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Genes Frequently Coexpressed with *Hoxc8* Provide Insight into the Discovery of Target Genes

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Identifying Hoxc8 target genes is at the crux of understanding the Hoxc8-mediated regulatory networks underlying its roles during development. However, identification of these genes remains difficult due to intrinsic factors of Hoxc8, such as low DNA binding specificity, context-dependent regulation, and unknown cofactors. Therefore, as an alternative, the present study attempted to test whether the roles of Hoxc8 could be inferred by simply analyzing genes frequently coexpressed with *Hoxc8,* and whether these genes include putative target genes. Using archived gene expression datasets in which *Hoxc8* was differentially expressed, we identified a total of 567 genes that were positively coexpressed with *Hoxc8* in at least four out of eight datasets. Among these, 23 genes were coexpressed in six datasets. Gene sets associated with extracellular matrix and cell adhesion were most significantly enriched, followed by gene sets for skeletal system development, morphogenesis, cell motility, and transcriptional regulation. In particular, transcriptional regulators, including paralogs of Hoxc8, known Hox co-factors, and transcriptional remodeling factors were enriched. We randomly selected *Adam19, Ptpn13, Prkd1, Tgfbi,* and *Aldh1a3,* and validated their coexpression in mouse embryonic tissues and cell lines following TGF-β2 treatment or ectopic Hoxc8 expression. Except for *Aldh1a3,* all genes showed concordant expression with that of *Hoxc8*, suggesting that the coexpressed genes might include direct or indirect target genes. Collectively, we suggest that the coexpressed genes provide a resource for constructing Hoxc8-mediated regulatory networks.

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INTRODUCTION

Hoxc8 is a homeodomain transcription factor that regulates pattern formation, cell migration, and differentiation (Lei et al., 2005; Pearson et al., 2005). Loss- or gain-of-function studies have suggested that Hoxc8 is essential for skeletal pattern formation, hematopoiesis, and cartilage differentiation during embryogenesis (Kruger and Kappen, 2010; Le Mouellic et al,. 1992; Shimamoto et al., 1999; Tiret et al., 1998; Yueh et al., 1998). To further elucidate the underlying mechanisms of developmental defects caused by *Hoxc8* mutation, it is necessary to identify the transcriptional target genes of Hoxc8. To date, several target genes have been identified, and several preliminary Hoxc8-mediated regulatory networks have been proposed. Hoxc8 directly downregulates *Opn*, *Zac1*, *Ncam*, and *Pedf*, and upregulates *Cdh11* in mouse embryonic fibroblast cells (Lei et al., 2005; 2006). We also previously identified *Pcna, ZNF804A,* and *Mgl1* as Hoxc8 direct target genes in mouse embryonic fibroblast cells (Chung et al., 2010; Min et al., 2010; Ruthala et al., 2011). In another study, although 15 of 21 target genes were upregulated by Hox genes (Pearson et al., 2005), more than half of the known Hoxc8 target genes were downregulated by Hoxc8. This bias in Hoxc8 regulation patterns might result from use of a single cell type, mouse embryonic fibroblast cells at specific developmental stage *in vitro*. To more accurately reflect *in vivo* Hoxc8 transcriptional specificity, more target genes need to be identified in diverse cell types and tissues throughout the stages of embryonic development.

Several characteristics of Hoxc8 make target gene identification difficult. First, it has low DNA binding sequence specificity. Hoxc8 proteins bind as monomers or multimers to specific sequence motifs (TAAT/ATTA, TTAT, and ATAA) in their target genes (Lei et al., 2006). However, other Hox proteins can potentially bind to the same elements, and the DNA-binding specificities are modified through interactions with cofactors, such as Pbx and Meis (Ladam and Sagerström, 2014, Mann, 1995; Mann and Chan, 1996; Mann and Affolter, 1998; Moens and Selleri, 2006;). Second, little is known about Hoxc8 cofactors. Interaction of HOXC8 with PBX1 has been reported in prostate cancer cells (Kikugawa et al., 2006) but in no other tissues. Interestingly, Hoxc8 interacts with Smad1 during osteoblast differentiation (Hullinger et al., 2001, Shi et al., 1999; Yang et al., 2000). This indicates that other unknown factors, even those without a homeodomain like Smads, might interact with Hoxc8 *in vivo*. Third, Hox members act as both transcriptional activators and repressors, depending on their cellular context (Pearson et al., 2005). Therefore, careful experimental designs, with

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attention to cell types, developmental stage, time, and location, are necessary to validate candidate genes.

In an attempt to identify more Hoxc8 target genes, we analyzed eight archived microarray datasets generated from diverse cell types and tissues throughout mouse embryonic development. We found that *Hoxc8* is often coexpressed with several transcription factors, including known cofactors *Pbx1* and *Meis*, and genes related to extracellular matrix (ECM) or organization. We then randomly selected five genes and assessed their likelihood of being Hoxc8 target genes.

MATERIALS AND METHODS

Gene expression data analysis

To identify genes with expression patterns similar to those of *Hoxc8* during mouse embryonic development, we used gene expression profiles from the GEO database (http://www.ncbi.nlm.nih.gov/geo/). We analyzed eight datasets (Table 1) and assessed the similarity of each gene expression pattern to that of *Hoxc8* based on Pearson's correlation coefficient (r-value > 0.75) and *t*-test (p-value < 0.001). For the *t*-test, we grouped samples based on differential Hoxc8 expression, as shown in Table 1. We selected genes with r-values greater than 0.75 in at least four out of the eight datasets. Genes with Pearson's correlation coefficients greater than 0.75 in at least four out of the eight datasets were subjected to gene set analysis with the Database for Annotation, Visualization, and Integrated Discovery (DAVID) (http://david.abcc.ncifcrf.gov/home.jsp) program. Gene Ontology (GO) annotations with an enrichment score > 1.0, p-value < 0.01, and false discovery rate (FDR) $\leq$ 5% were considered biologically significant. We used the rVista sequence analyzer (http://zpicture.dcode.org/) to search for Hox consensus binding elements present in the 5 kb promoter, first exon, and first intron of putative target genes. The gene network was analyzed with Ingenuity Pathway Analysis (IPA) (http://www.ingenuity.com/products/ipa).

Animal preparation

To obtain E14.5 embryos, male and female ICR mice were caged together for mating at around 6 pm. The next morning, when vaginal plugs were present, was defined as 0.5 days post-coitum (dpc) or as an E0.5 embryo. After 14 days, the pregnant female mice were sacrificed, and the E14.5 embryos were extracted. The maternal and extra-embryonic tissues, cervical region, internal organs, tail bud, and limbs were removed. The embryos were divided into three parts: brain, trunk anterior (somites 12-23), and trunk posterior (somites 24-41). Total RNA was isolated from each part. This study was carried out in strict accordance with the recommendations in the Guide for the Institutional Animal Care and Use Committee of Yonsei University College of Medicine. The protocol for obtaining embryonic samples was approved by the Committee on Animal Research at Yonsei University College of Medicine (permit number 2013-0174-1).

Cell culture and transfection

Three cell lines, MC3T3-E1, NIH3T3, and C3H10T1/2, were cultured in Dulbecco's modified Eagles medium (WelGENE Inc., Korea) supplemented with 10% fetal bovine serum (FBS; WelGENE Inc., Korea) and 100 μg/ml penicillin-streptomycin (WelGENE Inc., Korea) at 37°C inside a 5% CO_2 and 95% humidified incubator. The construction of the pcDNA3.1-Hoxc8 plasmid, harboring the murine Hoxc8 gene, has been previously described (Kwon et al., 2003). pcDNA3.1-Hoxc8 or pcDNA3.1 empty vector was transfected into MC3T3-E1 cells using Lipofectamine 2000™ reagent (Invitrogen, USA), as indicated by the manufacturer. To establish stable cell lines expressing Hoxc8, NIH3T3 and C3H10T1/2 cells were transfected with Lipofectamine 2000™ reagent and pcDNA3.1-Hoxc8 or control empty vector and selected in culture media containing 500 μg/ml G418 antibiotic (Invitrogen, USA). The media was changed every three days. Cells were subcultured when the cells reached 90% confluence.

MC3T3-E1 cells were seeded into 12-well dishes at 1×10^5 cells/well. After an overnight incubation, the media was replaced with fresh media supplemented with 10 ng/ml of TGF-β2 (R&D Systems, Inc., USA). Cells were harvested at time points 0 h, 6 h, and 12 h after treatment with TGF-β2, and total RNA was isolated using Trizol reagent (Invitrogen, USA).

Knockdown of Hoxc8 with short interfering RNA (siRNA)

The sequences of the *Hoxc8* and control siRNAs are as follows: *Hoxc8* sense, 5′-AGA CGC CUC CAA AUU CUA UTT-3′, *Hoxc8* antisense, 5′-AUA GAA UUU GGA GGC GUC UTT-3′, control sense, 5′-AUG AAC GUG AAU UGC UCA ATT-3′, and control antisense, 5′-UUG AGC AAU UCA CGU UCA UTT-3′ (Samchully Pharm Co., Ltd., Korea). MC3T3-E1 cells were seeded into 12-well dishes (1×10^5 cells/well) and incubated overnight. Then, 100 nM siRNA (final concentration) was transfected into the cells using HiPerfect transfection reagent (Qiagen, Germany), according to the manufacturer's instructions. Cells were harvested 60 h after transfection, followed by isolation of total RNA. All experiments were performed in triplicate and representative examples are shown.

RNA isolation and semi-quantitative RT-PCR

Total RNA was isolated using Trizol reagent, according to the manufacturer's instructions (Invitrogen, USA). Two micrograms (μg) of total RNA was reverse-transcribed with ImProm-II™ Reverse Transcriptase (Promega, USA) and poly $(dT)_{20}$, according to the manufacturer's instructions. Semi-quantitative RT-PCR was performed with hTaq DNA polymerase (Solgent, Korea) using the following thermo cycling conditions: initial denaturation for 5 min at 95°C, followed by 30-33 cycles of 94°C for 30 s (denaturation), 58°C for 30 s (annealing), and 72°C for 30 s (polymerization). We determined the highest PCR cycle numbers at which PCR products increased linearly and were detectable on agarose gel. Primer sequences are listed in Supplementary Table S1. The PCR products were analyzed on a 1.5% agarose gel containing ethidium bromide.

RESULTS

Hoxc8 was differentially expressed in several gene expression datasets

To select archived gene expression datasets in which *Hoxc8* was differentially expressed, the GEO database was analyzed. We selected datasets by focusing on two criteria: datasets associated with studies on mouse development and cell differentiation, as well as those with greater than two-fold differential *Hoxc8* expression under the given experimental conditions, tissue types, or cell types. Eight datasets (GDS2843, GDS2743, GDS1500, GDS2123, GDS2209, GDS2699, GDS2044, and GDS2421) met our criteria (Table 1). For example, Hoxc8 was differentially expressed during mammary gland development (GDS2843; Fig. 1). In addition, when different cell types were compared, *Hoxc8* was overexpressed in white adipocytes compared to brown adipocytes (GDS2743), spinal cord com-

Table 1. Gene expression datasets in which Hoxc8 was differentially expressed

GEO Data Set No.	Title	Summary	*Hoxc8* expression	Opn (r)	Fzd2 (r)
GDS2843	Mammary gland development	Analysis of mammary glands during pregnancy, lactation, and involution	Hoxc8 was sharply downregulated 7 days after pregnancy and maintained at a low level throughout lactation and involution	-0.64	0.9
GDS2743	Brown and white adipocyte differentiation	Comparison of brown and white preadipocytes at the undifferentiated and differentiating stages	Hoxc8 was overexpressed in white adipocyte compared to brown adipocyte, irrespective of differentiation status	-0.39	0.8
GDS1500	Mechanical stress effect on fibroblasts from various fetal tissues	Expression profiling of fetal fibroblasts from tendon, skin, and cornea following mechanical stimulation by fluid flow. Fibroblasts isolated from embryos at 19 days post conception	Hoxc8 was relatively overexpressed in skin fibroblasts compared to corneal or tendon fibroblasts isolated from embryos at 19 days post-conception	-0.76	0.17
GDS2123	Brown fat cell response to PGC-1alpha and PGC-1beta deficiency	Analysis of brown fat cells lacking PGC-1alpha or both PGC-1alpha and PGC-1beta. PGC-1alpha is required for the thermogenic function of brown fat cells, and PGC-1beta is the closest homolog of PGC-1alpha	Hoxc8 was remarkably downregulated in brown fat cells lacking PGC-1alpha or lacking both PGC-1alpha and PGC-1beta	-0.87	0.87
GDS2209	Spinal cord and dorsal root ganglion	Analysis of spinal cord (SC) and dorsal root ganglion (DRG). The central nervous system (CNS) comprises the brain and SC; the peripheral nervous system (PNS) includes spinal and cranial nerves along with their associated DRGs	Hoxc8 was upregulated in the spinal cord (SC), but it was downregulated in the dorsal root ganglion (DRG).	-0.83	-0.9
GDS2699	Mesenchymal and epithelial compartments of the developing intestine	Analysis of the mesenchymal and epithelial fractions of the embryonic small intestine. Crosstalk between the epithelium and the underlying mesenchyme is required for intestinal development	Hoxc8 was upregulated in mesenchymal cells compared to the epithelial fractions of the embryonic small intestine.	0.9	0.99
GDS2044	DNA demethylation effect on dermal fibroblasts	Analysis of primary dermal fibroblasts treated with the DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine (Aza-dC) for 96 hours	Hoxc8 was downregulated in primary dermal fibroblasts treated with the DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine (Aza-dC).	-0.17	0.97
GDS2421	Fibroblast growth factor 2 effect on embryonic fibroblast: dose response	Analysis of embryonic fibroblasts after treatment with fibroblast growth factor 2 (FGF2) at 4, 13, and 40 ng/ml for 24 hours. FGF2 promotes the self-renewal of embryonic stem cells (ESCs)	Hoxc8 was downregulated after treatment with fibroblast growth factor 2 (FGF2). FGF2 promotes the self-renewal of embryonic stem cells (ESCs)	-0.98	0.99

pared to dorsal root ganglion (GDS2209), mesenchymal cells compared to epithelial cells (GDS2699), and in skin fibroblasts among fetal corneal, skin, and tendon fibroblasts (GDS1500). Next, we examined the datasets in which the known *Hoxc8* target genes *Opn* and *Fzd2* were concordantly expressed with *Hoxc8* (Table 1). Representative data from the GDS2843 dataset, which originated from the developing mammary gland, are shown in Fig. 1. Therein, the gene expression levels of *Opn* and *Fzd2* were negatively and positively correlated with that of *Hoxc8,* respectively. In most datasets, the expression patterns of *Fzd2* were highly correlated with *Hoxc8* (Table 1) except in the GDS1500 and GDS2209 datasets.

Enriched functions of *Hoxc8* coexpressed genes

To search for genes coexpressed with *Hoxc8* in each dataset, we assessed the similarity of gene expression patterns between each gene and *Hoxc8* using Pearson's correlation coefficient (r-value > 0.75) and t-test (p-value < 0.001). A total of 567 genes that had r-values greater than 0.75 in at least four of eight datasets were selected (Fig. 2). Among these, 23 genes were found to have r-values greater than 0.75 in six of the eight datasets (Table 2). Among these 23 genes, Ncam1 and Fzd2

Table 2. Genes most frequently coexpressed with Hoxc8

Gene symbol	Name
Nfic	Nuclear factor I/C
Tgfbi	Transforming growth factor, beta induced
Anp32a	Acidic (leucine-rich) nuclear phosphoprotein 32 family, member A
Thbs2	Thrombospondin 2
Fzd2	Frizzled homolog 2 (Drosophila)
Ncam1	Neural cell adhesion molecule 1
Ldhb	Lactate dehydrogenase B; predicted gene 5514
Sparc	Secreted acidic cysteine rich glycoprotein; similar to Secreted acidic cysteine rich glycoprotein
Fbln1	Fibulin 1
Ift81	Intraflagellar transport 81 homolog (Chlamydomonas)
Timp3	Tissue inhibitor of metalloproteinase 3
P4ha2	Procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), alpha II polypeptide
Plat	Plasminogen activator, tissue
Tmem45a	Transmembrane protein 45a
Mylk	Myosin, light polypeptide kinase
Fyn	Fyn proto-oncogene
Clasp1	CLIP associating protein 1
Fbxo21	F-box protein 21
Pltp	Phospholipid transfer protein
Islr	Immunoglobulin superfamily containing leucine-rich repeat
Meis1	Meis homeobox 1
Sspn	Sarcospan
Cugbp2	CUG triplet repeat, RNA-binding protein 2

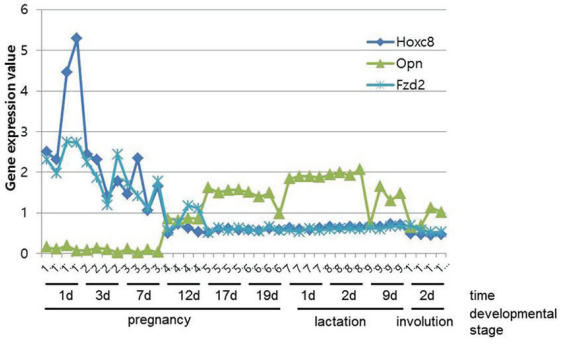

Fig. 1. Correlation of *Hoxc8* expression with expression of known downstream genes, *Opn* and *Fzd2*, during mammary gland development. Gene expression levels of *Hoxc8*, *Opn,* and *Fzd2* at each time point were obtained from the GEO database (GDS2843). For each time point, there were four biological replicates of the microarray experiments. *Hoxc8* expression gradually decreased until pregnancy day 7 and was maintained at low levels throughout lactation and involution. *Fzd2* exhibited similar behavior to *Hoxc8*. In contrast, *Opn*, which is negatively regulated by Hoxc8, was completely repressed until pregnancy day 7 and was then induced and maintained after 12 days of pregnancy.

are already known to be Hoxc8 target genes, and Meis1 is a known Hox co-factor. As the most of the known target genes are associated with ECM or cell adhesion, many of the identified genes were found to be associated with ECM (Isir, immunoglobulin superfamily containing leucine-rich repeat; Timp3; Sparc; Fbln1; Tgfbi**,** transforming growth factor beta-induced, 68kDa) or cell adhesion (Thbs2, thrombospondin 2; Fyn; Mylk, myosin light chain kinase).

To gain insight into the functional characteristics of *Hoxc8* coexpressed genes, we performed gene set analysis for 567 genes, using the DAVID program. Genes associated with the ECM were most significantly enriched (Fig. 2 and Supplementary Table S2). In addition to the 23 genes mentioned above, collagen family proteins (Col27a1, Col3a1, Col1a2, Col1a1, Lox, Col11a1, Col5a2, Col5a1, and Col4a5) and proteolysis metallopeptidases (Adam19, Adam9, and Adamts4, -5, and -12) were highly enriched. These genes are closely connected with the Hoxc8 subnetwork (Supplementary Fig. S1). Genes associated with the development of the skeletal system were also highly enriched. Of note, these included six other Hox genes: Hoxa5, Hoxa7, Hoxc6, Hoxc9, Hoxd8, and Hoxd9. These genes are neighboring genes localized next to Hoxc8 and its paralogues located in other clusters, with the exception of the Hoxb cluster. We also performed gene set analysis for genes that were negatively correlated with Hoxc8 at least four times in eight datasets (Supplementary Table S3). Most significantly, the enriched gene set was associated with the generation of precursor metabolites and energy, especially the TCA cycle and localization in the mitochondria.

Interestingly, 17.4% of the coexpressed genes were transcriptional regulators, such as transcription factors involved in a

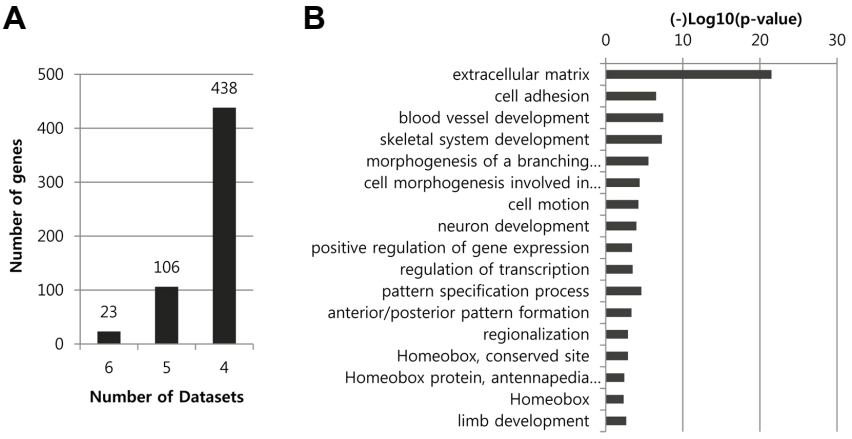

Fig. 2. Gene sets significantly enriched in *Hoxc8* coexpressed genes. (A) Number of genes that were repeatedly coexpressed with *Hoxc8* in at least four of eight datasets. (B) Gene set analysis was performed using the DAVID program. Several gene sets containing *Hoxc8* coexpressed genes, including those for extracellular matrix and cell adhesion, were significantly enriched.

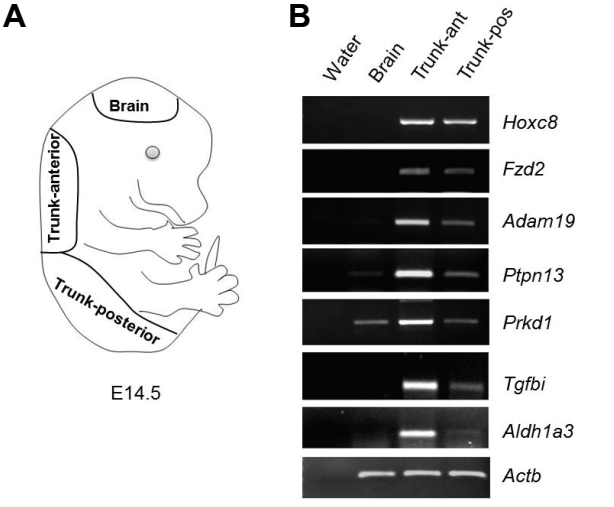

Fig. 3. Endogenous expression patterns of *Hoxc8* and its coexpressed genes in mouse embryonic tissues. (A) E14.5 mouse embryos indicating the location of the brain, trunk-anterior, and trunk-posterior tissues used for RT-PCR analysis. (B) Semi-quantitative RT-PCR was performed for *Hoxc8* and the 5 selected genes. *Fzd2* served as a positive control for *Hoxc8* expression, and *Actb* was used as a loading control. "Trunk-ant" and "trunk-pos" indicate the "trunk anterior" and "trunk posterior" regions, respectively. "Water" represents a negative control reactions containing water instead of template DNA.

pattern formation, Hox cofactors, and chromatin remodeling factors. In addition to Meis1, other well-known Hox cofactors, including Meis2, Pbx1, and Pbx3, belonging to the TALE (Three Amino acid Loop Extension) family, were concordantly expressed in four datasets. Zhx1, Prrx1, Zeb2, Pknox1, and Meox2 also have a homeobox domain, as do members of the TALE family. Zhx1 and Prrx1 are members of the zinc-finger and homeobox protein families, respectively, both of which act as repressors. However, whether they function as Hox cofactors is unknown. Although the tissues or cells used in the gene expression datasets are heterogeneous, gene set analysis of the coexpressed genes seemed to summarize Hoxc8 phenotypes.

Selected genes were endogenously coexpressed with *Hoxc8*

To assess the possibility that the 567 selected genes are coexpressed with *Hoxc8*, we selected five genes and compared their expression patterns with that of *Hoxc8* in mouse embryos and cell lines. We selected the five following genes showing different coexpression frequencies against *Hoxc8*: *Tgfbi* (transforming growth factor, beta induced), *Ptpn13* (protein tyrosine phosphatase non-receptor type 13), *Prkd1* (protein kinase D1), *Adam19* (a disintegrin and metallopeptidase domain 19), and *Aldh1a3* (aldehyde dehydrogenase 1a3). *Tgfbi* was the most frequently coexpressed (6 of 8 datasets) gene, while *Aldh1a3* was coexpressed in only three out of eight datasets. *Prkd1* and *Ptpn13* were coexpressed in five, and *Adam19* was coexpressed in four of the eight datasets (Supplementary Table S4). These proteins have been implicated in various biological systems, such as ECM and adhesion (*Tgfbi and Adam19*), phosphate metabolic processes (*Ptpn13 and Prkd1*), and retinoic acid biosynthetic processes (*Aldh1a3*).

First, the endogenous expression patterns of these five genes were analyzed in mouse embryos (Fig. 3). E14.5 embryos were dissected into three parts, brain, trunk anterior, and trunk posterior tissues, and then semi-quantitative RT-PCR was performed after isolating total RNA from each tissue. In agreement with previous reports (Kwon et al., 2005; Min et al., 2012; 2013), *Hoxc8* was strongly expressed in the trunk anterior and moderately expressed in the trunk posterior; it was not expressed in the brain (Fig. 3). All five of the examined genes, along with *Fzd2* as a control, exhibited similar expression patterns to those of *Hoxc8*, although *Prkd1* and *Ptpn13* were also weakly expressed in brain tissue (Fig. 3).

Since *Hoxc8* has been reported to be induced in preosteoblastic cells through the TGF-β signaling pathway (Li et al., 2006; Yang et al., 2000), we tested whether the putative target genes are also concordantly induced along with *Hoxc8* in the MC3T3-E1 preosteoblastic cell line. After treating the cells with TGF-β2, they were harvested at multiple time points (0 h, 6 h, and 12 h), RNA was extracted, and semi-quantitative RT-PCR was performed. As expected, expression of *Hoxc8 gradually* increased following TGF-β2 treatment (Fig. 4). Similarly, four genes of interest, *Adam19, Ptpn13, Prkd1,* and *Tgfbi,* were also induced (Fig. 4), although *Aldh1a3* was not detected at any time points (data not shown), probably due to gene silencing in MC3T3-E1 cells. Expression of the selected genes, except

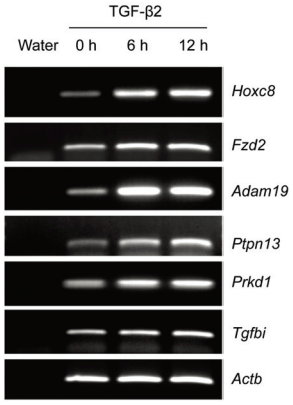

Fig. 4. TGF-β2 induces *Hoxc8* and its putative target genes. MC3T3-E1 cells were treated with TGF-β2 (10 ng/ml), and total RNA was isolated at the indicated time points (0-12 h). Semi-quantitative RT-PCR was then performed to determine the expression patterns of *Hoxc8* and its putative target genes. *Fzd2* and *Actb* were also analyzed as a positive control for *Hoxc8* downstream target gene expression and as an internal control, respectively. "Water" represents a negative control reactions containing water instead of template DNA.

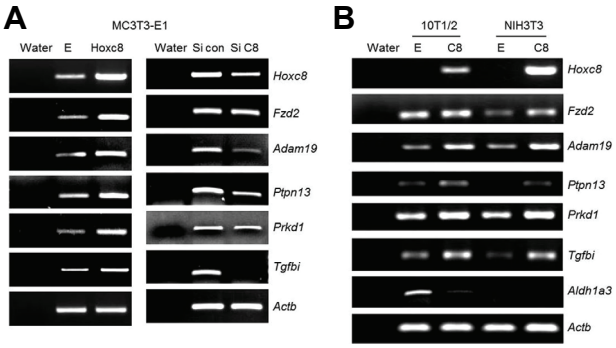

Fig. 5. The effects of *Hoxc8* overexpression or knockdown on putative target gene expression. (A) MC3T3-E1 cells were transfected with either empty (pcDNA3.1; E) or *Hoxc8* expression vector (pcDNA3.1-Hoxc8; Hoxc8) and harvested 48 h after transfection (left panel). Total RNA was isolated, and semi-quantitative RT-PCR was performed with the specific primers described in Supplementary Table 1. MC3T3-E1 cells were transfected with control (con) or *Hoxc8* siRNA (si C8) and harvested 60 h after transfection (right panel). *Fzd2* and *Actb* were analyzed as controls. (B) C3H10T1/2 (10T1/2) and NIH3T3 cells were transfected with either empty (pcDNA3.1; E) or *Hoxc8* expression vector (C8) and cultured in the selection media containing 500 μg/ml G418. Total RNA was isolated from stably transfected cell lines, and semi-quantitative RT-PCR was performed. *Fzd2* and *Actb* were analyzed as a positive control for *Hoxc8* downstream target gene expression and as an internal control, respectively. "Water" represents a negative control reactions containing water instead of template DNA.

Aldh1a3, was confirmed to be positively correlated with *Hoxc8* in mouse embryonic tissues and cell lines, in which the TGF-β signaling pathway was activated.

Hoxc8 positively regulates expression of the selected genes *in vitro*

To further confirm the correlation between *Hoxc8* and expression of the selected genes, overexpression and knockdown conditions of *Hoxc8* were generated in MC3T3-E1 preosteoblastic cells using a *Hoxc8* expression vector (pCDNA3.1-Hoxc8) and siRNA against *Hoxc8,* respectively. When *Hoxc8* was transiently overexpressed through introduction of the Hoxc8 expression vector into MC3T3-E1 cells (Fig. 5A), the expressions of *Adam19, Ptpn13, Prkd1*, and *Tgfbi* were concordantly increased. Likewise, the expression levels of the four genes decreased when Hoxc8 was knocked down (Fig. 5A). We also stably transfected the Hoxc8 expression vector into C3H10T1/2 and NIH3T3 cell lines, which lack any endogenous expression of Hoxc8. The basal expression levels of *Adam19, Ptpn13, Prkd1*, and *Tgfbi* were increased in the Hoxc8-expressing cell lines (Fig. 5B). Exceptionally, *Aldh1a3* was downregulated upon overexpression of Hoxc8 in C3H10T1/2 cells.

DISCUSSION

Microarray technology, in combination with chromatin immunoprecipitation (ChIP), has greatly enhanced the discovery of transcription factor target genes (Lei et al., 2005; 2006). Nonetheless, identification of Hox target genes, including Hoxc8, has yet to reach a point that would facilitate delineating their individual roles in specifying the identity of body segments, cell differentiation, migration, and proliferation (Hueber and Lohmann, 2008). Low DNA binding sequence specificity, context-dependent activation or repression, and unknown cofactors present extensive challenges for identification of Hox target genes. These potential pitfalls spurred us to undertake alternative methods for identifying Hox target genes. Genes that show similar expression patterns in multiple independent microarray datasets are considered to be highly functionally correlated (Lee et al., 2004; Price and Rieffel, 2004). Therefore, we hypothesized that genes coexpressed with *Hoxc8* might suggest plausible roles for Hoxc8, and that they might include cofactors or downstream target genes. In this study, 567 genes were found to be coexpressed with *Hoxc8* in at least four out of eight datasets; 23 genes were determined to be coexpressed in six datasets. Among these, genes associated with ECM and cell adhesion were most significantly enriched (Fig. 2 and Supplementary Table S2), irrespective of the tissue types studied. Since the functions of known Hoxc8 target genes are biased toward cell adhesion, it is not surprising that most significantly enriched genes are associated with ECM or cell adhesion. During vertebrate development, Hox genes are expressed during gastrulation, when epithelial mesenchymal transition is initiated at the midline of the embryos, the so-called primitive streak, in order to produce a new class of cells (mesodermal cells) between the epiblasts and hypoblasts. Eventually, *Hox* genes regulate morphogenesis, during which ECM and cell adhesion molecules play critical roles. Consequently, it is possible that ECM and/or cell adhesion molecules could be major targets of Hox proteins. Among the ECM genes and/or cell adhesion molecules identified (Supplementary Table S2), half of them are ECM structural constituents, like collagen, fibrillin, fibulin, and laminin. Interactions between ECM and cell adhesion molecules regulate cell migration and differentiation in cooperation with growth factors and hormones (Daley and Yamada, 2013). Enrichment of ECM genes has been reported as a significant feature of each dataset analyzed (LeDouxe et

al., 2006; Li et al., 2007; Timmons et al., 2007). For example, *Hoxc8* was overexpressed in white adipocytes compared to brown adipocytes (GDS2743) and in intestinal mesenchymal cells compared to epithelial cells (GDS2699). The transcriptomes of white adipocytes and mesenchymal cells were specifically enriched with ECM genes (Li et al., 2007; Timmons et al., 2007). Therefore, direct or indirect regulation of ECM genes might be a good signature of Hoxc8 activation.

In addition to ECM genes, significant enrichment of transcriptional regulators is also noteworthy. These genes include known Hox cofactors, transcription factors, and chromatin remodeling factors (Supplementary Table S2). The known Hox cofactors, such as the Pbx (Pbx1 and Pbx3) and Meis (Meis1 and Meis2) classes of TALE (Three Amino acid Loop Extension) homeodomain proteins (Moens and Selleri, 2006) suggest that Hoxc8 transactivates downstream target genes by interacting with these cofactors, as other Hox proteins do to overcome poor sequence specificity. Previously, our group identified Pcna, which harbors both Pbx1 and Hoxc8 binding sites in the promoter region, as a Hoxc8 target gene (Min et al., 2010). Other interesting genes analyzed here are those with homeodomains, such as Zhx1 and Prrx1, which function as transcriptional repressors. Zhx1 was found to interact with DNA methyltransferase (DNMT) 3B (Kim et al., 2007). Interestingly, Prrx1$^{-/-}$ mice showed limb bud and skeletal losses (Mann, 1995), and adipogenesis was inhibited (Du et al., 2013). Given that mutations in cofactors result in similar loss-of-function phenotypes of *Hox* (Moens and Selleri, 2006), Prrx1 might interact with Hoxc8 as a cofactor to repress genes during morphogenesis and adipogenesis. We also identified many other transcription factors that play important roles in embryonic development as Hoxc8 companion genes. This finding was not observed in a previous *in vitro* cell line experiment (Lei et al., 2006). However, about 50% of known Hox target genes have been reported to be transcription factors in *Drosophila* (Hueber and Lohmann, 2008). Thus, it is possible that Hoxc8 transactivates or represses other transcription factors, which in turn amplify signaling and construct Hoxc8-mediated regulatory networks.

To validate the genes analyzed *in silico*, five genes (*Tgfbi, Ptpn13, Prkd1, Adam19,* and *Aldh1a3*) among the 567 were selected at random, and their concordant expression patterns were analyzed with *Hoxc8*. We confirmed that their expression levels were positively correlated with that of *Hoxc8 in vivo* in embryonic tissues and *in vitro* in cell lines. As an *in vitro* model, we used a TGF-β-induced system in MC3T3-E1 cells and overexpression/ knockdown in different cell lines. Therein, we showed that four of the selected genes (*Tgfbi, Ptpn13, Prkd1, Adam19)*, except *Aldh1a3*, are coexpressed with *Hoxc8* and positively regulated by Hoxc8. This allowed us to make sure that *Aldh1a3* is, as we expected (coexpressed with *Hoxc8* in 3 out of 8 datasets), less relevant to *Hoxc8* network, whereas the other four genes, which showed coexpression in at least four of the eight datasets, sufficiently met the requirements to support our *in silico* methods. Evolutionally conserved Hoxc8 binding sites were found to be present in the promoter regions of the four genes (Supplementary Table S5). Practically, enrichment of Hoxc8 on Hoxc8 binding sites in *Adam19* and *Ptpn13* was confirmed by ChIP assays (Supplementary Fig. S2), suggesting that the list of genes reported by us include direct target genes. Based on the results, we assume that these genes can be directly or indirectly regulated by Hoxc8 and functionally linked. Especially, Adam19 and Tgfbi, whose functions are associated with ECM and cell adhesion, are likely to be Hoxc8 target genes. Adam19 is a membrane-anchored glycoprotein that plays key roles in embryo implantation, neurogenesis, cardiovascular morphogenesis, and the release of proteins, such as epidermal growth factor receptor ligands and osteoprotegerin ligand, a protein important in osteoclast differentiation and mammary gland development (Fata et al., 2000; Kong et al.,1999; Qi et al., 2009). Tgfbi is a secreted protein that is induced by treatment with transforming growth factor-β and inhibits cell attachment (Skonier et al., 1994). It is strongly expressed in the mesenchyme of numerous collagen-rich tissues throughout all stages of murine development (Ferguson et al., 2003; Schorderet et al., 2000), implicating its role in tissue morphogenesis. Together, our collective results suggest that the listed Hoxc8 companion genes are a worthy resource for exploration of Hoxc8 target genes and regulatory networks.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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