IVYSPRING
INTERNATIONAL PUBLISHER

International Journal of Biological Sciences

Short Research Communication

Hoxc Gene Collinear Expression and Epigenetic Modifications Established during Embryogenesis Are Maintained until after Birth

Hyehyun Min, Ji-Yeon Lee, Myoung Hee Kim✉

Department of Anatomy, Embryology Laboratory, Brain Korea 21 project for Medical Science, Yonsei University College of Medicine, Seoul 120-752, Korea.

✉ Corresponding author: E-mail: mhkim1@yuhs.ac. Tel: +82-2-2228-1647, Fax: +82-2-365-0700.

Abstract

The *Hox* genes, which are organized into clusters on different chromosomes, are key regulators of embryonic anterior-posterior (A-P) body pattern formation and are expressed at specific times and in specific positions in developing vertebrate embryos. Previously, we have shown that histone methylation patterns are closely correlated with collinear *Hox* gene expression patterns along the A-P axis of E14.5 mouse embryos. Since histone modification is thought to play a crucial mechanistic role in the highly coordinated pattern of collinear *Hox* gene expression, we examined the maintenance of the spatial collinear expression pattern of *Hoxc* genes and the corresponding histone modifications during embryogenesis and in early postnatal mice. *Hox* expression patterns and histone modifications were analyzed by semi-quantitative RT-PCR and chromatin immuno-precipitation (ChIP)-PCR analyses, respectively. The spatiotemporal expression patterns of *Hoxc* genes in a cluster were maintained until the early postnatal stage (from E8.5 through P5). Examination of histone modifications in E14.5 and P5 tissues revealed that level of H3K27me3 is only a weak correlation with collinear *Hoxc* gene expression in the trunk regions although diminished in general, however the enrichment of H3K4me3 is strongly correlated with the gene expression in both stages. In summary, the initial spatiotemporal collinear expression pattern of *Hoxc* genes and epigenetic modifications are maintained after birth, likely contributing to the establishment of the gene expression code for position in the anatomic body axis throughout the entire life of the organism.

Key words: *Hoxc* cluster genes; collinear expression: anterior-posterior body axis; mouse embryogenesis; histone modification.

Introduction

Hox genes are transcription factors that play a pivotal role in patterning of the anterior-posterior (A-P) body axis during embryonic development. In mammals such as mouse and human, 39 *Hox* genes are organized into 4 clusters on 4 different chromosomes, with each cluster containing 9 to 11 genes [1-4]. The genomic locations of *Hox* genes within a cluster correlate with spatiotemporal expression during em-bryogenesis, a phenomenon termed collinearity [3, 5]. *Hox* genes located at the 3' end in a cluster are expressed more anteriorly and earlier in development than genes located at the 5' end of a cluster. Sequential initiation of *Hox* gene expression in a cluster is driven by dynamic regulatory events, including retinoic acid (RA) [6], FGF signaling [7], and regulatory interactions between the *Hox* and *Cdx* gene families [8]. In

the developing mouse, *Hox* gene expression is initiated in the posterior region of the primitive streak during gastrulation [9]. The ontogenesis of *Hox*-mediated axial patterning during embryogenesis has been studied for decades. However, the molecular mechanisms underlying the collinear expression of *Hox* genes are not yet fully understood.

The Polycomb group (PcG) and trithorax group (TrxG) proteins, initially discovered in *Drosophila*, have been reported to be core epigenetic regulators of homeotic genes [10-12]. Polycomb Repressive Complex (PRC2), a class of PcG proteins, and tri-methylated H3K27 (H3K27me3) are essential for long-term repression of target genes [13, 14]. In contrast, TrxG protein complexes catalyze the tri-methylation of H3K4 (H3K4me3), which is generally associated with active transcription [15].

Recently, the antagonistic function of the PcG and TrxG complexes in regulating *Hox* gene expression during mammalian development has been actively investigated [16]. *In vitro* studies revealed that histone H3 modification is correlated with the maintenance phase of *Hox* gene transcriptional regulation in embryonic stem cells (ESC) and other types of cultured cells [17-20]. Soshnikova et al. (2009) reported the *in vivo* result that the organization of H3K4me3 and H3K27me3 chromatin marks was closely associated with the temporal activation of *Hoxd* genes in the developing mouse tail [16]. Through chromatin conformation capture (3C) and chromatin immunoprecipitation (ChIP) techniques, the loss of H3K27me3 and topological changes along the *Hox* cluster were shown to be prerequisites for collinear expression, and the concomitant acquisition of H3K4me3 was shown to be more closely associated with a collinear *Hox* gene expression pattern along the A-P body axis in E14.5 mice embryos [21].

As most spatial (A-P axis) collinear expression analyses have been performed in earlier stages including the mid-gastrulae stage of mouse embryos, *Hox* expression patterns in later embryonic stages are not fully understood. In this study, we analyzed *Hoxc* gene expression patterns during several embryonic stages (from E8.5 to E18.5) through the postnatal period (P5), as well as the dynamic changes in histone modifications along the A-P axis.

Materials and methods

Animal preparation

Pregnant Hsd:ICR (CD-1) mice were purchased from a pathogen-free laboratory animal company (Koatech, Gyeonggi-do, Korea). The embryos (E8.5, E11.5, E14.5, E16.5, and E18.5) and early postnatal mice (P1, P5) were dissected in PBS-containing plates on ice. After removing the internal organs, limbs and tail bud, each embryo or postnatal mouse was divided into five parts (B, brain; T1, somite number 5 to -14; T2, somite number 15 to -27; T3, somite number 28 to -32; T4, somite number 33 to the posterior end) along the A-P axis, except E8.5 embryo, which was divided into two parts, head (H, anterior end to somite number 6) and trunk (T, somite number 7 to the posterior end) as shown in Figure 1A. Experimental procedures were approved by the Animal Care and Use Committee of Yonsei University College of Medicine, Seoul, Korea.

RNA isolation and RT-PCR

Total RNA was isolated from freshly dissected samples using Trizol (Invitrogen, Carlsbad, CA, USA). Reverse transcription (RT) was performed with 1 µg of RNA using ImProm-ll™ Reverse Transcriptase (Promega, Madison, WI, USA). A triplicate set of PCR was performed using Taq DNA polymerase (Bioneer, Daejeon, Korea). PCR amplification was performed under the following conditions: the initial denaturation for 3 minutes at 94°C, then 30 cycles of 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds. At least three independent biological replicates were analyzed. All PCR primer sequences used for detecting *Hox* gene expression and β-actin were as previously described [22].

Chromatin immunoprecipitation (ChIP)-PCR

ChIP analysis was performed following the X-ChIP protocol from Abcam (Abcam, Cambridge, UK), with minor modifications. ChIP-grade Abcam antibodies, Anti-Histone H3 (tri methyl K4) antibody (ab1012) and Anti-Histone H3 (tri methyl K27) antibody (ab6002), as well as Santa Cruz normal mouse IgG (sc-2025) were used. Embryonic samples were cross-linked with 1% formaldehyde diluted in serum-free Dulbecco's modified Eagle's medium (WelGENE Inc., Daegu, Korea) for 10 min at room temperature with gentle shaking. This reaction was stopped by adding 0.125 M glycine for 5 min, and the samples were then washed three times with PBS at room temperature. The resulting cells (approximately 4×10^7 cells) were lysed in 600 µl of sodium dodecyl sulfate (SDS) lysis buffer for 10 min on ice and sonicated with 30 sets of 10-second pulses with a Vibracell™ sonicator (VCX130, Sonics & Materials Inc., Newton, CT, USA). The sonicated chromatin (500-1000 bp), salmon sperm DNA/protein coated A/G agarose beads (Santa Cruz, CA, USA), and appropriate antibodies were mixed and incubated overnight at 4°C with gentle shaking.

The following day, antibody-ChIP DNA complexes were washed, and the DNA was eluted and

purified using a PCR purification kit (Cosmogenetech, Seoul, Korea). PCR amplification was performed under the following conditions: initial denaturation for 5 minutes at 95°C, and then 32 cycles of 95°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds. The PCR primer sequences used were as previously described [21, 23].

Results and Discussion

Maintenance of the spatial collinear expression pattern of Hox genes during embryogenesis and in early postnatal period in mice

In order to investigate whether the collinear expression patterns of *Hox* genes are maintained throughout embryogenesis as well as after birth, tissues from embryos (E8.5, E11.5, E14.5, E16.5, and E18.5) and postnatal mice (P1 and P5) were used for RT-PCR analysis after isolating RNA from each segment along the A-P axis, as described in the Materials and Methods. In general, there were no *Hoxc* genes expressed in brain tissues in any of the developmental stages examined (E8.5 through P5), which was expected based on previous results [21, 24]. In trunk tissue, the spatial collinear expression patterns of *Hoxc* genes along the A-P axis were detected throughout embryogenesis, and this expression pattern was maintained until the postnatal period P5 (Fig. 1).

In E11.5 embryos, T1 (somite 5-14) and T2 (somite 15-27) tissues expressed only the 3' anterior *Hoxc* genes (*Hoxc4* to *Hoxc6* in T1, *Hoxc4* to *Hoxc10* in T2), but posterior gene expression was seen as development continued. In the T1 tissue of E14.5 embryos, expression of *Hoxc4* through *Hoxc9* was seen, and this expression pattern was retained until postnatal stage P5. In the case of T2 tissue, sequentially expanded *Hox*c genes to the 5' side were detected, up to *Hoxc11* at E14.5, *Hoxc12* at E16.5, and *Hoxc13* at E18.5 and after. This expanded expression pattern could be due to boundary elements and/or insulator-binding proteins implicated in the establishment of gene expression boundaries being driven to move to the 5' position within a locus, or the changing of the interaction/binding affinities of transcription factors. However, tissue (segment) contamination during the dissection process could be also possible. Posterior tissues, such as T3 and T4, showed expression of the entire *Hoxc* genes at stage E14.5 and maintained this pattern until P5. In early embryos such as E11.5, however, only the most posterior tissue, T4, showed expression of the entire *Hoxc* genes. Additionally, T3, which is anterior to T4, did not show expression of posterior genes; only the 3' genes *Hoxc4* to *Hoxc10* were expressed. In the case of E8.5 mouse embryos, anterior (H) and posterior (T) tissues were generated

by dissecting tissues between somite number 6 and 7. The anterior future brain region (H) did not express any *Hoxc* genes, whereas the posterior tissue (T) expressed the anterior *Hoxc* genes (*Hoxc4* to *Hoxc10*). Even though E8.5 usually has 8 -12 somites, T tissues expressed more posterior 5' *Hoxc* genes, than expected based on somite level, although very weakly, indicating that *Hox* genes seemed to be expressed prior to somite formation.

Expression levels of each *Hoxc* gene throughout development show that gene expression is relatively low at E8.5, increases to a peak between E11.5 and E14.5, and then decreases. Unusual expression of *Hoxc13*, a deviation from the collinear expression pattern of *Hox* cluster genes, was found in several tissues at different stages, as has been previously reported [23, 25, 26]. Although the mechanisms are not known, *Hoxc13* seems to escape the mechanisms regulating the collinear expression of *Hoxc* genes.

Interestingly, *Hox* genes are not only expressed during embryogenesis, but also widely expressed in adult human organ tissues, providing regional identities via differential and collinear expression along the A-P and proximal-distal axes of the body [27]. Even though there was a small extension of *Hoxc* gene expression in each trunk region following the mid-gastrula stage (E11.5), our results indicate that the spatial collinearity of the *Hox*c gene expression pattern along the A-P body axis is established during early gastrulation and persists until after birth.

Maintenance of epigenetic modifications at Hoxc loci along the A-P axis of postnatal mice

Although there have been several reports describing the functional relationship between histone marks such as H3K27me3 and H3K4me3 with the spatial collinear expression of *Hox* genes in embryos as well as in ES cells [19, 21, 24], little information is available regarding postnatal mice. To understand whether the maintenance of the collinear *Hoxc* gene expression pattern is accompanied by histone modifications up until the postnatal stage, we analyzed epigenetic modifications along the *Hoxc* cluster in P5 mice and the E14.5 embryo.

In E14.5 embryos, high levels of the repressive marker H3K27me3 were observed over the entire *Hoxc* locus in brain tissues that do not express *Hox*. However, the level of H3K27me3 was significantly reduced in all trunk (T1 to T4) tissues (Fig. 2A, B). There is only a weak correlation between the enrichment profile of H3K27me3 and the onset of collinear *Hox* gene expression in the trunk regions, which is in agreement with our previous report that the diminishment of H3K27me3 seemed to predate and was insufficient for the initiation of *Hox* gene expression

[21]. However, the enrichment of H3K27me3 was significantly reduced in T3 compared to that in T1 and T2, and furthermore was almost completely diminished in T4, especially in the genomic region of the posterior *Hoxc* genes. This could be due to the composition of T4 tissues, which have more or less homogenous populations of *Hox*-expressing cells, and thus the majority of cells are not enriched with H3K27me3. In the case of the active expression marker H3K4me3, the whole cluster in the brain tissue was unmarked with H3K4me3, whereas H3K4me3 modification was closely correlated with the collinear expression pattern of *Hoxc* genes in the trunk regions of E14.5 embryos (Fig. 2B), as described previously [21].

The epigenetic modifications in P5 mice were similar to those detected in E14.5 (Fig. 2C). A strong H3K27me3 mark was detected in the brain tissue, which lacks *Hoxc* gene expression, whereas less strong in the trunk tissues of postnatal P5 mice. In the case of H3K4me3 modification, a strong positive correlation was detected with the expression of *Hoxc* genes, as seen in E14.5 embryos. Compared to the *Hox*-silent brain tissue, H3K4me3 mark was detected in the 3' *Hox* genes (uC4 through uC8-uC9, Figure 2) in the anterior trunk tissue (T1), where *Hoxc*4 through *Hoxc*8 were expressed (Fig. 1). This positive H3K4me3 mark was detected in the entire cluster in the posterior trunk tissues, as the entire *Hoxc* cluster genes were expressed like in the E14.5 trunk tissues. This result indicates that positive and negative histone modifications, once established, seemed to be maintained throughout development and even after birth. We conclude that the basic mode of epigenetic mechanisms regulating *Hox* gene expression are intrinsically maintained throughout development and following birth, and probably over the lifetime of the organism, as previously implicated [27].
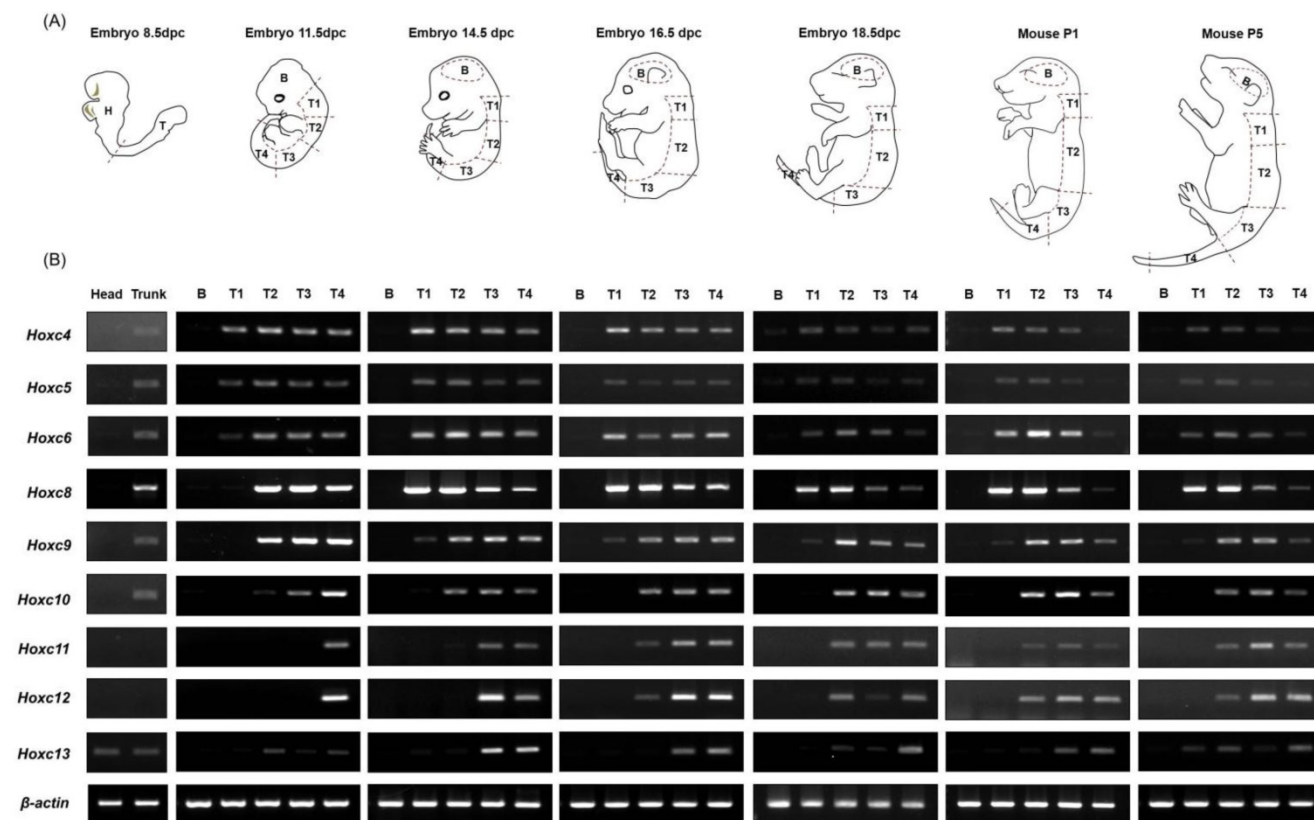
Fig 1. Expression pattern of *Hoxc* cluster genes during embryogenesis and in the early postnatal period along the anterior-posterior (A-P) body axis. (A) A diagram depicting mouse embryos of different developmental stages and the scheme of the embryo dissection. The dashed lines indicate the areas where each tissue was dissected. Each trunk region was carefully isolated on the basis of somite numbers (See Materials and Methods). (B) Expression patterns of *Hoxc* genes (*Hoxc4* through *Hoxc13*) in different tissues along the A-P axis. RT-PCR was performed using the primers specific to *Hoxc* genes. A pair of *β-actin* primers was used as a positive control for RNA integrity and cDNA synthesis. Selected data from three replicate experiments is shown.
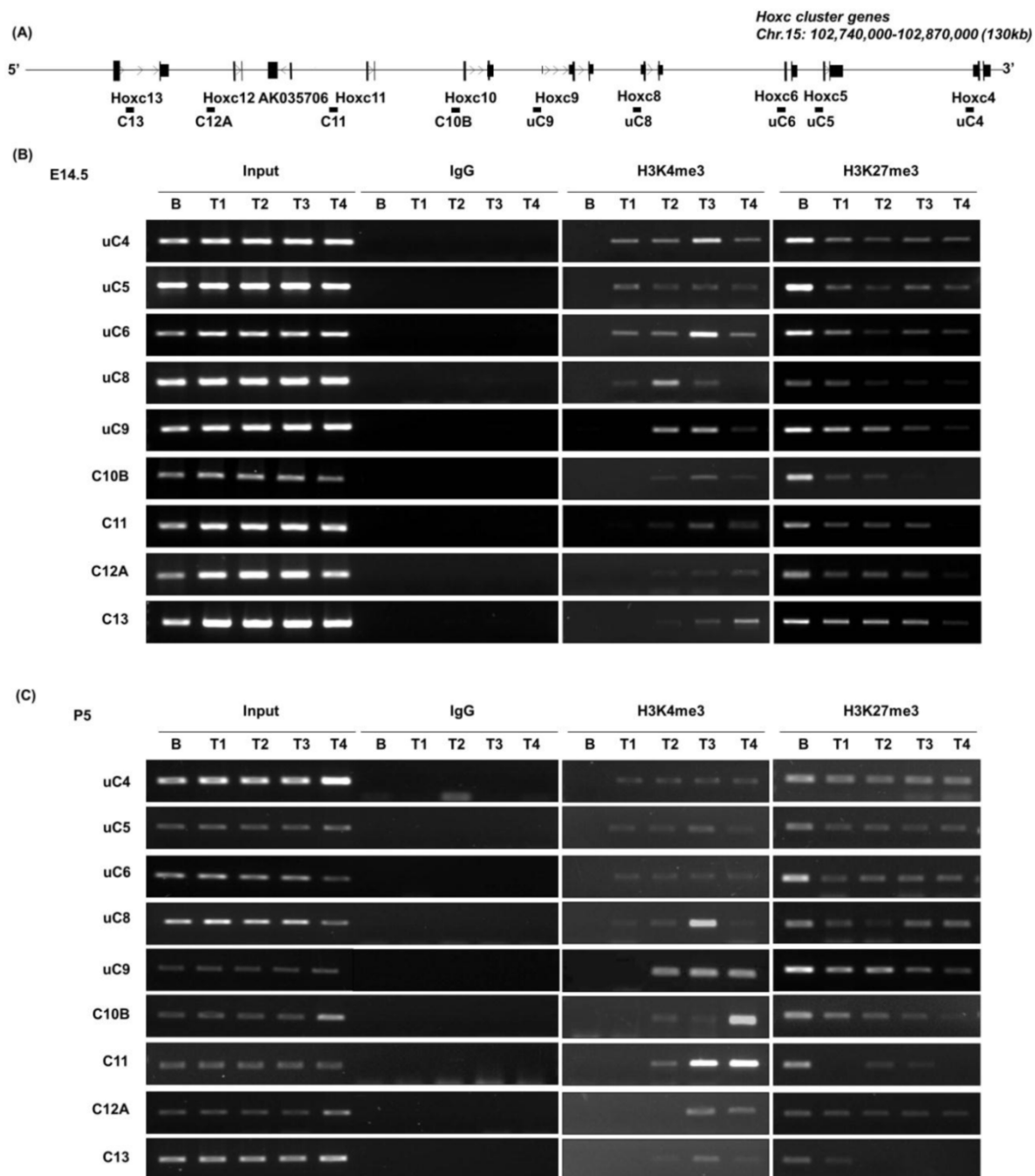
Fig 2. Histone modifications at *Hoxc* genomic loci in E14.5 mouse embryos and P5 mice. (A) A schematic representation of the *Hoxc* locus indicating the genomic position of each *Hoxc* gene and the amplicons used for ChIP-PCR. (B, C) The results of ChIP-PCR to detect histone modifications in the genomic region of the *Hoxc* cluster. Immunoprecipitated DNAs and input DNAs were derived from E14.5 (B) and P5 (C) brain and trunk (T1-T5) tissues using anti-H3K4me3 and anti-H3K27me3 antibodies. IgG ChIP DNA samples were amplified using the same primers as the negative control. The data is representative of three independent experiments on triplicate samples.

Competing Interests

The authors have declared that no competing interest exists.

References

1. McGinnis W and Krumlauf R. Homeobox genes and axial patterning. Cell 1992; 68: 283-302.
2. Kim MH and Kessel M. Homeobox genes as regulators of vertebrate development. AgBiotech News and Information 1993; 5: 189-194.
3. Duboule D and Morata G. Colinearity and functional hierarchy among genes of the homeotic complexes. Trends Genet 1994; 10: 358-364.
4. Krumlauf R. Hox genes in vertebrate development. Cell 1994; 78: 191-201.
5. Kmita MF, van Der Hoeven F, Zakany J, et al. Mechanisms of Hox gene colinearity: transposition of the anterior Hoxb1 gene into the posterior HoxD complex. Genes Dev 2000; 14: 198-211.
6. Simeone A, Acampora D, Arcioni L, et al. Sequential activation of HOX2 homeobox genes by retinoic acid in human embryonal carcinoma cells. Nature 1990;346: 763-766.
7. Partanen J, Schwartz L and Rossant J. Opposite phenotypes of hypo-morphic and Y766 phosphorylation site mutations reveal a function for Fgfr1 in anteroposterior patterning of mouse embryos. Genes Dev 1998;12: 2332-2344.
8. Young T and Deschamps J. Hox, Cdx, and anteroposterior patterning in the mouse embryo. Curr Top Dev Biol 2009;88: 235-255.
9. Duboule D. Temporal colinearity and the phylotypic progression: a basis for the stability of a vertebrate Bauplan and the evolution of morphologies through heterochrony. Dev Suppl 1994;: 135-142.
10. Francis NJ and Kingston RE. Mechanisms of transcriptional memory. Nat Rev Mol Cell Biol 2001; 2: 409-421.
11. Simon JA and Tamkun JW. Programming off and on states in chromatin: mechanisms of Polycomb and trithorax group complexes. Curr Opin Genet Dev 2002; 12: 210-218.
12. Orlando V. Polycomb, epigenomes, and control of cell identity. Cell 2003; 112: 599-606.
13. Czermin B, Melfi R, McCabe D, et al. Drosophila enhancer of Zeste/ESC complexes have a histone H3 methyltransferase activity that marks chromosomal Polycomb sites. Cell 2002; 111: 185-196.
14. Ebert A, Lein S, Schotta G, et al. Histone modification and the control of heterochromatic gene silencing in Drosophila. Chromosome Res 2006; 14: 377-392.
15. Bernstein BE, Mikkelsen TS, Xie X, et al. A bivalent chromatin structure marks key developmental genes in embryonic stem cells. Cell 2006;125: 315-326.
16. Soshnikova N and Duboule D. Epigenetic temporal control of mouse Hox genes in vivo. Science 2009; 324: 1320-1323.
17. Bernstein BE, Kamal M, Lindblad-Toh K, et al. Genomic Maps and Comparative Analysis of Histone Modifications in Human and Mouse. Cell 2005; 120: 169-181.
18. Bracken AP, Dietrich N, Pasini D, et al. Genome-wide mapping of Polycomb target genes unravels their roles in cell fate transitions. Genes & Development 2006; 20: 1123-1136.
19. Lee TI, Jenner RG, Boyer LA, et al. Control of Developmental Regulators by Polycomb in Human Embryonic Stem Cells. Cell 2006; 125: 301-313.
20. Barski A, Cuddapah S, Cui k, et al. High-resolution profiling of histone methylation in the human genome. Cell 2007; 129: 823-837.
21. Min H, Lee JY and Kim MH. Structural Dynamics and Epigenetic Modifications of Hoxc Loci along the Anteroposterior Body Axis in Developing Mouse Embryos. Int J Biol Sci 2012; 8: 802-810.
22. Lee JY, Min H, Wang X, Khan AA and Kim MH. Chromatin organization and transcriptional activation of Hox genes. Anat Cell Biol 2010; 43: 78-85.
23. Kong KA, Yoon H and Kim MH. Akt1 as a putative regulator of Hox genes. Gene 2013; 513: 287-291.
24. Noordermeer D, Leleu M, Splinter E, et al. The dynamic architecture of Hox gene clusters. Science 2011; 334: 222-225.
25. Cao R, Wang L, Wang H, et al. Role of histone H3 lysine 27 methylation in Polycomb-group silencing. Science 2002; 298: 1039-1043.
26. Negishi M, Saraya A, Miyagi S, et al. Bmi1 cooperates with Dnmt1-associated protein 1 in gene silencing. Biochem Biophys Res Commun 2007; 353: 992-998.
27. Rinn JL, Bondre C, Gladstone HB, et al. Anatomic demarcation by positional variation in fibroblast gene expression programs. PLoS Genet 2006; 2: e119.