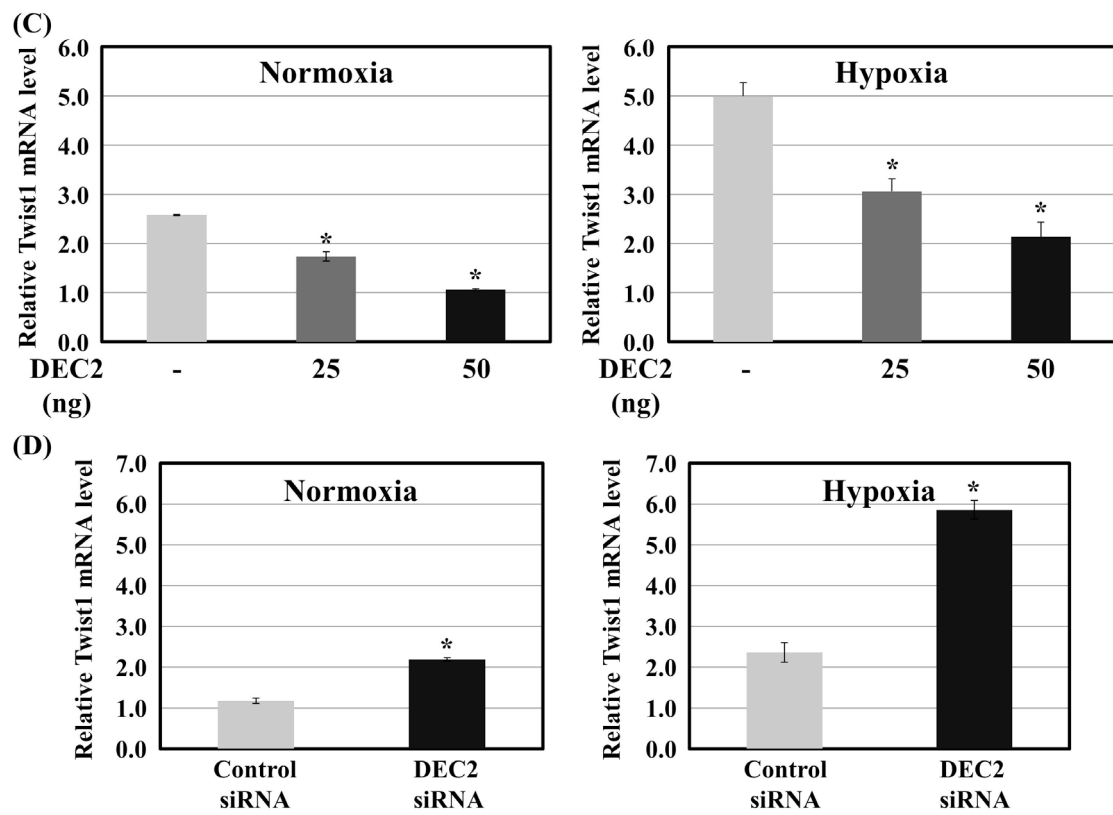


Figure 2

**Figure 2** DEC2 transrepresses the Twist1 promoter. **(A)** Luciferase reporter assay: HEK293 cells were transfected with Twist1-luc (firefly), Renilla luciferase and increasing amounts of DEC2 as noted. Following 48 h of culture, luciferase activity was measured and normalized to Renilla luciferase activity. The cells were transfected with two doses (25 and 50 ng) of DEC2 expression plasmid along with 25 ng of Twist1 promoter and various amounts of empty plasmid. Columns, mean of three independent experiments carried out in triplicate; bars, SE.  $*p < 0.05$ , by *t*-test. **(B)** A Twist1 E-box mutant-luc reporter was generated and was introduced into HEK293 cells with or without DEC2 as noted and the effect of an E-box mutation on the activity of the Twist1 promoter was noted as detailed for (A). **(C)** Twist1 mRNA expression was analyzed in transfected HEK293 cells by quantitative real-time RT-PCR. An empty luciferase reporter plasmid without reverse transcriptase (RT) was used as a negative control. Relative mRNA levels of cells in normoxia and in hypoxia were calculated as the ratio to beta-actin, and each bar represents the mean  $\pm$  SD for at least three independent experiments.  $*P < 0.05$ , by *t*-test. **(D)** DEC2 knockdown reverses the repression of Twist1. HSC-3 cells were transfected with a specific siRNA against DEC2 (DEC2 siRNA) or a control siRNA (scramble siRNA) under normoxic or hypoxic conditions. After 48 h of transfection, RNAs were prepared and subjected to quantitative real-time RT-PCR analysis. Each value represents the mean  $\pm$  SD for three independent experiments.  $*P < 0.05$ , by *t*-test.

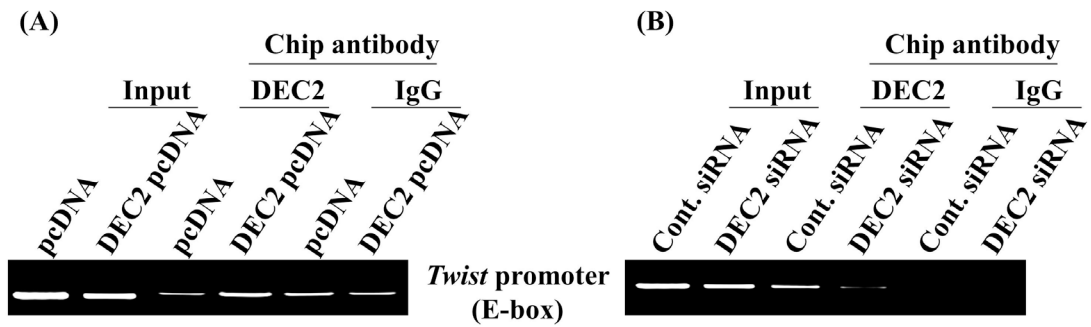
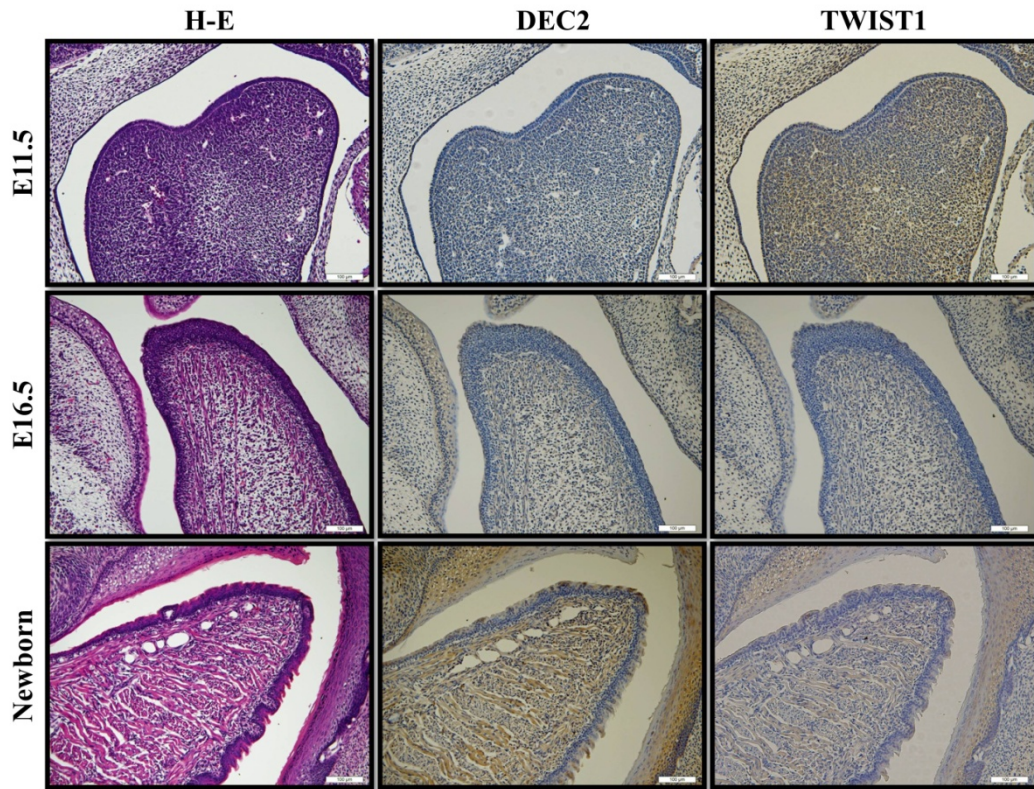


Figure 3

**Figure 3** DEC2 binds to the E-box in the Twist1 promoter. ChIP assays were carried out by over-expressing DEC2 in HEK293 cells (A) and with/without siRNA for DEC2 in HSC-3 cells (B). Twenty-four h post-transfection, cells were collected for the ChIP assays. PCR was performed with the eluted DNA fragments. Anti-rabbit IgG was used as an immunoprecipitation control.



**Figure 4**

**Figure 4** DEC2 and Twist1 expression during mouse tongue development. Immunohistochemical detection of DEC2 and Twist1 was performed in mouse embryonic tongue tissues at developmental times as noted using specific antibodies. The results are representative of three mice. Original magnification: x20; Scale bar: 100  $\mu$ M.