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Comparison of Expression Profiles of HOX Gene Family in Human Embryonic Stem Cells and Selected Human Fetal Tissues

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Abstract The HOX genes coding homeodomain proteins have been suggested as a candidate molecular switch that determines the fates of cells during embryonic development and patterning. It is believed that a set of differentiation-specific HOX genes enter into a turn-on state during tissue differentiation, in contrast to stem cell-specific HOX genes that enter into a turn-off state. However, comprehensive data of expression profiles of HOX genes in human embryonic stem cells (hESC) and differentiated embryonic tissues are not available. In this study, we investigated the expression patterns of all 39 HOX genes in hESC and human fetal tissues and analyzed the relationships between hESC and each tissue. Of the 39 genes, 18 HOX genes were expressed in stem cells, and diverse expression patterning was observed in human fetal tissues when compared with stem cells. These results indicate that HOX genes could be main targets for switching of stem cell differentiation into tissues.

Key words: HOX genes, expression patterns, human embryonic stem cells, fetal tissue

Human embryonic stem cells (hESC) are of great interest, because of their pluripotency to differentiate into many kinds of somatic cell types, which can substitute degenerated cells of the adult organism [12]. The process requires highly coordinated interactions of proper growth factors with intrinsic key factors including specific transcriptional modulators [9, 19]. Conceptually, it is assumed that the phenotypic setting of a certain undifferentiated cell to a specific differentiated cell type requires the turning-on or turning-off of a series of genes by regulatory molecular switches.

Homeoproteins, products of homeobox genes, are known to have functions of transcriptional regulators and mediate sequence-specific interactions with DNA elements [13]. In the human genome, at least 200 homeobox genes, including 39 HOX genes (*HOXA*, *HOXB*, *HOXC*, *HOXD*), have been identified and known to regulate developmental processes [15]. Although relatively few examples were identified, the homeoproteins have been suggested as a candidate molecular switch to determine the fates of cells by transcriptional modulation of target genes during embryonic development and patterning, because of their transcriptional modulator action [3]. However, recent studies on HOX gene expression profiles have been limited to embryonal carcinoma cells or certain human diseases, such as lung tissue with primary pulmonary hypertension, leukemias, or breast cancer [2, 17]. Consequently, little is known about the changes of expression patterns of HOX genes in hESC and developing human tissue. In this study, we investigated the expression profiles of the entire 39 HOX genes in hESC and eight types of human fetal tissue, including kidney, liver, small intestine, skin, lung, spleen, heart, and pancreas, and identified HOX genes that are either tissue-specific or stem cell-specific.

Materials and Methods

Stem Cell Culture

Human embryonic stem cells (SNU-hES3) were obtained from the Stem Cell Research Center at Seoul National University, Seoul, Korea. The cells were cultured on mitomycin C mitotically inactivated STO mouse embryonic fibroblast feeder layer (CRL-1503, ATCC, USA; used at 40,000 cells/cm^2) in 0.1% gelatin-coated tissue culture dishes, using Dulbecco's modified Eagle's medium F-12 (DMEM/F-12, with L-glutamine, 2.438 g/l sodium bicarbonate,

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pyridoxine hydrochloride; GIBCO, N.Y., U.S.A.) supplemented with 20% serum replacement (Knockout^{TM}SR; GIBCO, N.Y., U.S.A.), 0.1 mM β-mercaptoethanol, 1% nonessential amino acids, 50 units/ml penicillin, 50 μg/ml streptomycin (GIBCO), and basic fibroblast growth factor (bFGF, Invitrogen, CA, U.S.A.). The medium was changed every day. Six to eight days after plating, human stem cell colonies were mechanically removed by a glass micropipette from differentiated cell outgrowths and replated on fresh feeder layer. The stem cells were characterized by a panel of antibodies including alkaline phosphtase, SSEA-1, SSEA-3, SSEA-4, Tra-1-60, and Tra-1-81 (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.).

Tissue Preparation

A terminated fetus was donated by a patient who had taken heavy medications. Informed consent was obtained from the patient, and the study was performed after approval from the ethical committee at Hanyang University. The fetal tissues were immediately frozen during autopsy examination. For culture, parts of the fetal tissue were mechanically disaggregated, and then incubated in 0.05% trypsin-0.5 mM EDTA (GIBCO) or 0.25% trypsin at 37°C for 5–10 min, or incubated in a solution containing 0.01% hyaluronidase type V (Sigma, St. Louis, MO, U.S.A.), 0.1% collagenase type IV (Sigma), and 0.002% DNase type I (Sigma) at 37°C for 2 h. Each obtained cell was cultured and frozen until experiment.

Total RNA Isolation and Reverse Transcription (RT)

Frozen tissue or cultured fetal cells were homogenized in the Trizol reagent (Invitrogen, Carlsbad, CA, U.S.A.). Total RNA was isolated from each tissue and stem cells according to the manufacturer's instructions. cDNA was synthesized from 10 μg of total RNA using oligo (dT) primer with StrataScript^{TM} Reverse Transcriptase (Stratagene, La Jolla, CA, U.S.A.) in a total volume of 50 ml. The cDNA samples were aliquoted to 10 ml and stored at -20°C for the use of PCR amplification.

Sequences of Primers and Polymerase Chain Reaction (PCR)

To avoid amplification of genomic DNA of the *HOX* genes, we designed primer sets flanking intron(s) (Table 1). To test cDNA integrity, the GAPDH gene was amplified for each sample. The reaction mixture of polymerase chain reaction (PCR) contained 4 μl each of cDNA sample and 46 μl of a mixture containing $MgCl_2$ (2.5 mM), 10× buffer, 250 nM dNTPs (Promega, Madison, WI, U.S.A.), Taq polymerase (Promega), and 0.2 pmole each of both upstream and downstream PCR primers. Conditions for PCR were 94°C for 5 min, 40 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 5 min. PCR products were resolved by 1.5% agarose gel electrophoresis and stained with ethidium bromide. Densitometric analysis was performed with the Fluor-S^{TM} MultiImager using the Quantity One® program (Bio-RAD, Hercules, CA, U.S.A.).

RESULTS AND DISCUSSION

Expression of *HOX* genes during embryonic development has been known to occur temporally in accordance with both the position of a gene within its cluster and in a rostral-to-caudal manner [6]. Although there is functional overlap for even nonparalogous *HOX* genes, and *HOX* proteins mostly bind to the same set of target genes [7, 11], their functional effects seem to be distinct through their individual activation/repression domains or through interactions with different cofactors [1, 14]. To analyze the expression profile of *HOX* genes in hESC and a series of fetal tissue, we cultured SNU-hES3 cells on STO cells for 89 passages and characterized them by immnunocytochemically staining typical markers of hESC. As shown in Fig. 1, the hESC expressed cell surface

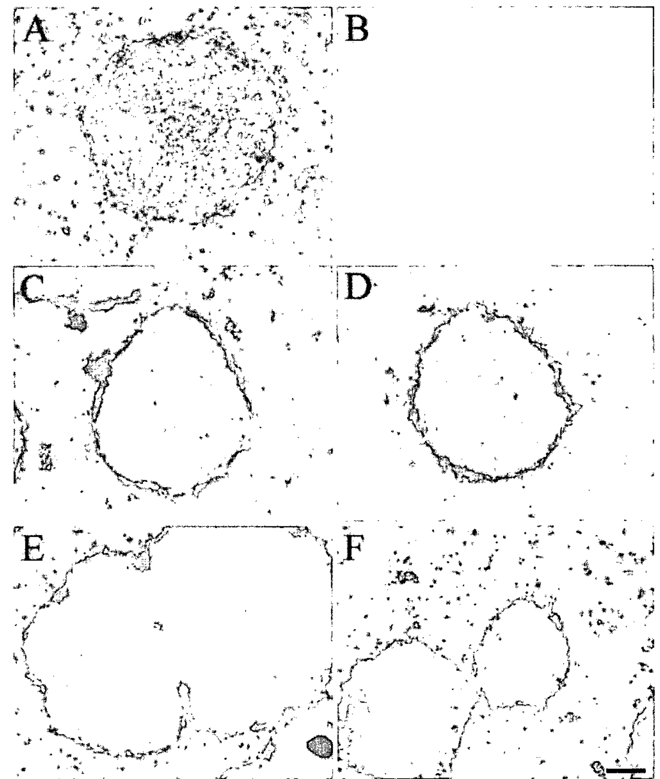

Fig. 1. Characterization of human embryonic stem cells.
SNU hES3 cells expressed alkaline phosphatase activity (A), and stage-specific surface markers of hESC and human EC cells were detected. SSEA-3 (C) was partially positive. SSEA-4 (D), Tra-1-60 (E), and Tra-1-81 (F) were strongly positive. SSEA-1 (B) was negative. (Scale bar: 100 μm)

Table 1. Sequence of PCR primers.

Gene	Sense primer	Antisense primer	GenBank Accession no.
HOXA1	TGGCTACGTATAATAACTCC	CAGATCTTCACTTGGGTCTC	NM_005522
HOXA2	AAAACCGCACTTCTGCCGGC	CCTTGCACTGGGTCTGCCTC	NM_006735
HOXA3	CTCAGAATGCCAGCAACAAC	CAGATTGGCCATCTCCACCC	NM_153631
HOXA4	GAAGAAGATCCATGTCAGCG	AGTTTGTGCTTTCCCTGGTG	NM_002141
HOXA5	AGCACCCACATCAGCAGCAG	CAATCCTCCTTCTGCGGGTC	NM_019102
HOXA6	GACTACCTGCACTTTTCTCC	GGAACCAGATCTTGATCTGG	NM_024014
HOXA7	CCTCCTACGACCAAAACATC	GCTCTTTCTTCCACTTCATG	AF032095
HOXA9	CAGTTGAAATCGTCTCCAGGTA	GCGCGCATGAAGCCAGTTGG	
HOXA10	TGGCTACTTC CGCCTTTCTC	CAGTTGGCTG CGTTTTCACC	AF040714
HOXA11	GGCGCTTCTCTTTGTTAATG	CGGCAGCAGAGGAGAAAGAG	AF071164
HOXA13	TCCCATGGAAAGCTACCAGC	TTCTGGTCGATTTCTCACCC	U82827
HOXB1	AACACCCTGCCCTTCAGAAC	GCGCTTCTTCTGCTTCATTC	NM_002144
HOXB2	AGAGACCCAGGAGCCAAAAG	ACCTGCCTTTCGGTGAGGTC	NM_002145
HOXB3	AGTGCCACTAGCAACAGCAG	AACTCCTTCTCCAGCTCCAC	NM_002146
HOXB4	CTGGATGCGCAAAGTTCACG	AGCGGATCTTGGTGTTGGGC	NM_024015
HOXB5	TTTCACCGAAATAGACGAGG	GAACCAGATCTTGATCTGGC	NM_002147
HOXB6	CCTTCTACCGCGAGAAAGAG	TCTTGATCTGCCTCTCCGTC	NM_018952
HOXB7	AGCCGAGTTCCTTCAACATG	TCTGTCTTTCCGTGAGGCAG	NM_004502
HOXB8	CGGCAATTTCTACGGCTACG	TCCACTTCATCCTCCGGTTC	NM_024016
HOXB9	GTACAGTTTGGAAACTTCGG	CATCCGCCGGTTCTGAAACC	NM_024017
HOXB13	TGGACGTGTCTGTGGTGCAG	GCCTCTTGTCCTTGGTGATG	NM_006361
HOXC4	AACCCATAGTCTACCCATGG	TAATGTCCTCTGCCCGTTGC	NT_009458
HOXC5	ACGAAGCGGCTCCTCTGAAC	GAGTGAGGTAGCGGTTAAAG	NT_009458
HOXC6	TGCAGACAAAACACCTTAGG	GAACCAGATTTGATCTGTC	NM_004503
HOXC8	ACCACGTTCAAGACTTCTTC	TGTAAGTTTGCCGTCCACTG	NM_022658
HOXC9	AGCGATTTTCCGTCCTGTAG	CGTACATGTAGTCCGGGTAG	XM_028620
HOXC10	AGCGATTTTCCGTCCTGTAG	GGCGATTCCAGATGTTCGGC	XM_028621
HOXC11	GAGTTCTCCACGGTCTCCTC	GTTGACTGAGGAGTAGAAGC	XM_012215
HOXC12	GAGTTCTCCACGGTCTCCTC	ACTAAGATTCAAGCGGTCTG	NT_009458
HOXC13	TCAGGTGTACTGCTCCAAGG	ATGAGGCGCTTTCGATTTGC	NT_009458
HOXD1	TTGTCTCAAAGCGTCAGCCG	GCAGTTGGCTATCTCGATGC	AF202118
HOXD3	TCTTCACCCACCAATCCTGG	CCGTGAGATTCAGCAGGTTG	NM_006898
HOXD4	AAGAAGGTGCACGTGAATTC	CATCGGCTGTAAATGCTGGC	NM_014621
HOXD8	ATATTGGCGAGGACCCAGAC	CTGTCTTCCTCCAGCTCTTG	NM_019558
HOXD9	CTCGTGCAACTCGTTCCTGC	AGGTTGAGAATCCTGGCCAC	NM_014213
HOXD10	CCACCAAAGTCTCCCAGGTG	TGCCTGTCGGTGAGGTTAAC	NM_002148
HOXD11	GCCTCCAACTTCTACAGCGC	TTGAGCATCCGAGAGAGTTG	NM_021192
HOXD12	TGCAGTGGCTGCTCTCAAAG	CGTTGACGAGGAATTCGTTC	NM_021193
HOXD13	TTCTACCAGGGCTATACGAG	TCCAGTTCTTTAAGCTGCAG	NM_000523
GAPDH	CAACTACATGTTTACATGTTC	GTCCTTCCACGATACCAAAG	BC029618

markers, including stage-specific embryonic antigen (SSEA)-3, SSEA-4, Tra-1-60, Tra-1-81, and alkaline phosphatase, but it was negative for SSEA-1.

Specific oligonucleotides corresponding to each of 39 *HOX* genes (*HOXA*, *HOXB*, *HOXC*, and *HOXD* cluster) were designed for use in RT-PCR (Table 1). To obtain an independent experimental result, we additionally screened succeeding cells after transfer from the original stem cells. Considering that most of the organs are derived from stem cells, several organs from human fetus (pancreas, heart, kidney, liver, small intestine, skin, lung, and spleen) were analyzed and compared with hESC. Figure 2 shows the expression profiles of the *HOX* genes in SNU-hES3 and fetal kidney tissue samples. Of the 39 genes tested, 18 *HOX* genes were expressed in hESC, 5 genes (*HOXA1*, *HOXA3*, *HOXA4*, *HOXA7*, and *HOXA10*) in cluster A, 4 genes (*HOXB1*, *HOXB3*, *HOXB7*, and *HOXB13*) in cluster B, 6 genes (*HOXC4*, *HOXC6*, *HOXC8*, *HOXC10*, *HOXC11*, and *HOXC13*) in cluster C, and 3 genes (*HOXD1*, *HOXD10*, and *HOXD13*) in cluster D. Nineteen *HOX* genes were expressed in fetal kidney tissue. Interestingly, 8 genes (*HOXA2*, *HOXA6*, *HOXA11*, *HOXB4*, *HOXB9*, *HOXC5*,

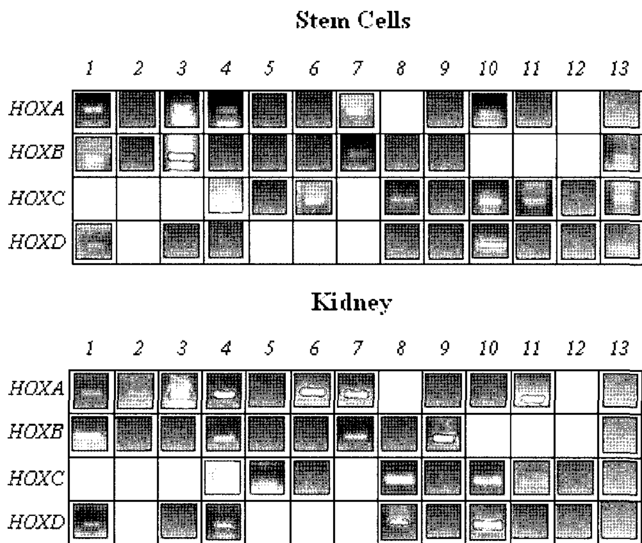

Fig. 2. Expression patterns of HOX genes in human embryonic stem cells and fetal kidney.

The level of expression was qualitatively assessed by RT-PCR technique, as described in Materials and Methods. The alphabetical order denotes 3' to 5' orientation in the HOX clusters.

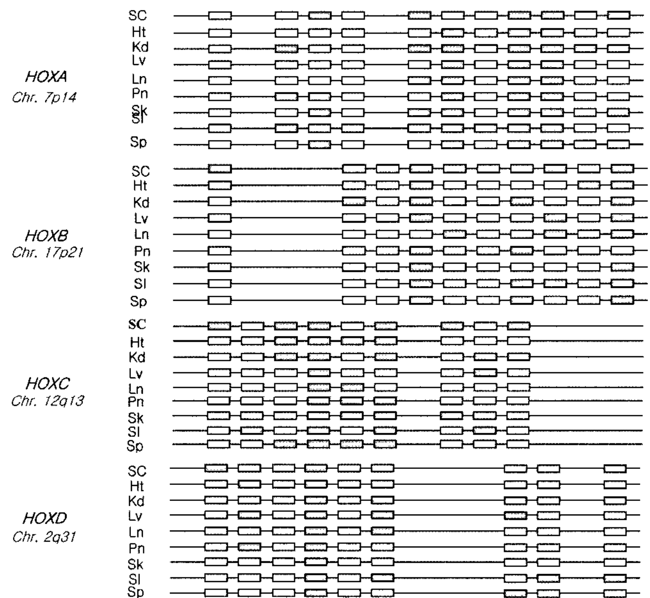

Fig. 3. Expression pattern of 39 HOX genes in human embryonic stem cell (SC), heart (Ht), kidney (Kd), liver (Lv), lung (Ln), pancreas (Pn), skin (Sk), small intestine (SI), and spleen (Sp).

Numbers denote gene numbers organized in the chromosome from a 5' to 3' direction.

HOXD4, and *HOXD8*) were not expressed in hESC, but they were newly detected in kidney tissue. On the contrary, 6 genes (*HOXA10*, *HOXB3*, *HOXB13*, *HOXC6*, *HOXC13*, and *HOXD13*) expressed in hESC were not found in kidney. The expression profile of *HOX* genes in the fetal kidney is largely similar to previous Northern blot data [4, 5]. The expression of *HOXB1*, *HOXC10*, and *HOXD1* in the present study were reported to be not expressed in renal tissue, and therefore, they may represent the developing nature of the fetal kidney. The results suggest that switch-on and switch-off of a series of *HOX* genes occur during renal development.

Similarly, several differences in the expression of *HOX* genes were observed between other organs and hESC (Fig. 3). In the fetal heart, 12 genes were expressed, in which 4 *HOX* genes (*HOXA6*, *HOXB2*, *HOXC9*, *HOXD12*) were newly expressed and 10 genes disappeared. In the fetal liver, lung, pancreas, skin, small intestine, and spleen, 16, 11, 12, 13, 17, and 13 *HOX* genes were respectively expressed, in which 7 genes (*HOXA2*, *HOXA6*, *HOXA11*, *HOXC5*, *HOXD4*, *HOXD8*, *HOXD12*), 4 genes (*HOXA6*, *HOXB6*, *HOXC9*, *HOXD8*), 4 genes (*HOXA6*, *HOXA11*, *HOXB4*, *HOXC9*, *HOXD12*), 2 genes (*HOXA6*, *HOXC9*), 8 genes (*HOXA5*, *HOXA6*, *HOXA11*, *HOXB4*, *HOXC5*, *HOXC12*, *HOXD3*, *HOXD8*) and 4 genes (*HOXA6*, *HOXB4*, *HOXC9*, *HOXD4*) were respectively newly expressed, and 7 to 11 *HOX* genes disappeared.

As shown in Fig. 3, among the 39 genes tested, 5 genes (*HOXA3*, *HOXA4*, *HOXB7*, *HOXC10*, and *HOXD10*) were constitutively expressed in hESC and all tissues analyzed, whereas *HOXA9*, *HOXA13*, *HOXB5*, *HOXB6*, *HOXB8*, *HOXD9*, and *HOXD11* seemed to be not expressed. To clarify whether these genes were silent in hESC and all tissues tested, we performed PCR with the same oligonucleotides as in genomic DNA, and RT-PCR with another primer set newly designed, as shown in Table 1. By using PCR for genomic DNA, we found the presence of PCR products for each gene, but newly designed primer sets were not able to detect the genes.

Interestingly, we observed that 7 genes (*HOXA5*, *HOXB2*, *HOXB9*, *HOXC12*, *HOXC13*, *HOXD3*, and *HOXD13*) were expressed specifically in only a single organ. For example, *HOXA5* was expressed only in small intestine, *HOXB2* in heart, *HOXB9* in kidney, *HOXC12* in small intestine, *HOXC13* in hESC, and *HOXD3* in small intestine, whereas *HOXD13* was expressed only in stem cells. Although the expression level of these genes was very low, except *HOXB9*, we noted that these genes might show tissue-specific nature during stem cell differentiation into each organ. On the other hand, 3 *HOX* genes (*HOXB13*, *HOXC13*, and *HOXD13*) were expressed only in stem cells, suggesting the association of these genes with hESC maintenance.

Although variety of the *HOX* gene in hESC seems to be important for differentiation, some reports suggest that all *HOX*-encoded proteins regulate identical or functionally equivalent downstream target genes; therefore, the quantity of *HOX* is more important than the quality of *HOX* [8]. Consequently, we analyzed the relative quantities of the *HOX* genes in the hESC and fetal tissues (Fig. 4).

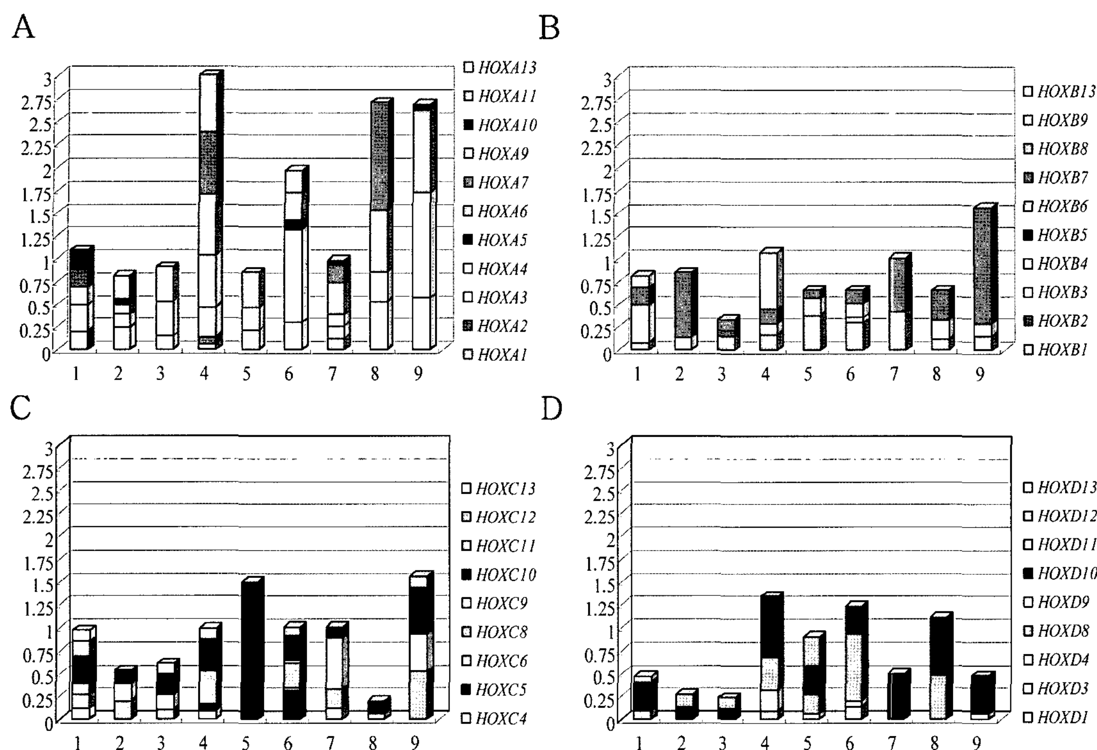

Fig. 4. Quantitative comparison of HOX gene expression in human stem cells with fetal tissues.
Arbitrary expression unit of HOX genes (**A**: HOXA; **B**: HOXB; **C**: HOXC; **D**: HOXD) was calculated by normalizing with the density of GAPDH expression volume in stem cells (**1**), pancreas (**2**), heart (**3**), kidney (**4**), liver (**5**), small intestine (**6**), skin (**7**), lung (**8**), and spleen (**9**). Each vertical axis represents an arbitrary expression unit.

Since stem cell research would provide fundamental information to understand cellular diversity and cell fate, many attempts have been made to identify genes that regulate stem cell differentiation. As a result, mainly transcription factors, such as GATA-2, Tcf4, Oct4, Mash1, LH2, and SCL/tal-1, have been found to regulate stem cell development [16, 18]. Consistent with this notion, our data suggest a possibility that *HOX* genes serve as intrinsic regulators, as transcription factors for stem cell development. Furthermore, identification of the difference in *HOX* expression patterns between hESC and fetal tissue may provide useful tools to generate specialized cell types from hESC through gene regulation.

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