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Two Enhancers Regulate *HoxB* Genes Expression During Retinoic Acid-Induced Early Embryonic Stem Cells Differentiation Through Long-Range Chromatin Interactions

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Homeobox B cluster (*HoxB*) genes play important roles in retinoic acid (RA)-induced early embryonic stem cells (ESCs) differentiation. Knowledge of regulation network of *HoxB* is important to further unveil the mechanism of ESCs differentiation. In this study, we identified two enhancers that were activated by RA treatment and 4C data showed long-range interactions between *HoxB* genes and the two enhancers. CRISPR/Cas9-mediated individual or compound deletion of the two enhancers significantly inhibits *HoxB* gene expression, and transcriptome analysis revealed that RA-induced early ESCs differentiation was blocked in the enhancer KO cells. We propose new mechanism by which two enhancers regulate *HoxB* gene expression by different regulation modes during RA-induced early ESCs differentiation through long-range chromatin interactions.

Keywords: enhancers, *HoxB,* long-range chromatin interactions, ESCs differentiation

Introduction

Retinoic acid (RA) is a product of vitamin A metabolism, which has been reported to play an important role during embryogenesis [1–3]. RA functions as ligand for RA receptors (RARs), RA-RAR can regulate transcription of several key developmental genes [4]. Proper RA signaling is essential for embryonic stem cells (ESCs) differentiation and maintenance of tissue-specific gene expression [2,3]. *HoxB* genes are reported playing important role in RA signaling [4,5]. RA regulates the spatial and temporal activation of *Hox* genes in vivo and in vitro [1,2,6] and induces rapid increase of *HoxB* genes in a collinear manner [7,8].

Previous studies have shown that RA regulates *HoxB* genes by RA response element (RARE) [9]. Binding of RA-RAR and RARE recruits coactivators, further activating *HoxB* genes [4,5,9]. Early studies report that RA directly regulates *Hoxb1* expression through RARE during early neural differentiation [10]. Precise regulations of *HoxB* genes result in accurate ESCs differentiation, further studies on the regulation network of *HoxB* genes are essential to help understand the mechanism of RA-induced ESCs differentiation.

Studies on 3D chromatin structures indicate complicated gene regulation network through long-range chromatin interactions [11]. Recent studies identified highly dynamic change of 3D genome architecture during early embryonic development [12–14], which suggests that long-range chromatin interactions may play important roles in ESCs differentiation.

Chromatin conformation capture techniques such as 3C, 4C, and Hi-C increased our understanding of 3D chromatin structure in many biological processes [15–18]. Early studies have reported long-range regulation of genes during ESCs differentiation [19,20] and indicated that 3D chromatin structure is critical for activation of *Hox* genes [21–23]. Decondensed chromatin conformation is along with *HoxB* clusters gene activation [24]. However, during RA-induced ESCs early differentiation, the detailed function of long-range enhancer regulation network of *HoxB* genes induced by 3D chromatin structure is still not very clear.

Here in this study, we noticed that two enhancers (E1 and E2) were activated during RA-induced ESCs early differentiation. E1 is located in the *HoxB* cluster and E2 is about 209 kb away from *HoxB* genes, which may function as a distal enhancer. 4C assay indicates long-range interactions between E1/E2 and *HoxB* clusters, which suggest possible regulation of *HoxB* genes by E1 and E2 through long-range chromatin interactions. In addition, CRISPR/Cas9-mediated individual and compound deletion of these

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two enhancers significantly inhibit expression of *HoxB* genes and affect ESCs early differentiation. We suggest E1 and E2 regulate *HoxB* genes during ESCs early differentiation through long-range chromatin interactions.

Materials and Methods

Cell culture and RA-induced ESCs differentiation

Mouse E14 cells were cultured in culture dishes coated with 0.1% gelatin (Sigma) in medium consisting of Dulbecco's modified Eagle's medium (DMEM), 100 nM nonessential amino acids (Gibco), 200 mM glutamate (Gibco), 1% penicillin-streptomycin (Gibco), 15% fetal bovine serum (FBS; AusGeneX), 10 ng/mL LIF (ESGRO), 1% sodium pyruvate (Gibco), 50 μM-mercaptoethanol (Sigma), 3 μM CHIR99021 (a GSK inhibitor; Selleckchem), and 1 μM PD0325901 (a MEK inhibitor; Selleckchem). Cells were cultured at 37°C in a 5% CO_2 incubator and passaged every 2–3 days [20]. RA-treated ESCs were induced to differentiate by LIF/2i withdrawal and addition of 2 μM RA (Sigma), medium was replaced every day.

RNA extraction, reverse transcription, and quantitative real-time PCR

Total RNA was extracted from cells using TRIzol reagent (Life Technologies). PrimerScript™ RT reagent Kit and gDNA Eraser (TaKaRa) were used to synthesize cDNA according to the manufacturer's instruction. Quantitative real-time PCR (qRT-PCR) were performed using Hieff™ qPCR SYBR Green Master Mix (YEASEN) and a Bio-Rad CFX Connect Real-Time system. PCR cycling conditions were as follows: 95°C for 5 min, 40 cycles of 95°C for 15 s, 60°C for 15 s, and 72°C for 30 s. The quantification of target genes is normalized to *Gapdh* expression and qRT-PCR Primer sequences are listed in Supplementary Table S1.

Chromatin immunoprecipitation sequencing

ChIP-seq assay was performed as previously described protocol with minor modification [25]. In brief, 1×10^7 cells were treated with 1% formaldehyde and harvested in lysis buffer [50 mM (pH 7.5) tris-HCl, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, and 1% Triton X-100] with protease inhibitor cocktail and 1 mM phenylmethylsulfonyl fluoride. Cross-linked chromatin was sonicated to fragment DNA to 100–500 base pairs (bp).

Samples were precleared for 1 h with 40 μL of protein A/G agarose beads (Thermo Fisher Scientific). Precleared samples were immunoprecipitated with 5 μg of H3K27ac antibody (Abcam; ab4729) or H3K4me3 antibody (Abcam; ab8580) and 60 μL of protein A/G agarose beads. The precipitated DNA and input were sequenced using an Illumina NovaSeq 6000 sequencer. Reads were mapped to the mm10 reference genome using HISAT2 [26] and analyzed with HOMER (Hypergeometric Optimization of Motif EnRichment) package [27].

4C-Seq

4C libraries were prepared as previously described with minor modifications [28]. In brief, 1×10^7 cells were trypsinized and resuspended as single cells in 9.376 mL culture medium. For crosslinking, 0.624 mL 16% formaldehyde was added, and fixation was quenched with 1.425 mL 1 M glycine. Then cells were lysed in 5 mL cold lysis buffer (10 mM Tris, pH7.5, 10 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EGTA, 0.2% NP-40, protease inhibitors) for 10 min at 4°C with rotating. Lysed nuclei were resuspended in 60 μL restriction enzyme buffer and incubated with 0.3% SDS for 1 h at 37°C with shaking and then incubated with 2% Triton X-100 for 1 h. DpnII restriction enzyme were added and incubated at 37°C overnight. Digested samples were then diluted in ligation buffer with 400U T4 DNA ligase.

Samples were incubated at 16°C for 4 h and then at 25°C for 30 min. The ligated chromatin was digested by proteinase K and then incubated with RNaseA. DNA was purified by phenol-chloroform extraction and ethanol precipitation. DNA was further digested by *Nla*III and circularized using T4 DNA ligase. After purification, three parallel PCR were performed. 4C Primers are listed in Supplementary Table S2. The barcoded DNA libraries were sequenced as 150 bp paired-end reads using the Illumina HiSeq2500 platform. 4C data were analyzed using the published method [28].

CRISPR/Cas9-mediated enhancer deletion

Guide RNAs (sgRNA) were designed using the online tools (http://crispr.mit.edu/). SgRNAs were cloned into a Cas9-puro vector for Enhancer knockout. SgRNA plasmids were transfected into ESCs using Lipofectamine 3000 (Life Technologies) following the manufacturer's instructions. Then the cells were treated with 5 μM puromycin. Cell colony was selected and verified by PCR and subsequent Sanger DNA sequencing. Guide RNA sequences are listed in Supplementary Table S3 and genotyping PCR primers are listed in Supplementary Table S4.

RNA-seq analysis

Total RNA was extracted from ESCs using TRIzol reagent (Life Technologies). Barcoded RNA-seq libraries were sequenced and clean reads were mapped to Ensemble mm10 mouse genome using Hisat2 with default parameters. Fold changes were analyzed as the log2 ratio of normalized reads per gene using DEseq2 R package [29]. Gene expression with $|\log2(\text{foldchange})| \geq 1$ ($P < 0.05$) was considered as significantly changed expression. Heatmaps were drawn with heatmap2.

Gene ontology biological process and KEGG pathway analyses

Gene ontology biological process and KEGG pathway were performed by using the DAVID Functional Annotation Bioinformatics Microarray Analysis Tool (http://david.abcc.ncifcrf.gov)[30,31].

Published data used in this study

The following published datasets were used in our analysis: GSM881349 for H3K27ac ChIP-seq analyses and GSM881354 for H3K4me3 ChIP-seq analyses in mouse ESCs [32]. Raw reads were aligned using bowtie to build version mm10 of the mouse genome. MACS2 was used to call peaks using a default *P*-value cutoff of 1e-5 [33].

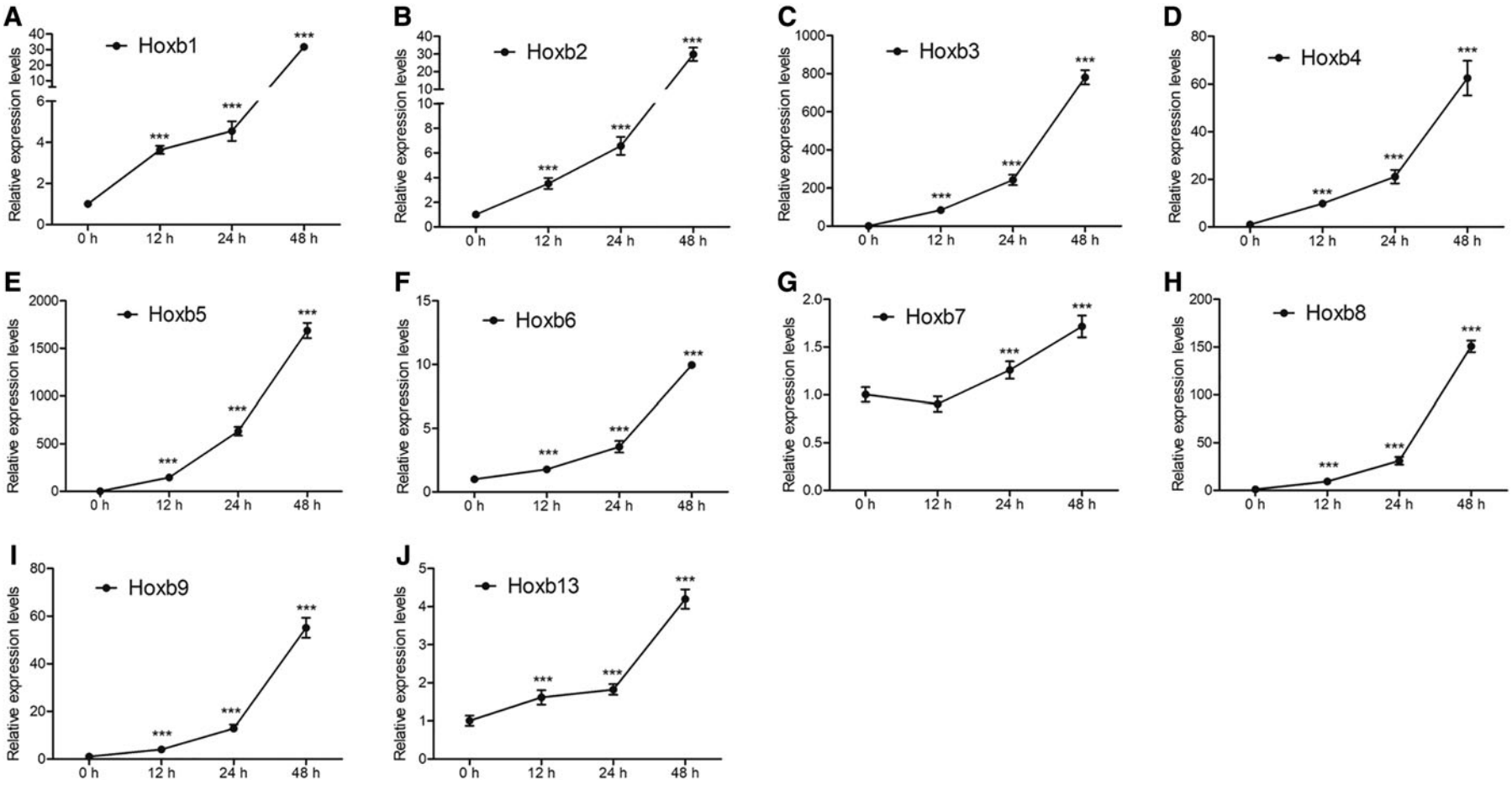

FIG. 1. RA treatment-induced upregulation of *HoxB* genes. **(A–J)** qRT-PCR analysis of mRNA levels of *Hoxb1–9* **(A–I)** and *Hoxb13* **(J)** in ESCs and ESCs treated with RA for 12, 24, or 48 h. Data represent means ± SD of three biological replicates. $^{***}P < 0.001$ compared with ESCs without RA treatment. qRT-PCR, quantitative real-time PCR; RA, retinoic acid; ESC, embryonic stem cell.

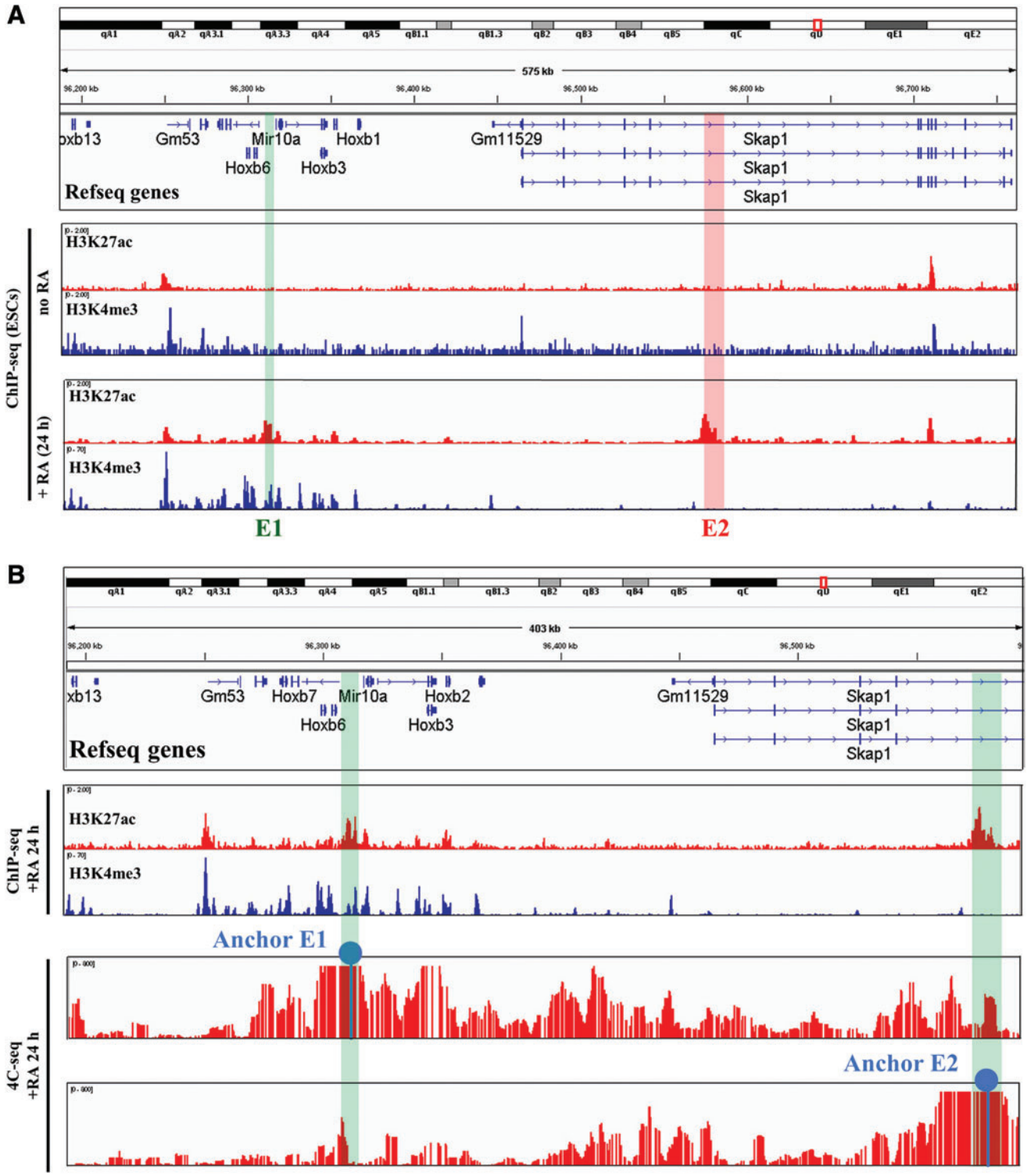

FIG. 2. Enhancer 1 (E1) and Enhancer 2 (E2) interact with *HoxB* genes in RA-treated ESCs. **(A)** H3K27ac and H3K4me3 ChIP of the region containing E1, E2, and *HoxB* genes in ESCs and ESCs treated with RA for 24 h. E1 is shaded in *green* and E2 is shaded in *red*. **(B)** 4C-seq profiles generated in ESCs treated with RA are shown around E1, E2, and *HoxB* genes. E1 and E2 are shaded in *green*. 4C-seq experiments were performed using E1 (up, ''Anchor E1'') or E2 (down, ''Anchor E2'') as viewpoints. Color images are available online.

Statistical analyses

Data were analyzed by Student's *t*-test (Two-tailed) unless otherwise specified. Statistically significant *P*-values are indicated in figures as follows: $*P<0.05$, $**P<0.01$, $***P<0.001$.

Data availability statement

The raw data reported in this article for the RNA-seq and 4C data have been deposited in NCBI Gene Expression Omnibus (GEO; www.ncbi.nlm.nih.gov/geo/) under the accession number GSE165725.

Results

Long-range chromatin interactions between HoxB *genes and distal enhancer*

Twenty-four hours after RA-treatment was verified to be important time point to characterize the early transcriptional and epigenetic events during RA-induced early differentiation of ESCs [20,34]. qRT-PCR data showed upregulation of *HoxB* genes in 24 h RA (2 μM)-treated ESCs (Fig. 1). Ten *HoxB* genes (*Hoxb1–9* and *Hoxb13*) were significantly upregulated in ESCs with RA treatment. H3K27ac ChIP-seq showed significant H3K27ac signal enrichment at two loci (E1 and E2) in ESCs that were treated with RA for 24 h, while not in ESCs without RA treatment (Fig. 2A). H3K27ac is a well-characterized marker of active enhancer [35], thus the ChIP-seq data indicate two possible RA-activated enhancers (E1 and E2, Fig. 2A) at the two loci.

One of the enhancers (E1) is between *Hoxb4* and *Hoxb5*, while the other one (E2) is about 209 kb away from the *HoxB* cluster. To further investigate if E1 and E2 involved in *HoxB* genes regulation, 4C was performed using E1 and E2 region as bait to detect the interactions between these two enhancers and the *HoxB* genes. 4C data showed significant interactions between E1 and *HoxB* genes, E2 and *HoxB* genes, and also E1 and E2 (Fig. 2B). 4C data suggest possible regulations of *HoxB* genes by E1 and E2.

Individual or compound deletion of E1 and E2 inhibits RA-induced HoxB expression

To determine whether E1 and E2 regulate *HoxB* gene expression, we used CRISPR/Cas9 technique to conduct

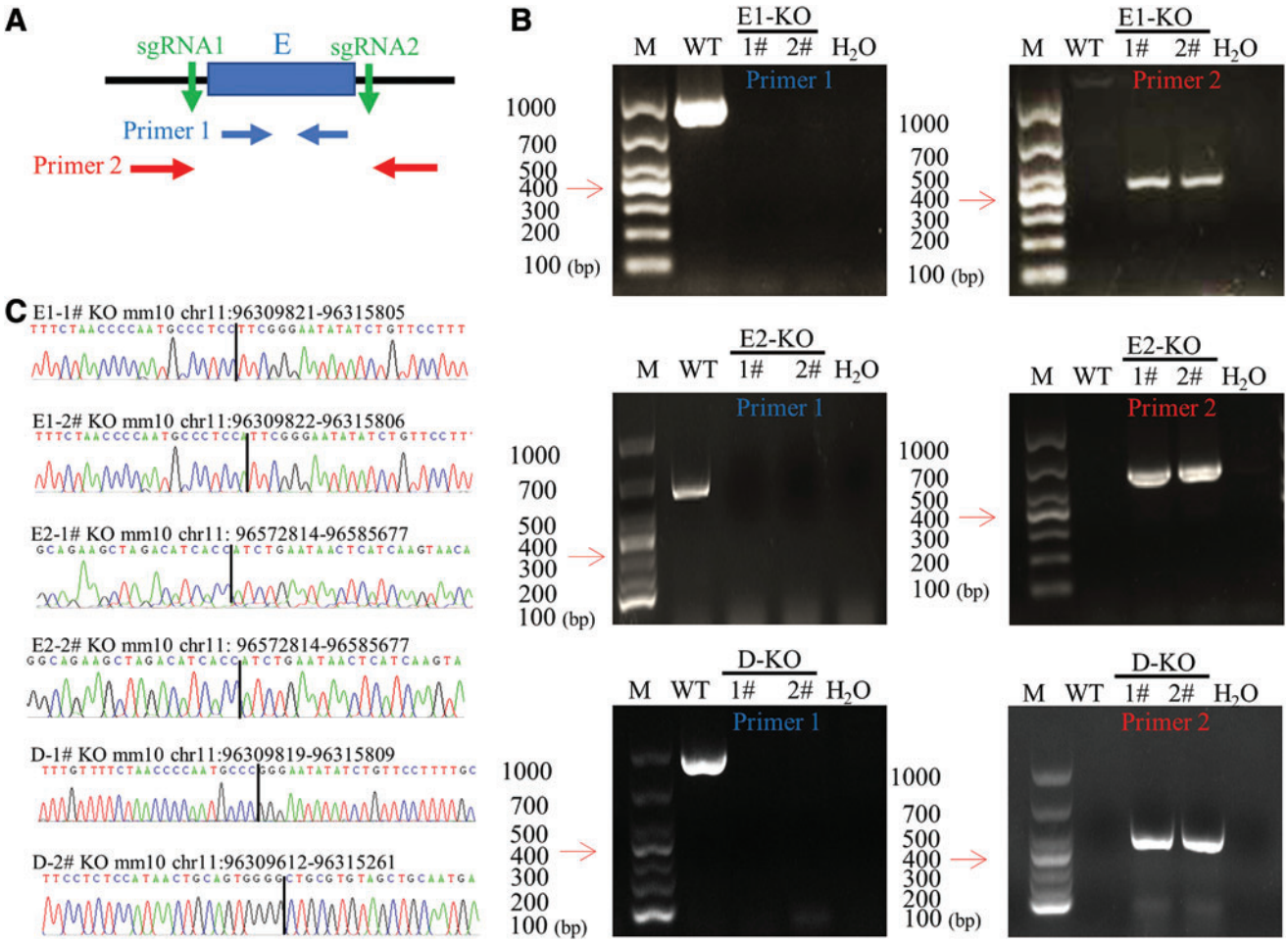

FIG. 3. CRISPR/Cas9-mediated deletion of E1 and E2. **(A)** Schematic showing the primers that were used to detect the deletion of E1 and E2. **(B)** PCR analysis used to identify the individual or compound E1 and E2 KO cell lines. PCR product of the E1 or E2 region was shown in the WT lane. M: marker; H_2O: negative control. E1-KO, E2-KO: individual deletion of E1 or E2. D-KO: compound deletion of E1 and E2. PCR product of E1 region was detected to verify the D-KO cells due to the D-KO cell lines that were derived from the verified E2-KO cell lines. **(C)** Sequencing of the E1 region in the E1-KO and D-KO cell lines and sequencing of E2 region in the E2-KO cell lines. Color images are available online.

individual or compound deletion of E1 and E2. Homozygous knockout cell lines were characterized by PCR (Fig. 3A, B) and DNA sequencing (Fig. 3C). Two E1 knockout cell lines (E1–1# KO and E1–2# KO), two E2 knockout lines (E2–1# KO and E2–2# KO), and two E1 and E2 compound knockout cell lines (D-1# KO and D-2# KO) were used in the following experiments (Fig. 3A–C). qRT-PCR data showed significant inhibition of *Hoxb1–9* expression in RA-treated E1 or E2 individual knockout cell lines compared with RA-treated WT ESCs (Fig. 4A–I). In RA-treated E1 or E2 individual knockout cell lines, *Hoxb13* did not show significant inhibition (Fig. 4J). But E1 and E2 compound deletion significantly inhibits *Hoxb13* in RA-treated ESCs (Fig. 4J).

E1 and E2 compound deletion induced more significant inhibition of *Hoxb2* and *Hoxb5–9* expression than E1 and E2 individual deletion (Fig. 4B, E–I). However, E1 individual deletion induced more significant downregulations of *Hoxb1* and *Hoxb3* than E1 and E2 compound deletion (Fig. 4A, C). Furthermore, E1 and E2 compound deletion induced significant decrease of *Hoxb4* than E2 individual deletion but not E1 (Fig. 4D). These data suggest synergistic or redundant function of E1 and E2 in regulating some of *HoxB* genes' expression. Given that E2 is about 209 kb away from *HoxB* genes, the long-range regulation of *HoxB* genes by E2 may achieve through long-range chromatin interactions, which is verified by 4C data (Fig. 2B).

RA-induced ESCs early differentiation is inhibited in E1 and E2 KO cells

RA-induced ESCs early differentiation is associated with *HoxB* expression. To investigate whether the two RA-induced enhancers function in ESCs early differentiation, RNA-seq was performed to analyze transcriptome in WT and enhancer-KO cell lines. RNA-seq data show global alteration of genes in the enhancer KO cell lines. In RA-treated E1-KO cells, 1265 genes were upregulated ($|\log2$ (fold change)$|\geq1$, $P<0.05$), while 1451 genes were downregulated ($|\log2$ (fold change)$|\geq1$, $P<0.05$) (Fig. 5A). In RA-treated E2-KO cells, 1156 genes were upregulated ($|\log2$ (fold change)$|\geq1$, $P<0.05$), while 1517 genes were downregulated ($|\log2$ (fold change)$|\geq1$, $P<0.05$) (Fig. 5B).

In E1 and E2 compound KO cells, 804 genes were upregulated ($|\log2$ (fold change)$|\geq1$, $P<0.05$), while 1128 genes were downregulated ($|\log2$ (fold change)$|\geq1$, $P<0.05$) (Fig. 5C). Pluripotency and differentiation genes were selected to construct a heatmap, and it shows upregulations of several pluripotency genes in E1 and E2 individual or compound deletion cell lines (Fig. 5D), which suggests inhibition of ESCs early differentiation. In addition, most of endodermal genes were downregulated in E1 and E2 individual or compound deletion cell lines (Fig. 5D), which is

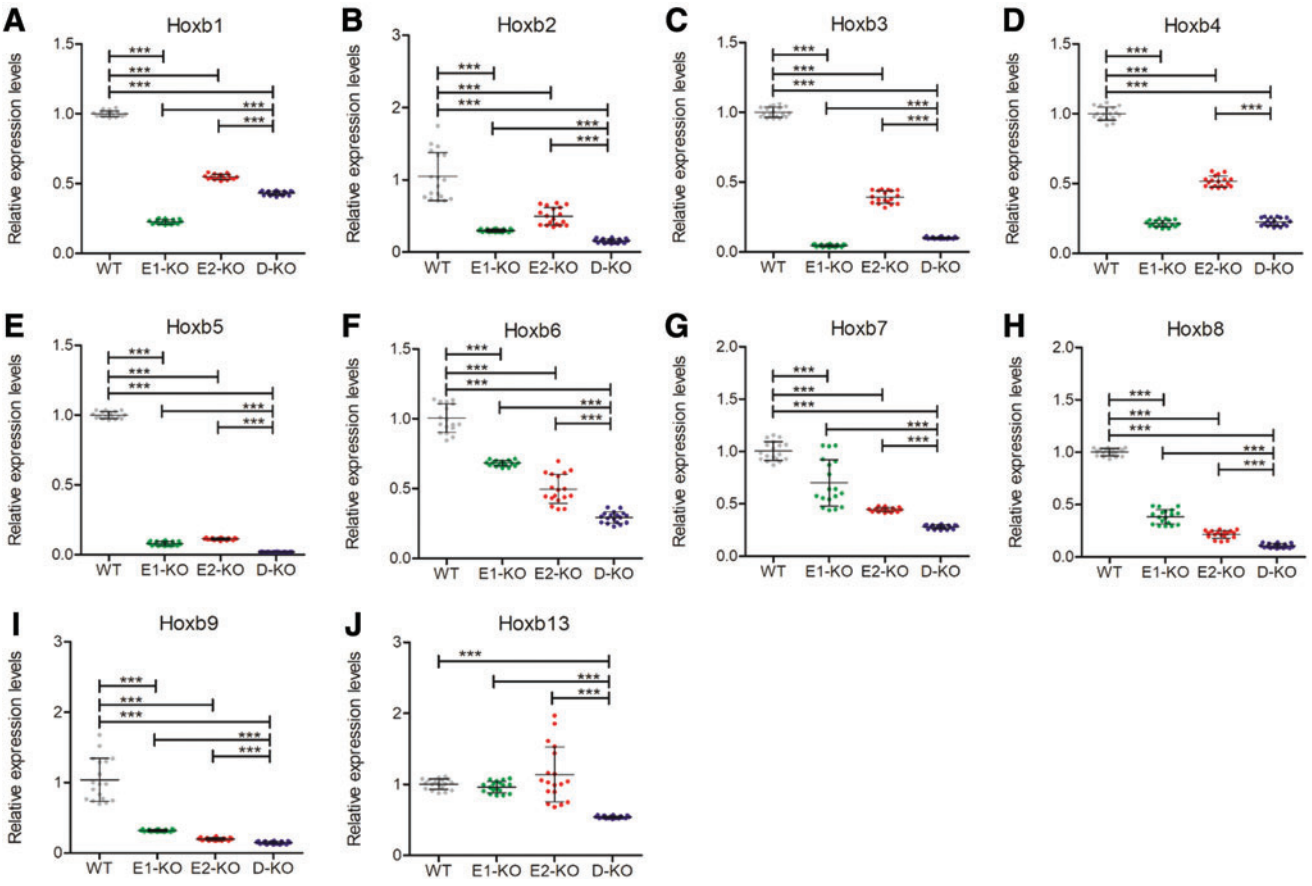

FIG. 4. Individual or compound deletion of E1 and E2 induced downregulation of HoxB genes. **(A–J)** qRT-PCR analysis of mRNA levels of *Hoxb1–9* **(A–I)** and *Hoxb13* **(J)** in ESCs and enhancer KO cells (E1-KO, E2-KO, D-KO) treated with RA for 24 h. Data represent means ± SD of three biological replicates. $^{***}P<0.001$ compared with WT ESCs. Color images are available online.

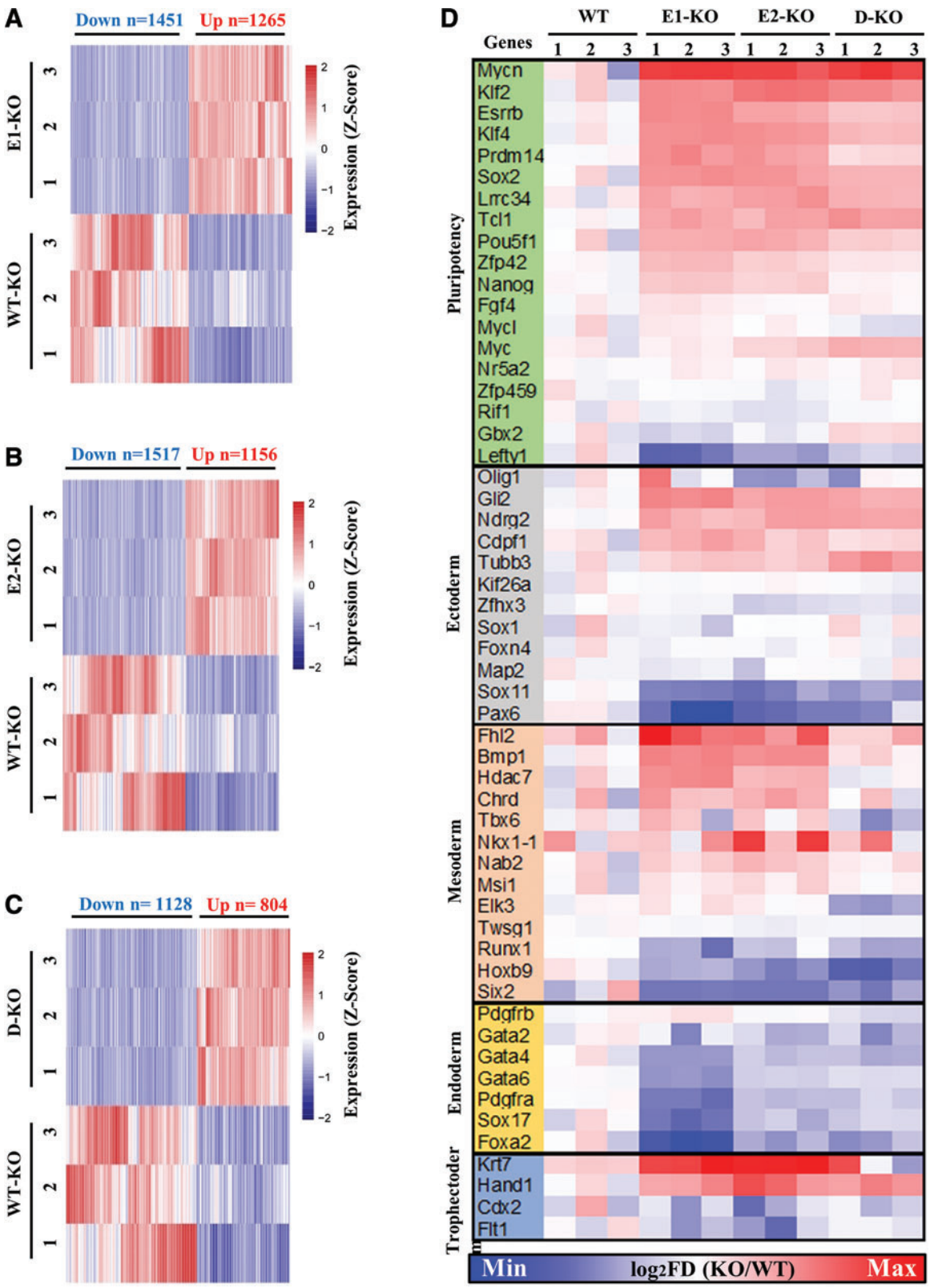

FIG. 5. Individual or compound deletion of E1 and E2 induced change of transcriptome. **(A–C)** Heatmap shows gene expression changes (fold-change ≥2, and $P < 0.05$) and clustering of differentially expressed genes in WT and E1-KO cells treated with RA **(A)**, WT and E2-KO cells treated with RA **(B)**, and WT and D-KO cells treated with RA **(C)**. **(D)** Heatmap shows RNA-seq results of pluripotency and differentiation genes. Color images are available online.

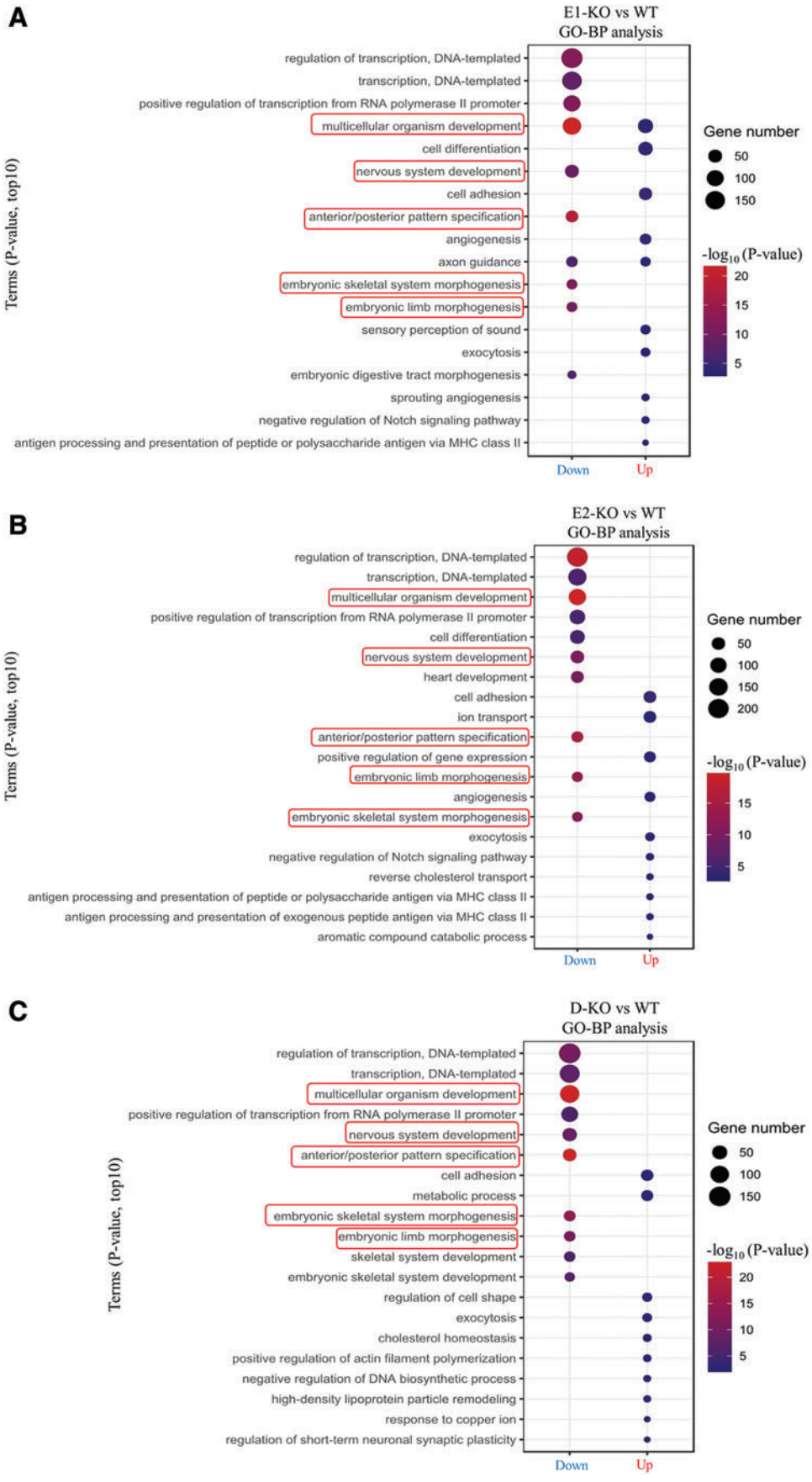

FIG. 6. GO-BP analyses suggest that individual or compound deletion of E1 and E2 are associated with ESCs early differentiation. **(A–C)** GO-BP analyses indicated differentially expressed genes in E1-KO cells (**A**), E2-KO cells (**B**), and D-KO cells (**C**). GO-BP, gene ontology biological process. Color images are available online.

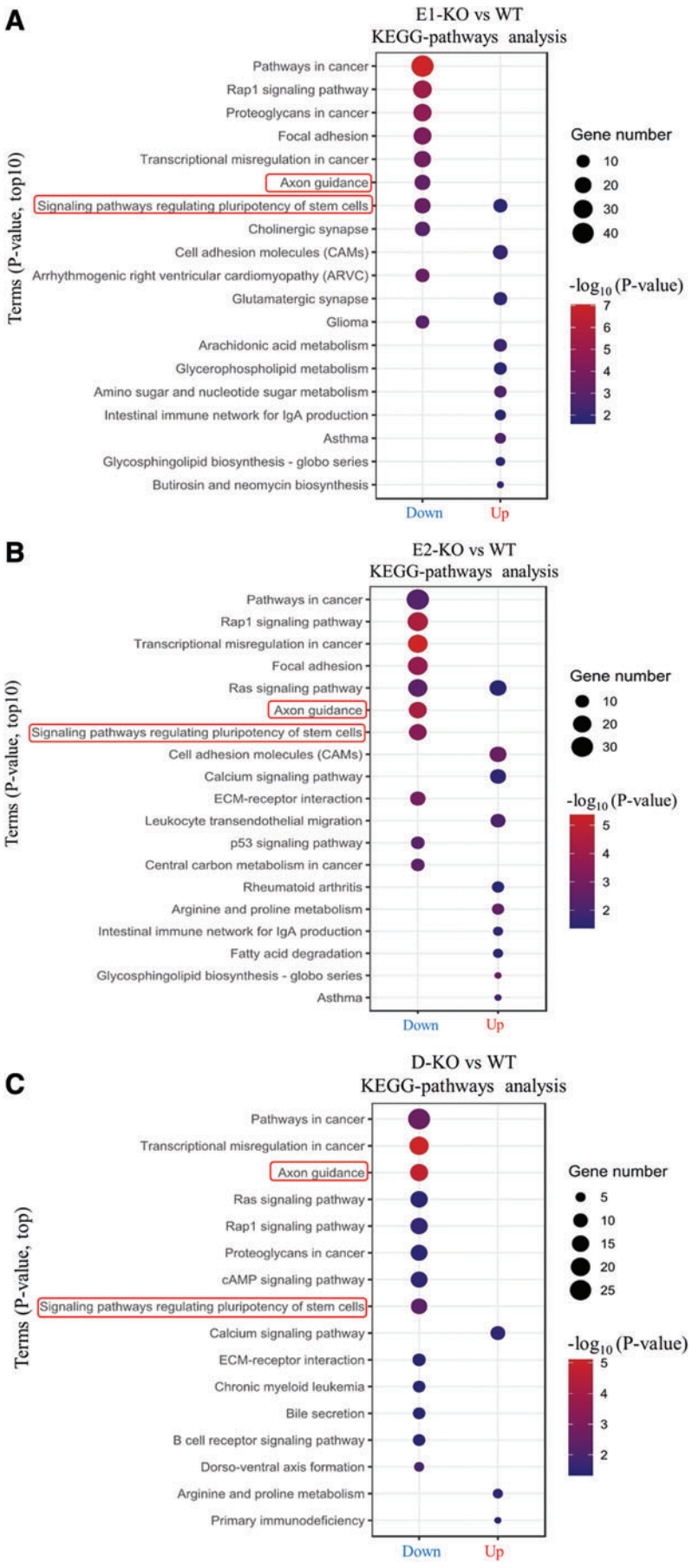

FIG. 7. KEGG pathway analyses suggest that individual or compound deletion of E1 and E2 are associated with ESCs early differentiation. **(A–C)** KEGG pathway analyses of indicated differentially expressed genes in E1-KO cells **(A)**, E2-KO cells **(B)**, and D-KO cells **(C)**. Color images are available online.

consistent with previous studies that show that *HoxB* genes are associated with endoderm development [36,37].

BP-GO analysis shows similar gene ontology patterns in E1 and E2 individual and compound deletion cell lines. Upregulated and downregulated genes in RA-treated E1-KO, E2-KO, and D-KO cells are mainly associated with organism development, nervous system development, anterior/posterior pattern specification, and other biological processes that are related to ESCs differentiation (Fig. 6A–C). KEGG pathway analysis also indicates that upregulated and downregulated genes in RA-treated E1-KO, E2-KO, and D-KO cells are involved in axon guidance pathway and signaling pathways regulating pluripotency of stem cells (Fig. 7A–C). Both BP-GO and KEGG pathway analysis of the RNA-seq data suggest that individual and compound deletion of E1 and E2 affect RA-induced ESCs early differentiation.

Discussion

Long-range regulation of target genes by distal gene regulatory elements could regulate cell proliferation, cancer cells' drug sensitivity, and stem cell differentiation [19,25, 38,39]. Long-range chromatin interactions play important roles in several biological processes.

Early study reported that a distal enhancer maintain *Hoxa1* expression through long-range interaction loop and regulate ESCs early differentiation [20]. Zhao et al. found that lncRNA *5430416N02Rik* promotes proliferation of ESCs by regulating *Mid1* through long-range chromatin interactions [40], while Yin et al. verified that *Haunt* locus provides enhancers to long-range regulate *HOXA* during ESCs differentiation [41], which is similar with the long-range regulation pattern of *HoxB* genes reported in our study. However, they also found that lncRNA transcript *Haunt* participate in the regulation of *HOXA* by the long-range enhancer to prevent aberrant *HOXA* expression. These studies suggest that long-range chromatin interactions induce gene regulation network and play an important role in ESCs differentiation and proliferation.

Chromatin decondensation and nuclear reorganization of the *HoxB* locus was reported regulating the expression of the *HoxB* genes [24,42], which indicates that long-range chromatin interactions play an important role in the regulation of *HoxB* genes. Further study on the detailed function of how gene regulatory elements orchestrate *HoxB gene expression* through long-range interactions is essential to reveal the regulation network of *HoxB* genes.

Our study identified two RA-induced enhancers synergistically regulate *HoxB* genes through long-range chromatin interactions and are involved in the regulation of ESCs early differentiation (Fig. 8), which reveals new regulatory mechanism of *HoxB* genes and ESCs early differentiation. 4C data also showed interactions between E1/E2 and the gene desert region between *Hoxb2* and *Skap1*. However, ChIP-seq data in RA-treated ESCs did not show H3K27ac enrichment in this region. Previous study reported that there are regulatory elements of *HoxB* genes in this region and they function in early heart and endoderm development [43]. Enhancers in this gene desert region may function in specific stages of development. In short-term RA-treated ESCs, interactions between E1/E2 and this region may not have specific functions.

Early studies show that some poised enhancers may be activated during differentiation to support new developmental state [35,44]. Based on the H3K27ac ChIP-seq data (Fig. 2A), we noticed that H3K27ac signal was not enriched at E1 and E2 loci in ESCs without RA treatment. By contrary, in ESCs following RA treatment, H3K27ac signal was significantly enriched at E1 and E2 loci.

Creyghton et al. identified several poised enhancers in ESCs that became specifically activated in neural progenitors, while not in liver or proB cells, *Neuroglycan-C* and *Neurophilin-2* are two genes that harbor poised enhancers in ESCs that become active in neural progenitors [35]. Cruz-Molina et al. found that poised enhancers are necessary for the induction of major anterior neural genes during stem cell differentiation and these poised enhancers physically contact their target genes in ESCs [45]. Furthermore, previous studies report that RA and RARs can actively open the chromatin structure with recruited histone acetyltransferases [46,47], which suggests possible change of histone modifications and state of enhancers during RA-induced differentiation.

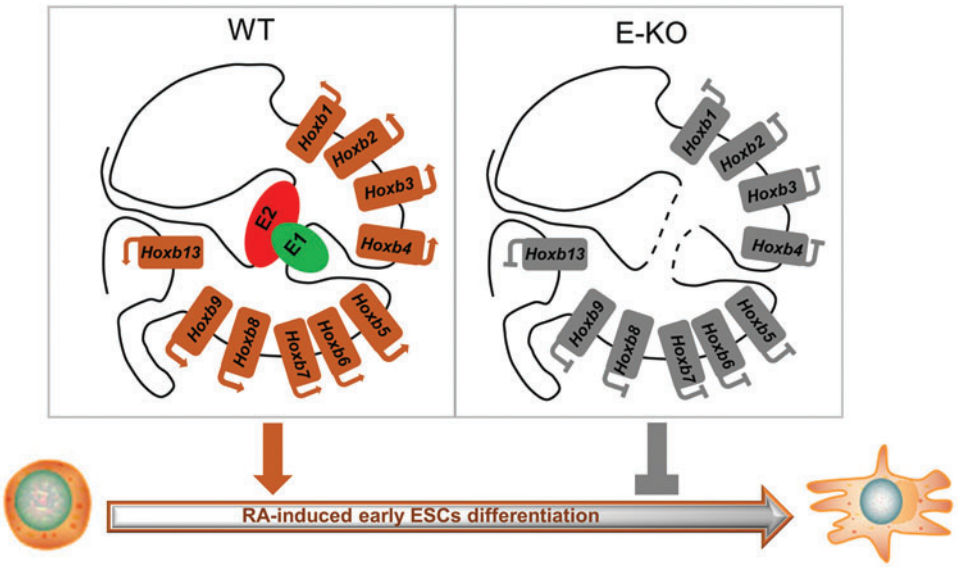

FIG. 8. Schematic drawing of the two enhancers (E1 and E2) synergistically regulate *HoxB* genes expression during RA-induced early ESCs differentiation through 3D chromatin structure. In WT ESCs, the two enhancers interact with *HoxB* genes and regulate the expression of *HoxB* genes and thus maintain proper RA-induced early ESCs differentiation (*Left*). In E1 and E2 KO ESCs, expression of HoxB genes is inhibited and induced impairing RA-induced ESCs early differentiation (*Right*). Color images are available online.

Therefore, based on the data in our study, it is possible that the two enhancers, E1 and E2, might be activated by RA signaling and function as poised enhancers to support ESCs early differentiation.

E1, in this study, is located at the same position of DERARE in human hematopoietic stem cells, which is previously reported as a retinoid-dependent cis-regulatory element functions in maintaining normal hematopoiesis and inhibits leukemogenesis [48]. Loss of DERARE abrogates *HoxB* expression and induces loss of hematopoietic stem cells self-renewal and reconstitution capacity, which is similar with the E1' function we found in this study. It is possible that E1 or DERARE could regulate *HoxB* genes in different kind of stem cells and both of them are involved in regulation of stem cell self-renewal or differentiation.

Genes could be regulated by several different enhancers. Many of the genes controlling anterior-posterior patterning contain multiple enhancers that initiate the *Drosophila* embryo segmentation [49]. These enhancers function together to produce authentic patterns of gene expression during *Drosophila* embryo segmentation. In addition, expression of *Hoxd* Genes in digits reportedly requires several enhancers that are dispersed throughout the nearby gene desert. Also, each of the enhancers contributes either quantitatively or qualitatively to *Hox* gene transcriptions [23].

Several enhancers were reported located within a *HoxA* 3′subtopologically associated domain, and the proximal cassette of these enhancers are Wnt-dependent [50] and response to the earliest Wnt signal in the gastrulating embryo, which suggests a precise activation of *HoxA* during development. Regulation of *HoxB* by several enhancers may also function as a precision process to control accurate expression of *HoxB* genes during stem cell differentiation.

Previous studies also report that some enhancers function as shadow enhancers or secondary enhancers [51,52], they function redundantly on regulation of one specific gene. Simultaneous deletion of two shadow enhancers induce impaired activation of HoxA, while individual deletion of them did not result in significant decrease of *HoxA* [53], and in our study, either E1 or E2 knockout did not induce significant decrease of *Hoxb13*, while E1 and E2 compound knockout induced markedly decrease of *Hoxb13*. Thus, E1 and E2 may regulate *Hoxb13* redundantly as shadow enhancers. On the contrary, E1 and E2 compound knockout resulted in more significant downregulation of *Hoxb2* and *Hoxb5–9* than E1 or E2 individual knockout. Thus, E1 and E2 may regulate *HoxB* genes synergistically instead of redundantly.

At the same time, we noticed that compared with E2 individual knockout and E1 and E2 compound knockout, E1 individual knockout induced more significant decrease of *Hoxb1* and *Hoxb3* (Fig. 4A, C). We reckon that enhancer E2 may also regulate other genes that could regulate *Hoxb1* or *Hoxb3*, thus E2 knockout simultaneously induced expression changes of other genes regulated by E2, and the expression changes of these genes may more or less rescue the decrease of *Hoxb1* and *Hoxb3*. Furthermore, E1 and E2 compound knockout induced more significant downregulation of *Hoxb4* than E2 individual knockout, but not E1 individual knockout (Fig. 4D). This may due to a more stronger function of E1 than E2 on regulation of *Hoxb4*, E1 individual knockout is enough to induce relatively complete reduction of *Hoxb4* (Fig. 4D).

In summary, E1 and E2 synergistically or redundantly regulate some of *HoxB* genes, and there are also other target genes that could be regulated by E2 that may simutaneously regulate *HoxB* genes. E1 and E2 are important component of the *HoxB* regulation network. It is possible that gene regulation by multiple enhancers is to provide an inherent flexibility or precise control of embryonic development.

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Author Disclosure Statement

No competing financial interests exist.

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Supplementary Material

Supplementary Table S1
Supplementary Table S2
Supplementary Table S3
Supplementary Table S4

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