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# Histone modification-mediated Lhx2 gene expression

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ABSTRACT

Lhx2, a member of LIM homeobox transcription factors, plays a key role in central nervous system (CNS) and embryonic tissue development. However, molecular mechanism of Lhx2 gene regulation remains largely unknown. Here, we identified and characterized a regulatory region of Lhx2 gene which mediates responses to two different signals such as inhibition of HDAC3 and stimulation by E2F1. In particular, the promoter region of −229 to −126 was responsible not only for basal expression but also for a inhibitor of histone deacetylase, trichostatin A (TSA)-mediated activation of Lhx2 gene. Intriguingly, transcription factor E2F1 also activates Lhx2 gene via direct binding to the same −229 to −126 region. Based on these observations, we could have demonstrated that E2F1 is necessary for TSA-mediated activation of Lhx2 gene and acetylation of histone 3 is involved in this event. This study provides evidence that the histone modification and E2F1 binding are integral parts of the mechanism for Lhx2 gene expression.


## 1. Introduction

The LIM homeobox genes are widely expressed in various developmental and physiological processes including embryogenesis, development of central nerve system (CNS) and endocrine regulation [1–5]. LIM homeobox genes encode proteins having a LIM homeodomain and two zinc-finger domains, which act a transcription factor [6]. Lhx2, a subfamily member of LIM homeobox genes, was originally identified in pre-B cell lines and was then found to bind to the αGSU (glycoprotein hormone α-subunit) promoter, allowing pituitary-specific expression of the gene [7,8]. Lhx2 plays a pivotal role in embryogenesis, as $Lhx2^{-/-}$ mouse embryos develop liver hypoplasia and die at embryo day E15–E17 [9]. Lhx2 is also required for the development of eye, forebrain, and erythrocyte [10]. In addition, it is involved in the formation of hair follicle [11,12]. We and others have shown that Lhx2 can enhance the expression of the thyroid stimulating hormone β-subunit (TSHβ) gene and follicle stimulating hormone β-subunit (FSHβ) gene in murine thyrotroph and gonadotroph cells, respectively [13,14]. Recently, it was shown that formation of hippocampus and maintenance of thalamic development during neurogenesis specifically require Lhx2 [15]. While Lhx2 is an integral part of the developmental and physiological regulation, upstream regulators of the Lhx2 expression itself remain to be identified.

Histone modifications (i.e., acetylation, methylation and phosphorylation, etc.) have been shown to play critically important roles in transcriptional regulation of the cell differentiation and proliferation. Histone modifications modulate chromatin structure and thereby control gene transcription [16]. In particular, histone acetylation regulates histone-DNA interaction via two different families of enzymes: histone acetyltransferases (HATs) and histone deacetyltransferases (HDACs) [17,18]. HATs acetylate histone tails and enhance gene transcription. In contrast, HDACs remove acetyl groups from the lysine side chains of histones and repress gene transcription [18,19]. HATs and HDACs can modify gene expression through interaction with sequence-specific transcription factors and regulate fundamental biological processes including cellular differentiation, proliferation, and survival [18]. HDACs are grouped into Class I (HDAC1, 2, 3 and 8), Class II (HDAC4, 5, 6, 7, 9 and 10) and Class III (Sirt1–7) [20]. While Class I HDACs seems to be expressed ubiquitously, class II and III HDACs are more restrictedly expressed [21]. In particular, it was claimed that HDAC1 and HDAC3 are involved in the regulation of LIM homeobox gene in developmental and endocrine processes [22–25]. We have focused on understanding the molecular mechanism by which Lhx2 expression is changed in an acetylation-mediated manner. In order to examine Lhx2 expression upon histone acetylation, trichostatin A (TSA) has been used, which is the most well known form of a general HDAC inhibitor [26].

In this study, we first identified a transcription start site of the Lhx2 gene and then promoter constructs were tested for the expression of Lhx2 gene. Indeed, TSA induced Lhx2 transcription and this effect was mediated by a −229 to −126 proximal promoter region via binding of E2F1 transcription factor. These results suggest that epigenetic modification may be one of the molecular

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mechanisms responsible for Lhx2 expression in developmental and endocrine processes.

## 2. Material and methods

### 2.1. Mouse tissue

C57/B6 mice were anesthetized with isoflurane and sacrificed by decapitation. Tissues were immediately removed and frozen in liquid nitrogen.

### 2.2. Cell culture, transfection, plasmids and siRNAs

αTSH, TαT1, αT3-1, LβT2, HepG2, PANC-1, HaCat, NIH3T3, C6 and αTN-4 cells were maintained in DMEM (Invitrogen) containing 10% heat-inactivated fetal bovine serum (FBS). AGS cell was maintained in RPMI 1640 (Invitrogen) containing 10% heat-inactivated FBS. Cells were seeded at $3 \times 10^5$ cells/ml into 6-well or 100 mm culture plates and were grown for 24 h. Cells were transfected using Lipofectamin 2000 (Invitrogen) according to the manufacturer's protocols. The expression vectors for E2F1 (pcDNA3.1-E2F1 and pCMV2-Flag-E2F1), HDAC1-pcDNA3.1, HDAC3-pcDNA3.1 and HDAC4-pcDNA3.1 have been described previously [27,28]. Transient transfection of siRNA was performed using siGENOME SMARTpool reagents from Dharmacon according to the manufacturer's protocol. Depletion of E2F1 was performed using specific siRNA targeted to E2F1. A non-targeting 20–25 nucleotide siRNA, scrambled siRNA (scRNA) from Dharmacon were served as a negative control.

### 2.3. RNA preparation, RT-PCR and quantitative real-time PCR

Total RNA was isolated using RiboEX Reagent (GeneAll). RT-PCR was performed using Superscript III RNase $H^-$ reverse transcriptase (Invitrogen) according to the manufacturer's protocol. PCR primers used for quantification of endogenous genes were as follows. Lhx2 forward (5′-TCTTCCCTACTACAACGGCGTG-3′) and reverse (5′-AGTAAGACTTCATTGTCCGAAGCT-3′) primers were designed to amplify a 189 bp fragment. E2F1 forward (5′-CATCCAGCTCA TTGCCAAGAA-3′) and reverse (5′-GTCCGGTCCTCCCCAGAGGATC-3′) primers were to amplify a 445 bp fragment. GAPDH forward (5′-TGATGACATCAAGAAGGTGGTGAAG-3′) and reverse (5′- TCCTT GGAGGCCATGTAGGCCAT-3′) primers were to amplify a 229 bp fragment. Real-time PCR was performed using the Roter-Gene real time system (Corbett research) according to the manufacturer's protocol. All reactions were performed under identical condition using 32 cycles of amplification with denaturation at 95 °C for 30 s, annealing 58 °C for 30 s and elongation at 72 °C for 30 s. The specificity of products generated by each set of primers was examined using gel electrophoresis and was further confirmed by a melting curve analysis.

### 2.4. Western blot analysis

Whole cell extracts were prepared using protein extraction reagent (Pierce) supplemented with a protease inhibitor mixture (Roche). 20–40 μg of total protein was loaded per lane on SDS–polyacrylamide gel. Following electrophoresis, proteins were transferred to a polyvinylidene fluoride (PVDF) membrane, which was then probed with primary antibodies to α-tubulin (mouse monoclonal, Sigma), Flag (mouse monoclonal, Sigma), Lhx2 (rabbit polyclonal, Santa Cruz), or HDAC3 (rabbit polyclonal, Cell Signaling). Blots were developed using an ECL detection system (Millipore) and exposed to film.

### 2.5. Identification of a transcription start site of Lhx2 gene, construction of luciferase reporter plasmids and luciferase reporter assay

5′-RACE (5′-rapid amplification of cDNA ends) analysis was performed using a CapFishing Full length cDNA kit (Seegene) according to the manufacturer's protocol. cDNAs were generated from ployadenylated mRNA of αT3-1 cell. Gene specific primer, 5′-GGAACAGCATCGCGGCGGGACCGGGG-3′, was designed based on sequences of the coding region of Lhx2 gene. Genomic DNA isolated from mouse αT3-1 cells was used to amplify a 5′-flanking region of the Lhx2 gene. The 5′-GTCAGAGAGGTCGGTACCGAGTGG TGTAC-3′ forward and 5′-GCTAGCACGTCTCTTCCCCCCGGGGTGTG-3′ reverse primer set was used to amplify −1847 to +20 region of the Lhx2 gene. A series of 5′ deletion mutants (−887, −407, −229 and −126) were prepared using PCR with following primers: 5′-GGATCCTCCAGACTGCAGACT-3′ forward primer for −887 deletion mutant; 5′-GGATCCTCTCTCCGCTCCTCT-3′ forward primer for −407 deletion mutant; 5′-GGATCCGAGGCTGAGCTCGGC forward primer for −229 deletion mutant; 5′-GGTACCTCCTCCGCCGCCG CC-3′ forward primer for −126 deletion mutant. A same reverse primer (5′-GCTAGCACGTCTCTTCCCCCCGGGGTGTG-3′), which target +20 region of the Lhx2 gene, was used with each forward prime described above. Products specific for the Lhx2 gene were digested with KpnI and NheI, and then subcloned into a promoterless luciferase expression plasmid, pLuc-link2. P1 region (−229 to −126) was PCR-amplified with 5′-GGATCCGAGGCTGAGCTCGGC-3′ forward primer and 5′-GCTAGCGGCGGCGGCGGAGGAGGT-3′ reverse primer. P2 region (−126 to −1) was also PCR-amplified with 5′-GGTACCTCCTCCGCCGCCGCC-3′ forward primer and 5′-GCTAGC GTGTTCCCAACCAAAGGCGA-3′ reverse primer. PCR products specific for P1 or P2 region of the Lhx2 gene were digested with KpnI and NheI, and then subcloned into a pMinimal-Luc vector which contains only a TATA box of the human thymidine kinase gene linked to a luciferase reporter gene. Luciferase activities were measured using an LB 953 Autolumat (EG&G Berthold) as previously described [13]. β-Galactosidase assay was performed colorimetrically by a standard protocol. Luciferase activities were normalized based on the expression of Rous sarcoma virus-β-galactosidase plasmid.

### 2.6. Chromatin immunoprecipitation (ChIP)

ChIP assay was performed with anti-HDAC3 (Cell Signaling), anti-acetylated H3 (Cell Signaling) or anti-E2F1 (Santa Cruz) antibody using a ChIP assay kit (Upstate Biotechnology Inc.) according to manufacturer's protocol. DNAs recovered from precipitated complexes were subjected to PCR for the Lhx2 promoter region. Primer pairs for each Lhx2 promoter region were as follows; 5′-CCGAGGCTGAGCTCGGCGCGCGCTT-3′ forward primer and 5′-GGCGGCGGCGGAGGAGGTGGAGGA-3′ reverse primer for −229 to −126 region and 5′-GACTGCAGAGCTTTGGAGAAAT-3′ forward primer and 5′-TCTGGGGCGCTACTCTCTGCGCTG-3′ reverse primer for −887 to −700 region.

### 2.7. Site-directed mutagenesis

E2F1 binding site in the Lhx2 promoter was mutated using a QuickChange Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer's instructions. The mutagenic primer that was used is as follows (only the sense primer is shown; changes are in boldface and underlined): 5′-GCTTGGGTAGCGTT**ATAT**GCGGCTGG GCCCGCGGC-3′.

## 3. Results and discussion

### 3.1. TSA increases the recruitment of acetylated histone H3 to the proximal Lhx2 promoter

In an effort to elucidate the regulatory mechanism of Lhx2 gene, we first examined the expression pattern of Lhx2 gene in mouse tissues using RT-PCR (Fig. 1A, upper panel). Lhx2 gene was highly expressed in brain, while other tissues showed moderate or very low expression. Consistent with the *in vivo* data, high levels of Lhx2 mRNA were observed in C6 cell which is derived from brain tissue as well as in pituitary cell lines (αTSH, αT3-1 ,TαT1, and LβT2), but not in any other cell lines (Fig. 1A, lower panel).

Histone-modifying enzymes cause changes in gene expression, thereby affecting various cellular processes. Here, we investigated the effects of HDAC inhibition on Lhx2 expression. Trichostatin A (TSA), which interacts at nanomolar concentration with the catalytic site of class I and II HDACs, was employed as a competitive inhibitor. The Lhx2 expression was increased at both the mRNA and protein levels by a TSA treatment in HepG2 cells where

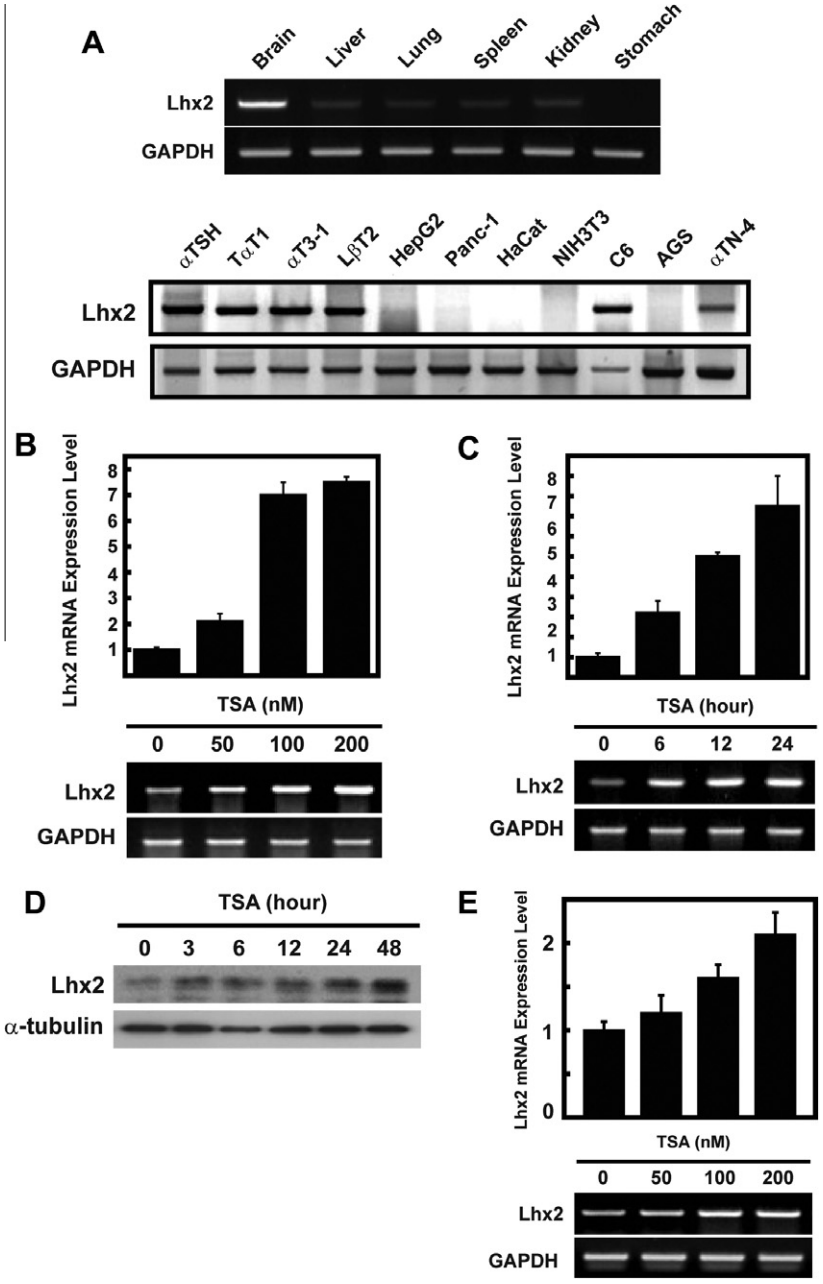


**Fig. 1.** TSA induces Lhx2 gene expression. (A) Expression of Lhx2 gene in mouse tissues (upper panel) and various cell lines (lower panel). mRNAs were prepared from tissues and cell lines derived from mouse thyrotroph (αTSH, TαT1), gonadotroph (αT3-1, LβT2), human hepatocelluar carcinoma (HepG2), human pancreatic carcinoma (PANC-1), human keratinocyte (HaCat), mouse embryonic fibroblast (NIH3T3), rat glioma (C6), human gastric carcinoma (AGS) and mouse lens cells (αTN-4) and then subjected to RT-PCR for Lhx2 gene. (B) Dose-dependent effect of TSA on Lhx2 mRNA level. HepG2 cells were treated with TSA at each concentration for 24 h. Lhx2 mRNA level was quantified using real-time PCR and RT-PCR. Values were normalized to the internal standard GAPDH. (C) Time-dependent effect of TSA on Lhx2 mRNA level. HepG2 cells were treated with 200 nM TSA for 0, 6, 12, 24 h. Lhx2 mRNA level was quantified using real-time PCR and RT-PCR. Values were normalized to the internal standard GAPDH. (D) Time-dependent effect of TSA on Lhx2 protein level. Lhx2 and α-tubulin levels in HepG2 cells treated with 200 nM TSA for each designated time were determined by Western blot analysis. (E) Dose-dependent induction of Lhx2 mRNA level by TSA. αT3-1 cells were treated with TSA at each concentration and Lhx2 mRNA level was quantified using real-time PCR and RT-PCR. Values were normalized to the internal standard GAPDH. All results are expressed as means ± S.D. for three independent experiments.

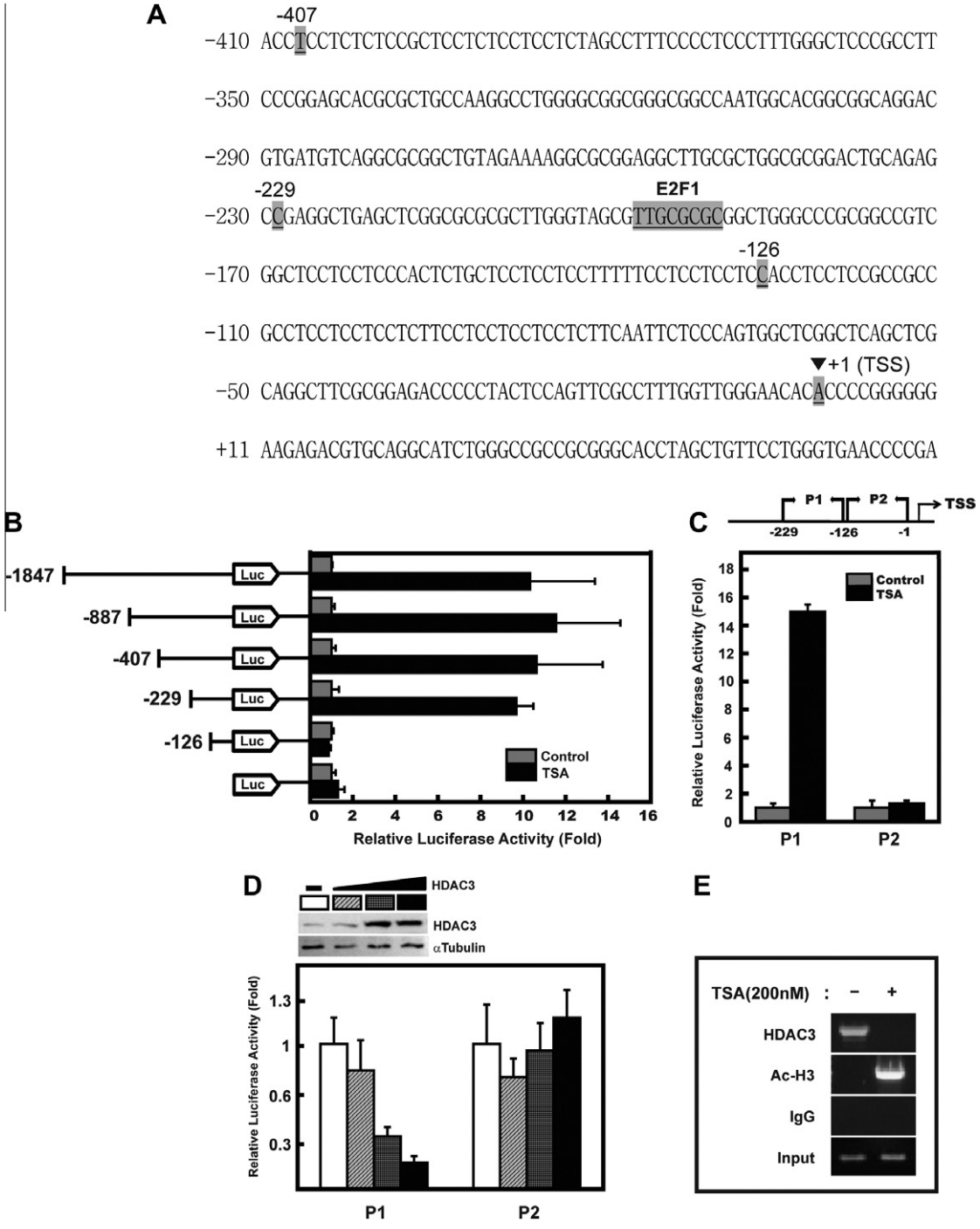


**Fig. 2.** Induction of Lhx2 by TSA is associated with the recruitment of acethylated histone 3 to the Lhx2 promoter. (A) Sequences of a proximal 5′-flanking region of the Lhx2 gene showing a transcription start site (TSS) and a putative E2F1 binding site. (B) Effect of TSA on expression of 5′-deletion constructs of Lhx2 promoter. Each promoter region was fused to a luciferase reporter vector. αT3-1 cells were transiently transfected with the 5′- deletion constructs and then either treated or not treated with 200 nM TSA for 12 h. (C) Determination of a TSA-response region in Lhx2 promoter. P1 and P2 represent −229 to −126 and −126 to −1 regions, respectively. The P1 or P2 region was fused to pMinimal-Luc vector and transfected into the αT3-1 cells which were then treated with 200 nM TSA for 12 h. (D) Repression of Lhx2 gene by HDAC3. αT3-1 cells were transfected with the P1- or P2-minimal promoter construct together with the increasing amount of HDAC3 expression vector (0, 10, 100, 500 ng). Western blot analysis was performed with antibody against HDAC3 or α-tubulin to check its expression at the protein level. (E) Recruitment of acetylated histone H3 (Ac-H3) to the TSA-induced Lhx2 promoter. αT3-1 cells were treated with 200 nM TSA for 12 h and then ChIP assay was carried out using antibody against HDAC3 or Ac-H3. Primers used in PCR were to amplify the P1 region specifically. All results are expressed as means ± S.D. for three independent experiments.

endogenous Lhx2 expression is low (Fig. 1B–D). αT3-1 cells, in which endogenous Lhx2 expression is high, also exhibit same TSA-stimulated Lhx2 expression pattern (Fig. 1E). These observations suggest that Lhx2 expression is affected by histone acetylation in various cell types, regardless of lineage.

Although genetic function of Lhx2 has been well established, molecular mechanism governing its transcription remain poorly understood. Thus, we first performed a 5′-RACE analysis to identify a transcription start site (TSS) of the mouse Lhx2 gene. 1.8-kb promoter region of the mouse Lhx2 gene was also amplified and

the sequences analyzed (Fig. 2A). No recognizable TATA box was evident, as was expected in other developmentally regulated genes. In order to determine the region required for transcriptional regulation, −1848 to +20 bp fragment of the Lhx2 gene was fused to a luciferase reporter plasmid and a series of the 5′-deletion constructs were prepared as described in Section 2. DNA-mediated gene transfer studies with these deletion mutant constructs in αT3-1 cells clearly showed that deletions up to position −126 completely abolished the stimulatory effect of TSA on Lhx2 promoter (Fig. 2B). In order to examine whether the −229 to −126 region is sufficient to confer TSA-mediated regulation, −229 to −126 (P1) or −126 to −1 (P2) region was subcloned in pMinimal-Luc vector (see Section 2) and examined for their responsiveness to TSA in transient transfection experiments. Indeed, P1 but not P2 region conferred TSA-mediated stimulation upon minimal promoter (Fig 2C). Taken together, these results indicate that P1 region is necessary and sufficient for TSA-mediated Lhx2 activation.

To determine which type of HDACs is responsible for repression of the Lhx2 promoter, P1- or P2-minimal promoter fusion construct was transfected into αT3-1 cells with increasing amount of HDAC1, HDAC3 or HDAC4 (one of class II HDACs) expression vector. Indeed, overexpression of HDAC3 decreased P1-mediated promoter activity, while P2-mediated promoter activity was not affected by HDAC3 at all (Fig. 2D). HDAC1 and HDAC4 had nothing to do with the Lhx2 repression (data not shown). These results indicate that Lhx2 gene is basically repressed by HDAC3 via P1 region. It could be plausible that HDAC3 be recruited to the P1 region and TSA regulate HDAC3 recruitment to P1. To test this possibility, αT3-1 cells were treated with or without TSA and then ChIP assay was carried out using anti-HDAC3 or anti-acetylated Histone 3 (Ac-H3) antibodies. TSA treatment decreased the levels of HDAC3 recruitment to P1 and increased the levels of Ac-H3 recruitment to P1 (Fig. 2E), indicating that TSA-mediated upregulation of Lhx2 gene is associated with the acetylation status of histone H3.

### 3.2. E2F1 activates Lhx2 expression

The TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html) software program was run to predict the binding sites of transcription factors which might influence on Lhx2 gene expression. It was of interest to find the E2F1-binding consensus sequence, TTGCGCGC, in P1 region (Fig. 2A, shaded box). To test whether Lhx2 gene is regulated by E2F1 via putative E2F1 binding site in P1 region, 5′-deletion constructs were transfected into αT3-1 cells together with a Flag-E2F1 expression vector. Stimulatory effect of E2F1 on the Lhx2 promoter was completely abolished upon deletion of P1 region (Fig. 3A). A site-directed mutation was

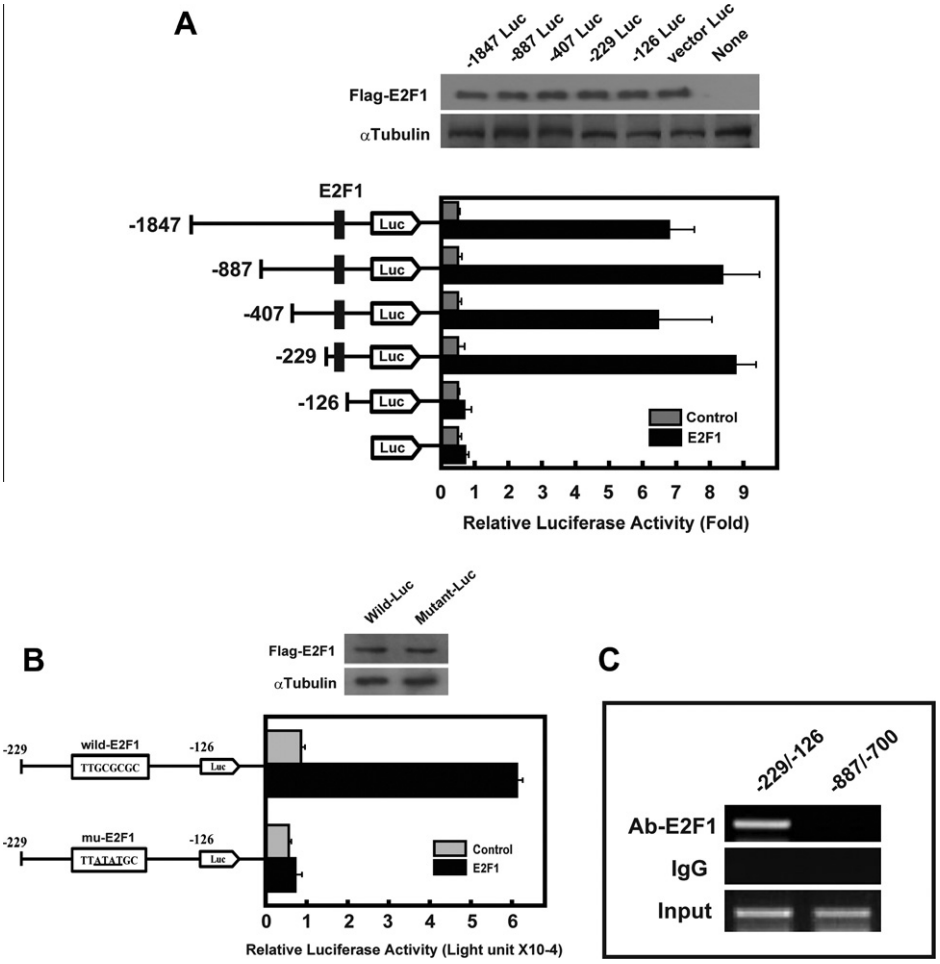


**Fig. 3.** E2F1 stimulates Lhx2 promoter via TSA-response region. (A) Effects of E2F1 overexpression on Lhx2 promoter activity. αT3-1 cells were transiently transfected with Lhx2 deletion constructs, together with either Flag-E2F1 or mock expression vector. Western blot analysis was performed with antibody against Flag or α-tubulin to check its expression at the protein level. (B) Effect of mutation in putative-E2F1 binding site on E2F1 response of Lhx2 promoter. Either wild-type or mutated-type promoter construct was transiently transfected into αT3-1 cells, together with either Flag-E2F1 or mock expression vector. Western blot analysis was performed with antibody against Flag or α-tubulin to check its expression at the protein level. (C) Binding of E2F1 to P1 region of the Lhx2 promoter *in vivo*. ChIP assay was carried out using E2F1 antibody, followed by PCR with primers amplifying −229 to −126 or −887 to −700 regions. All results are expressed as means ± S.D. for three independent experiments.

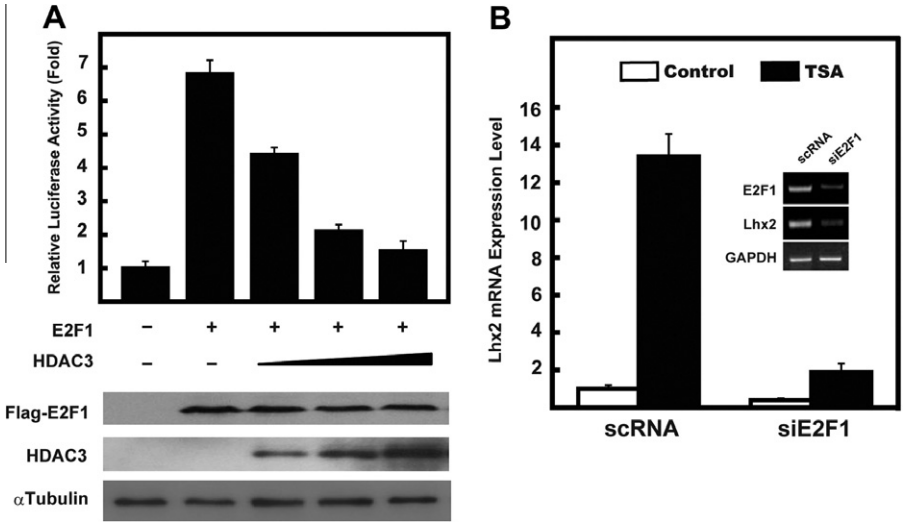


**Fig. 4.** Knockdown of E2F1 blocks TSA-induced Lhx2 expression. (A) Effect of HDAC3 on E2F1-induced Lhx2 promoter activity. αT3-1 cells were transfected with P1 region-containing Lhx2 promoter construct, together with E2F1 expression vector and increasing amount of HDAC3 expression vector. Western blot analysis was performed with antibody against Flag, HDAC3 or α-tubulin to check its expression at the protein level. (B) Effect of E2F1 knockdown on TSA-induced Lhx2 mRNA level. αT3-1 cells transfected with the scrambled RNA (scRNA) or E2F1 siRNA (siE2F1) for 24 h were treated with 200 nM TSA for another 24 h. Real-time PCR was employed to quantitate the Lhx2 mRNA level. Values were normalized to the internal standard GAPDH mRNA. All results are expressed as means ± S.D. for three independent experiments.

introduced in E2F1 binding site to confirm the above observation. As expected, the construct with mutated E2F1 binding site was not stimulated by E2F1 (Fig. 3B). Furthermore, ChIP assay using anti-E2F1 antibody demonstrated a binding of E2F1 *in vivo* to P1 region (−229 to −126) but not to any other regions of Lhx2 promoter in αT3-1 cells (Fig. 3C). Therefore, it is highly likely that Lhx2 gene is activated by a direct binding of E2F1 to the P1 region.

### 3.3. E2F1 is involved in the activation of Lhx2 by TSA

A key point emerging from the results obtained so far was that same P1 region of the Lhx2 promoter mediates the response to the two different signals such as TSA and E2F1. Target promoters regulated by E2F1 were reported to advance from a hypoacetylated to a hyperacetylated state as cell cycle progresses [29]. In addition, E2F1 tightly regulates progression of cell cycle via retinoblastoma (Rb) protein which binds to the class I HDACs [29]. Since Lhx2 expression is inhibited by HDAC3 (Fig. 2D), the E2F1-mediated Lhx2 expression was examined in the presence of HDAC3. As expected, increasing amount of HDAC3 reduced the E2F1-driven Lhx2 expression dramatically (Fig. 4A). To further test whether down-regulation of E2F1 expression could decrease the endogenous Lhx2 levels, αT3-1 cells were transiently transfected with E2F1 siRNA or scrambled (sc) RNA as a negative control. As shown in inside of Fig. 4B, the levels of E2F1 and Lhx2 mRNA expression were definitely reduced by E2F1 siRNA, indicating that E2F1 transcription factor acts as an upstream regulator of Lhx2 expression. Next, the role that endogenous E2F1 takes for TSA-induced Lhx2 expression was investigated using E2F1 siRNA in αT3-1 cells. Cells were transiently transfected with E2F1 siRNA or scrambled siRNA and then treated with TSA for 24 h. siRNA targeting E2F1 mRNA significantly inhibited TSA-induced Lhx2 mRNA level, which was not affected by scrambled siRNA (Fig. 4B). Taken together, these results demonstrated that E2F1 is a key regulator which is necessary for TSA-induced Lhx2 expression.

Epigenetic changes through histone modifications, especially acetylation and deacetylation, have been claimed to be a pivotal regulatory mechanism for development and function of certain tissues. In this reports, we have shown that inhibition of HDAC can potentially alter Lhx2 transcription in a number of cell types and that such effect is mediated through the conserved E2F1 binding site. E2F1 transcription factor contributes to the regulation of Lhx2 gene via HDAC3, a subfamily member of Class I HDACs. HDAC3 and E2F1 have also been reported to influence through histone aceylation on p15 (INK4b), p21 (WAF1/cip1) and Runx2 genes which are associated with cell cycle progression and tissue development [30,31]. HDAC3, but not other HDACs of class I, has been shown to predominantly modulate a critical role in the development and proliferation of hematopoietic stem cells [32]. Interestingly, Lhx2 first identified in pre-B cells is able to immortalize multipotent hematopoietic progenitor/stem cells for the formation of blood [33]. Although only two restricted cell types such as gonadotroph- and hepatocyte-derived cell lines were examined in this study, regulation of Lhx2 gene by histone acetylation seems to be a general mechanism applicable to various cell types. The present study underlines the need to explore the regulatory mechanism of Lhx2 gene, as Lhx2 is required for the development of various organs and tissues.

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