# Spatiotemporal expression pattern of *Hoxc8* during early mouse embryogenesis and the plausible down stream genes of *Hoxc8*

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# Spatiotemporal expression pattern of *Hoxc8* during early mouse embryogenesis and the plausible down stream genes of *Hoxc8*

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This certifies that the Master's Thesis of

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# 감사의 글

논문을 쓰면서 졸업을 실감할 수 있었습니다. 그리고 처음 실험실에 발을 들였을 때의 모습과 지금의 나를 잠시 생각해볼 수 있는 시간이었습니다. 많이 모자란 지식으로 마음만 앞섰던 제가 이젠 그동안 배운 것들을 안고 밖으로 나가 사회의 한 귀퉁이에서 작은 보탬이 되려고 합니다.

저는 지금 여기서 저에게 그 동안 많은 도움을 주신 소중한 분들의 이름을 불러보려고 합니다. 먼저, 김명희 교수님. 시끄럽게 웃어대는 사고뭉치에게 많은 것을 가르쳐주셨어요. 특히 교수님의 진지하면서도 늘 즐겁게 학문하시는 모습은 앞으로도 잊지 않으려고 해요. 박형우 교수님, 역시 실험실 생활하는 데 있어서 여러 방면으로 다양한 가르침 주신 것 감사 드립니다. 그리고 허만욱 교수님, 자주 뵙지는 못했지만 찾아 뵐 때마다 관심 가져 주시고 조언해 주신 것들 소중히 하겠습니다. 감사 드립니다.

실험실에 처음 왔을 때 쥐뿔도 모르면서 고집만 센 저에게 실험 가르쳐 주시느라 고생하신 부처님 반 토막 같은 설은영 선생님, 죄송하고 감사 드려요. 신재승 선생님, 물심양면으로 잔뜩 도와주셨죠. 덕분에 덜 배고팠고 덜 힘들었고...무지 감사 드려요. 박성도 선생님, 2년이 넘는 오랜 시간 등대고 앉아서 제 성격 다 받아주신 거, 조언해 주신 거 늘 고맙게 생각했어요. 유병기 선생님께선 늘 친절하셨고 김병규 선생님도 바쁘셔두 구원 요청하면 늘 도와주시고 윤보연 선생님도 덜렁대는 저 항상 챙겨주시고 너무 고마워요, 정말. 공경아 선생님이랑 정명섭 선생님도 짧은 시간이었지만 여러 가지로 고마웠구요.

다음으로, 힘들 때도 즐거울 때도 많은 부분을 함께 나눠준 소중한 나의 친구들아! 머리 쥐어뜯고 싶을 땐 위로해주고 기쁠 땐 축하해주고 힘들 땐 때를 가리지 않고 도와준 현정이랑 원정이, 많은 부분에서 도움이 되어주어 항상 고마운 정현이랑 완이, 연락은 뜸하지만 다정한 지이랑 윤주, 멀리 있어도 여전히 가까이 있는 것 같은 세영이, 상당히 틱틱대도 술맛나는 영재, 비슷한 고민으로 힘이 되어준 영조, 항상 나 걱정해주고 맛있는 것도 많이 사주어 날 행복하게 한 나영이랑 가방 끈 늘이기를 함께 해 날 외롭지 않게 해준 보윤이랑 윤정이. 정말 너희들은 나의 소중한 보물이야. 모두모두 내가 아주 많이 고마워한단다.

마지막으로 우리 가족들, 멀리서 제가 하는 것들 많이 걱정되셨을 텐데 믿고 지켜봐 주신 거 정말 감사 드려요. 지금의 제가 있을 수 있는 건 모두 아빠, 엄마, 오빠가 있었기 때문이에요. 표현은 이렇게 상투적일 수밖에 없지만 제 마음은 ... 알죠? 아빠, 엄마, 오빠, 그리고 우리 뽀순이 모두 너무 사랑해요.

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권 윤 정

# TABLE OF CONTENTS

# LIST OF FIGURES



# LIST OF TABLES

## ABSTRACT

### Spatiotemporal expression pattern of *Hoxc8* during early mouse embryogenesis and the plausible downstream genes of *Hoxc8*

*Hox* gene expression can be seen along the dorsal axis (in the neural tube, neural crest, paraxial mesoderm, and surface ectoderm) from the anterior boundary of the hindbrain through the tail. The different regions of the body from the midbrain through the tail are characterized by different constellations of *Hox* gene expression and the pattern of *Hox* gene expression is thought to specify the different regions. In this study, spatiotemporal distribution of *Hoxc8* in mouse embryo was studied by using *in situ* hybridization on tissue sections and whole mounts. Furthermore, influences of *Hoxc8* on early embryogenesis were discussed. Next, 2-DE and MALDI-TOF were performed to identify the plausible downstream genes of *Hoxc8*, which were followed by categorization of downstream genes of *Hoxc8* based on their functions.

Expression pattern of *Hoxc8* during mouse embryogenesis has been extensively studied by using RNA *in situ* hybridization. There is a general agreement about the overall expression domain of *Hoxc8* in mesodermal derivatives of the thoracic region and in the neural tube at cervicothoracic levels. Previously reported data by *in situ* hybridization had difficulty in analyzing time dependent expression pattern due to their flatten image, whereas whole mount *in situ* hybridization had advantages in examining spatiotemporal expression of *Hoxc8* three dimensionally in embryos from 7.5 to 12.5 day p.c.. Expression of *Hoxc8* was first observed at day 8.5 p.c. and regulated in ectoderm and mesoderm respectively. Regarding ectoderm, the distinct anterior boundary of *Hoxc8* expression within the neural tube was established at the $10^{th}$ somite in embryos at day 10.5 p.c.. In the other hands, *Hoxc8* was detected in the paraxial mesoderm within an anterior boundary at the $16^{th}$ somite at day 9.5 p.c. and this expression pattern was maintained through later stages. At day 12.5 p.c., forward progression of the expression pattern was observed and stain was weakened. Comparing expression pattern of *Hoxc8* transcripts in this study with expression pattern of Hoxc8 protein reported previously showed strong correspondence.

Collectively, This data suggested that *Hoxc8* expression is controlled at the level of transcription

Frozen section of embryos at day 10.5-11.5 p.c. showed that expression predominant in the ventral horn of the neural tube expanded in the ventral and mediolateral region of the mantle layer. This ventrolateral expression is associated with the onset and progression of neural differentiation. Moreover, *Hoxc8* was detected strongly in sclerotome cells on the way to notochord and neural tube. This result showed the potential role of *Hoxc8* in forming vertebrae and rib. Additionally mesonephros also expressed *Hoxc8*. Considering that the previous report of *Hoxc8* expression in the kidney, our data suggest that *Hoxc8* might be involved in differentiation into kidney.

Target genes of *Hoxc8* were also analyzed by using 2-Dimentional Elelctrophoresis (2-DE). Categorization of analyzed putative downstream genes of *Hoxc8* was as follows; 1) cytoskeleton and motility, 2) folding, modification and degradation of protein, 3) metabolism, 4) transcription factors and general binding proteins. Among genes analyzed here, carbonic anhydrase II (CAII) decreased by overexpression of *Hoxc8* in this study and CAII deficiency cause osteopetrosis due to defects in osteoclasts. Considering the reverse data reported previously that *Hoxc8* induce differentiation of osteoclasts, *Hoxc8* might be involved in antagonistic mechanism in osteoclast differentiation. Another interesting downstream genes was proliferation cell nuclear antigen (PCNA) which upregulated by *Hoxc8*. Since *Hoxc8* has been reported to be involved in cell proliferation, it could be partly explained by the increased expression of PCNA.

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*Hoxc8*, *in situ* hybridization, anterior boundary, mesonephros, 2-DE, downstream genes, carbonic anhydrase II, proliferating cell nuclear antigen

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## I. INTRODUCTION

Starting as a fertilized egg with a homogeneous appearance, an embryo made of skin, muscles, nerves and other tissues gradually arises through the division of cells. Long before most cells in the emerging body begin to specialize, however, a plan that designates major regions of the body –the head, the trunk, the tail and so on-is established. This plan helps seemingly identical combinations of tissues arrange Starting as a fertilized egg with a homogeneous appearance, an embryo made of skin, themselves into distinctly different anatomical structures, such as arms and legs.

Recently embryologists have made great progress in uncovering the mechanisms that control this once mysterious process. In the past decade the powerful techniques of molecular biology have made it possible to isolate and characterize individual genes that mediate some of the developmental decisions involved in establishing the embryonic body plan. The key is a family of genes, known as homeobox genes, that subdivides the early embryo into fields of cells with the potential to become specific tissues and organs.[1] Analysis of genes essential to *Drosophila* morphogenes showed that these genes were all homologous to one another and containing a common DNA motif of 183 base pairs coding for DNA-binding homeodomain,[2] which were named Homeobox by Gehring *et al*.

Long before the discovery of the homeobox,[3] *Drosophila* workers knew that homeotic

genes are special. In the late 1940s, Ed Lewis began a study of mutations that produces almost magical transformations; he made flies with four wings instead of two wings and two halters, and from then until now he has worked on the bithorax complex (BX-C), a group or ruling genes that help design the fly. It was Lewis who discovered the vitally important fact that the wild-type function of each homeotic gene is restricted to a specific region of the developing insect. He and others showed that the developmental pathway followed by each cell depends on the set of BX-C genes active within it. By studying genetic mosaics, these researchers showed that each cell differentiates autonomously.[4] Thus, homeotic genes like the BX-C are responsible for determination, the internal molecular and genetic state of a cell that makes it stably different from other cells.

Subsequently, *Hox* genes, homeobox genes homologous to the homeotic genes in *Drosophila*, have been found in vertebrate. There are 39 *Hox* genes organized into 4 separated chromosome cluster[5] and 39 genes are subdivided into 13 paralogous groups on the basis of duplication of an ancestral homeobox cluster.[6] Each paralog group has been demonstrated to be responsible for the morphogenesis of a particular embryonic domain or structure.[5]

The *Hox* genes are key regulators of regional pattern formation along the antero-posterior (A-P) and other embryonic axes. These genes have been involved in transducing positional information to the precursors of embryonic axial and paraxial structures since before the ancestors of insects and vertebrates diverged form each other. In vertebrates, the precise restricted localization of each *Hox* gene product is crucial for correct patterning of tissues surrounding the rostral expression boundary of therefore essential to the proper orchestration of embryonic morphogenesis.[7]

Expression of *Hox* genes in the various body regions is collinear with their genomic organization in such a way that genes at the 3' end of the cluster are expressed more anteriorly, and those at the 5' end more posteriorly. The physical position of a *Hox* gene in the cluster not only corresponds to its expression domain along the primary or secondary

body axes (spatial collinearity),[8-11] but also to its time point of activation (temporal collinearity)[12] and its response to the morphogen retinoic acid (RA).[13] In general, *Hox* genes located at the 3' end of the cluster are expressed earlier, are more anterior and are more sensitive to induction by RA than those in a more 5' position.

Expression pattern of *Hoxc8* during mouse embryogenesis has been extensively studied with RNA *in situ* hybridization.[14-19] There is a general agreement about the overall expression domains of *Hoxc8* in mesodermal derivatives of the thoracic region and in the neural tube at cervicothoracic levels. The distribution of *Hoxc8* was also examined with monoclonal antibodies against *Hoxc8* in early and mid-gestation embryos.[20]

In this study, *in situ* hybridization on tissue sections and whole mounts were used for a detailed study of the spatiotemporal distribution of *Hoxc8* transcripts in mouse embryos and influences of *Hoxc8* on early embryogenesis were discussed.

Next, homeobox genes are a family of regulatory genes containing homeobox and coding for specific nuclear protein that act as transcription factors.[21] The homeobox sequence itself encodes a 61 amino acid domain, the homeodomain, responsible for recognition and binding of sequence-specific DNA motifs[22] and gene regulation relevant to cell proliferation and differentiation. The specificity of this binding allows homeoproteins to activate or repress the expression of batteries of downstream target genes[23] that mediate developmental decisions involved in establishing the embryonic body plan. *Hox* target genes are presently poor and most of them have been identified in the *Drosophila* so *Hox* target genes as well as their functions still remain to be elucidated.

In mammals, there are few *Hox* genes whose functions have been identified. The *HOXA5* controlled activation of the progesterone receptor gene[24] and *HOXC13* has been reported to be involved in control of hair keratin expression during early trichocyte differentiation.[25] In addition, it was reported that *HOXA7* is inversely related to keratinocyte differentiation by attenuating transglutaminase I induction.[26] Furthermore, it

has been demonstrated that synpolydactyly, an inherited human abnormality of the hands and feet, is caused by expansions of a polyalanine stretch in the amino-terminal region of *HOXD13*. These mutations could lead to change truncated protein missing 20 C-terminal amino acids.[27-28] Additionally a deletion in *HOXA13* leads to hypodactly.[29-30]

In the case of *Hoxc8*, which is one of three members of paralog VIII, the function has been examined using null mutant mice or *Hoxc8* overexpressing cells. Bending and fusion of the ribs, anterior transformation of the vertebrae, and abnormal patterns of ossification in the sternum were observed in adult *Hoxc8* null mouse.[31] Tissue specific overexpression of *Hoxc8* transgene inhibited chondrocyte maturation and stimulated chondrocyte proliferation.[32] Recent study demonstrated that *Hoxc8* mediated osteoclast differentiation by repressing osteoprotegrin (OPG) in response to bone morphogenetic protein (BMP) stimulation.[33] Furthermore, it was also suggested that the interaction of Hoxc8 with Smad1 induced osteoblast differentiation and bone cell formation.[34-35]

Here, 2-DE and Matrix Assisted Laser Desorption Ionization – Time Of Flight (MALDI-TOF) were performed to identify the putative downstream target genes of *Hoxc8* using mouse teratocarcinoma stem cells overexpressing *Hoxc8*. This study was followed by categorization based on their functions.

## II. MATERIALS AND METHODS

1. Analysis of spatiotemporal expression pattern of *Hoxc8*

A. Preparation of embryos

For mating, pairs of ICR mice (Samtako, Osan, Republic of Korea) were caged together in the late afternoon and the female was examined for the presence of vaginal plug in the following morning that was defined as day 0.5 p.c. (post coitum). The pregnant females were sacrificed at the required gestational stages by cervical dislocation and the embryos were dissected free of maternal and extraembryonic tissue in PBS. The embryos were fixed in 4% paraformaldehyde (PFA)/PBS at 4 ℃ for 1-12 hr depending on the age of the embryos (7.5-12.5 day p.c.).

(A) For whole mount *in situ* hybridization; embryos were washed with PBT, 25%, 50%, 70% MetOH/ PBT and 100% MetOH, then stored at -20 ℃.

(B) For frozen section; embryos were transferred to 30% sucrose overnight at 4°C, then mounted in tissue tek (Miles Inc. Diagnostic division, Elkhard, IN, USA) and covered with Tissue Freezing Medium (Triangle Biomedical Science, Durham, N.C., USA). Embryos were stored at -70°C

B. Subcloning of *Hoxc8* into pGEM-7zf

(A) Generation of pGEM:C8

pBS:C8 in which mouse *Hoxc8* cDNA containing 726 base pair (b.p.) coding 242 amino acid (a.a.) had been subcloned into pBluscript $SK^+$ (Stratagene, La Jolla, California, USA) was donated by Dr. Gruss from Max Planck Institute of Biophysical Chemistry (Goettingen, Germany). To generate sense and antisense RNA probes of *Hoxc8*, *Hoxc8* coding region was subcloned into pGEM-7zf (promega, Fitchburg, Wisconsin, USA) that has both T7 and SP6 promoters at

each end of the multicloning site. So *Hoxc8* fragment isolated from the pBS: C8 after digestion of *Bam*HI/*Eco*RI restriction endonuclease was ligated into the same enzyme sites of pGEM-7zf.

(B) *In vitro* transcription

To synthesize antisense and sense RNA as probes, pGEM-7zf:C8 plasmids were linearized with *Bam*HI and *Eco*RI enzymes each and then followed by the polymerization with T7 and SP6 RNA polymerases (Boehringer Mannheim, Mannheim, Germany), respectively, in the presence of nucleotide mixture containing digoxygenine labeled CTP (Boehringer Mannheim, Mannheim, Germany) for 2 hr at 37°C. After precipitation with LiCl and EtOH, RNAs were used as probes for *in situ* hybridization on whole mounts as well as tissue sections.

C. Whole mount *in situ* hybridization and paraffin section

Dehydrated embryos stored at -70°C were washed with 75%, 50%, 25% MetOH/ PBT and PBT for rehydration and bleached with 6% $H_2O_2$/PBT for 1 hr then, treated with 10 μg/ml proteinase K/PBT for 15 min. Next, 2 mg/ml glycine was added to inactivate proteinases, and 0.2% glutaraldehyde/4% paraformaldehyde/PBT was treated for refixation. After PBT washing, embryos were incubated in prehybridization solution [50% formamide, 5xSSC (pH 4.5), 50 μg / ml yeast tRNA, 1% SDS, 50% μg/ml heparin] at 70°C for 1 hr and then, incubated in hybridization solution including 1 μg/ml digoxygenine-labelled RNA probe 70°C overnight. Following day, Post-hybridization wash was carried out with soln. I [50% formamide, 5xSSC (pH 4.5), 1% SDS] at 70°C for 30 min twice, soln. I: soln II = 1: 1 at 70°C for 10 min and soln. II [0.5M NaCl, 10mM Tris-Cl (pH 7.5), 0.1% Tween 20] 3 times. After that, incubation in 100 μg/ml RNaseA/soln. II was followed at 37°C 30 min twice. After washing with TBST, preblocking in 10% sheep serum/ TBST for 90 min was followed by incubating embryos with serum and anti-digoxygenine antibody (Boehringer Mannheim, Mannheim, Germany) at 4°C for 1 hr. Next, Post-antibody wash was performed with TBST for 5 min 3 times and NTMT for 1 hr 5 times and

embryos were incubated with NTMT containing 4.5 µl NBT (Boehringer Mannheim, Mannheim, Germany), 3.5 µl BCIP (Boehringer Mannheim, Mannheim, Germany) per ml in the dark. When color was developed to the desired extent, embryos were washed twice with PBT. After photography, clearing with benzene followed dehydration with EtOH. Embedding with paraplast was performed and embryos were cut at 7 µm for detection.

D. *In situ* hybridization of frozen section

Frozen section was prepared on a Microm microtome using disposable blades at -20°C. Sections were cut at 6-12 µm and transferred to pretreated slides (Fisher Superfrost Plus, Pittsburgh, PA, USA). The slides were equilibrated to room temperature (RT) before removing them from the container. All steps were performed at RT unless noted otherwise. The sections were rehydrated in PBS and post fixed in cold 4% PFA/PBS for 10 min then, washed 3 times with PBS for 10 min each. Acetylation [295 ml $H_2O$, 4 ml triethanolamine, 0.525 ml HCl, 0.75 ml acetic anhydride] was followed by washing 3 times with PBS. After applying 500 µl of prehybridization buffer [the same solution used in whole mount *in situ* hybridization slides were incubated in a humidified chamber containing 5xSSC for 2 hr. Then, Solution was changed into hybridization solution containing 200-400 ng/ml RNA probe at 80°C for 5 min and slides were further incubated in a humidified chamber containing 5xSSC and 50% formamide at 72°C overnight. After removing coverslips by submerging in 5xSSC at 72°C, samples were incubated in 0.2xSSC for 3hr, 0.2xSSC for 5min, and Buffer B1 [0.1M Tris pH 7.5, 0.15M NaCl] including levamisole for 5 min. Then, anti-Digoxygenine antibody (1:5000 dilution in B1 buffer) was added on slides, and incubated in the humidified chamber overnight at 4°C. Washing with B1 buffer 3 times for 5min each was followed by equilibration with B3 buffer [0.1M Tris pH 9.5, 0.1M NaCl, 50mM $MgCl_2$]. After applying 60-70 µl of B4 solution [4.5 µl/ ml NBT, 3.5 µl/ ml BCIP, 0.24 mg/ml levamisole in B3], slides were covered with parafilm. Then, slides were incubated for about 6 hr-3 days in humidified chamber in dark and reaction was stopped with washing with PBS.

After air-dry, slides were mounted with Universal Mount (Huntsville, AL, USA).

2. Analysis of downstream target genes

A. Subcloning of *Hoxc8*

(A) Generation of pEGFP: C8

In order to follow the localization of Hoxc8 protein, pEGFP:C8 expressing EGFP (enhanced green fluorescent protein) fused Hoxc8 was generated. A 780 bp *Bam*HI/*Kpn*I fragment harboring *Hoxc8* cDNA was isolated from pBS:C8 and ligated into the plasmid pEGFP-C1 (Clontech, Palo Alto, CA, USA) to give rise pEGFP:C8.

(B) Generation of pcDNA: C8

To generate *Hoxc8* overexpressing stable cell line, 780 bp of *Hoxc8* fragment was subcloned into the *Bam*HI/*Xho*I site of expressing vector, pcDNA3 having CMV promotor (Invitrogen, Carlsbad, California, USA). Before transfections, pcDNA:C8 was linearized with *Bgl*II restriction endonuclease.

B. Cell culture

(A) Transient transfection

Mouse F9 teratocarcinoma cell was purchased from ATCC (American Type Culture Collection). Cells were cultured *in vitro* in Dulbeccos Modified Eagles Medium(DMEM : Gibco BRL, Carlsbad, California, USA) containing 10% fetal calf serum (FCS : Gibco BRL, Carlsbad, California, USA), 60 μg/ml penicillin (Gibco BRL, Carlsbad, California, USA), and 100 μg/ml streptomycine (Gibco BRL, Carlsbad, California, USA), and incubated with 5% $CO_2$ at 37°C. $1x10^5$ cells/well were grown in 12 well plate for about 1 day and transfected with pEGFP: C8. Lipofectamin plus reagent (GibcoBRL, Carlsbad, California, USA) was used for transfection according to manufacturer's manual. Next day, cells washed with 1xPBS were fixed with formaline solution for 10 min before

detection with fluorescent microscope.

(B) Staining nucleus

1 x $10^5$ cells/well were grown in 12 well tissue culture plates. After 24 hr incubation, cells were washed with 1xPBS and fixed with 4% paraformaldehyde/1xPBS for 15 min. Hoechst 33258(1 mg/ml)/1xPBS was added for 15 min to stain nucleus. After washing with 1xPBS, cells were examined under the fluorescent microscope (Olymphus, Melville, NY, USA).

(C) Generation of stable cell line

F9 teratocarcinoma cells were used and cultured as described above. $2.5x10^5$ cells were grown in 6 well plate. When cells reached about 60% confluence, pcDNA:C8 was transfected by lipofectamine plus according to the manual. Blank vector was also transfected as a control. After 1 day, cells were transferred into 100Ø dish and selected with Geneticin (Gibco BRL, Carlsbad, California, USA).

C. RNA preparation and RT-PCR

RNA zol B (LPS industries inc., New jersey, NY, USA) was used for RNA preparation. Reverse transcription (RT) was performed with 2 g of RNA. 2/25 (vol/vol) of RT reaction was used for PCR amplification. Starting with denaturation for 5 min at 95 °C, 40 cycles (95°C for 1 min, 55°C for 1 min, 72°C for 1 min) of PCR reaction were performed. β-actin was also used for RT-PCR as a control. *Hoxc8* (sense, 5'-CAC GTC CAA GAC TTC TTC CAC CAC GGC; antisense, 5'-CAC TTC ATC CTT CGA TTC AAC C)and β-actin primer (sense, 5'-CAT GTT TGA GAC CTT CAA CAC CCC; antisense, 5'-GCC ATC TCC TGC TCG AAG TCT AG) were purchased from Bionia (Taejon, Korea).

D. 2-Dimentional electrophoresis (2-DE) and MALDI-TOF

*Hoxc8* overexpressing cells were grown in 150Ø dish until the confluence

reached more than 80 %. Vector transfectants were incubated as a control. Cells were harvested and washed with PBS. After resuspending cells in PBS, lysis of cells was carried out by sonication 10 times for 1 min each. After centrifugation (4℃ 12,000 rpm), 20% TCA (trichloroacetic acid)/Acetone was added to the supernatant and kept at -20°C for protein precipitation. To remove TCA and salt, centrifugation (4℃ 12,000 rpm) and washing with acetone were repeated until pH became 7. Then, Air-dry was followed by resolving in lysis buffer [(9.5 M urea, 2% TritonX-100, 2% DTT (w/v), 2% IPG Buffer (Amersham pharmacia, Piscataway, NJ, USA)). IPG (immobilized pH gradient gel) strip (BIO-RAD, Hercules, California, USA) was rehydrated in the protein lysis solution containing 1-1.5 mg protein in reswelling tray (Amersham Pharmacia, Amersham pharmacia, Piscataway, NJ, USA) for more than 18 hr and transferred to multiphorII electrophoresis unit (amersham pharmacia, Amersham pharmacia, Piscataway, NJ, USA). After that, first-dimension isoelectric focusing was carried out according to the manufacture's manual. After the first dimension, the IPG gel was equilibrated for 15 min with equilibration buffer consisting of 50 mM Tris-HCl (pH 6.8), 6M urea, 3% SDS, 50 mM DTT, and 0.01% BPB]. Next, IPG strip was transferred to SDS-polyacrylamide gel for second-dimension SDS-PAGE, which was stopped when bromophenol blue reached the bottom of the gel. Gels were washed with distilled water for 5 min 3 times and incubated in Bio-Safe Coomassie Brilliant Blue (CBB) (Bio-Rad, Hercules, California, USA) with shaking for at least 1 hr. After that, another washing with distilled water was carried out 3 times for 30 min each.

After gels were scanned, spot were analyzed by using PDQuest$^{TM}$ software (Huntington Station, NY, USA). Spots of interest were excised and washed with 30% MeOH until coomasie blue was removed completely. (if color was remained, 50% acetonitril/10 mM Ammonium Bicarbonate was added until blue color was gone). After 100% acetonitrile was added, gel particles were incubated at RT for 10 min, and dried compeletly by using speed vac. By

incubation in buffer containing 50 mM of ammonium bicarbonate. Gels were reswelled and 0.5 mg trypsin was applied for 16 hr at 37°C. After another incubation in 50 mM ammonium bicarbonate at 37°C for 1 hr with shaking, supernatant was transferred. Gel particles were incubated in 100% acetonitrile at 37°C for 10 min and supernatant was also transferred. After repeating these steps two times more, total supernatant was dried completely by speed vac and analyzed with MALDI-TOF.

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## III. RESULTS

### 1. Dynamic expression pattern of *Hoxc8* during embryonic development

Whole mount *in situ* hybridization was performed to examine the distribution of *Hoxc8* transcripts in murine embryos from day 7.5 to 12.5 p.c.. Expression of *Hoxc8* was first observed in the posterior epiblast at 8.5 day p.c. in mouse embryos when the embryos had initiated the turning sequence. Expression was diffused in the unsegmented region from the base of allantois (Figure 1A).

In embryos at day 9.5 p.c., *Hoxc8* was detected in the paraxial mesoderm with an anterior boundary at the $16^{th}$ somite. Posterior expression boundary due to downregulation of *Hoxc8* and expression at the tail bud were detected. The resulting expression domain in the paraxial mesoderm spanned 5-6 somits. Expression was robust from somite 16-20 (Figure 1B,C). This distribution of *Hoxc8* in the paraxial mesoderm was maintained through later stages of embryonic development (Figure 1D-I).

At day 10.5 p.c., the downregulation was not complete, as residual *Hoxc8* was observed at the tip of the tailbud and the stain in the paraxial mesoderm was intensified. Expression in the neural tube was strong in the $10^{th}$-$13^{th}$ somites, and spreading gradually towards more posterior levels (Figure 1D,E). At this stage, *Hoxc8* exhibited the characteristic expression described for mouse *Hox* genes with a more anterior expression boundary in the neural tube than in the mesoderm.

Previous expression pattern was maintained through 11.5 day p.c. and the downregulation in the tail bud was completed (Figure 1F,G).

Forward progression of the expression pattern was observed at the day 12.5 p.c. and resulted in an expression up to the anterior boundary of forelimb in the neural tube and posterior boundary of forelimb in the mesoderm (Figure 1H,I).

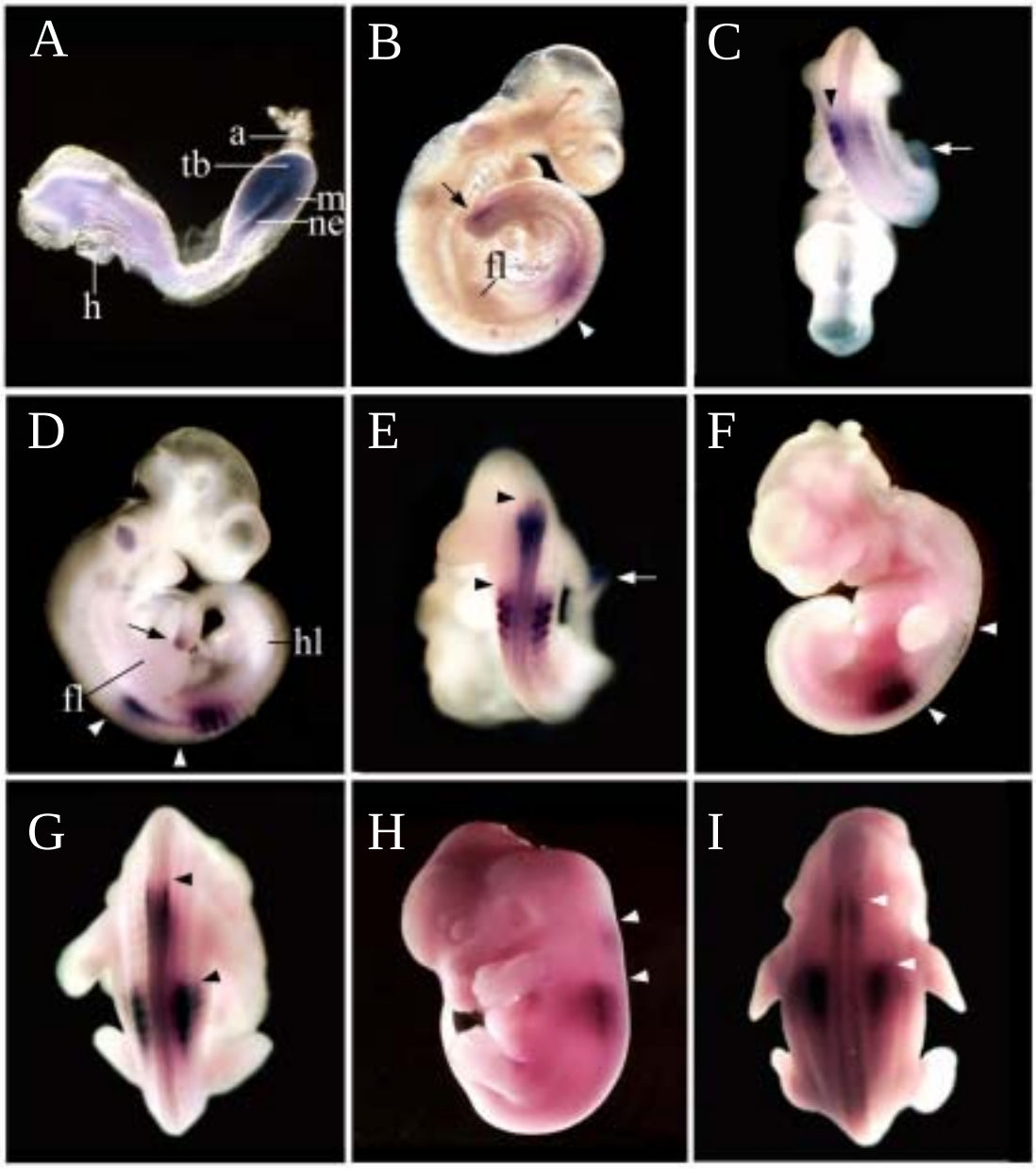


**Figure 1.** Expression of *Hoxc8* in early mouse embryos. Whole mount *in situ* hybridization of *Hoxc8* mRNA at 8.5 day p.c. (A), 9.5 day p.c. (B, C), 10.5 day p.c. (D, E), 11.5 day p.c. (F, G), 12.5 day p.c. (H, I). Arrow heads indicate anterior expression boundaries of *Hoxc8* in the neural tube and paraxial mesoderm. Arrow: expression of *Hoxc8* in tail bud. a, allantois; fl, forelimb bud; h, heart; hl, hindlimb bud; m, mesoderm; ne, neurectoderm; tb, tailbud.

**2. Lineage specific expression pattern of *Hoxc8*: Strong expression of *Hoxc8* in the ventral horn within the neural tube and sclerotome of the mesoderm and weak expression in the intermediate mesoderm**

For an analysis at higher resolution, paraffin section was performed for embryos at day 8.5 p.c. (Figure 2D-F) and *in situ* hybridization on frozen tissue sections was performed with embryos at day 10.5 and 11.5 p.c. (Figure 2G-I).

*Hoxc8* was distributed over the entire neural plate in the unsegmented region at 8.5 day p.c. (Figure 1B,C). At day 9.5 p.c., expression in the neural tube was not detected. However, the strong expression was detected at subsequent days to 11.5 day p.c and expression pattern changed dramatically (Figure 2B,C). In contrast to the expression of *Hoxc8* over the entire neural tube at day 8.5 p.c., ventral horn in the neural fold strongly expressed *Hoxc8* at 10.5 day p.c., whereas expression was not detected in ependymal layer, floor plate and roof plate (Figure 2H).

At day 8.5 p.c., expression of *Hoxc8* was found in mesodermal derivatives including the paraxial and intermediate mesoderm (Figure 2F). According to the result of whole mount *in situ* hybridization, expression pattern in mesoderm was established at day 9.5 p.c. and maintained through 11.5 day p.c.(Figure 1B-G). In this study, the embryos at day 11.5 p.c. were examined with frozen section. While neural tube, dorsal root ganglia and notochord were all *Hoxc8* negative at the level of $16^{th}$-$20^{th}$ somites, sclerotome rather than dermomyotome were positive. In addition, weak stain at intermediate mesoderm derivatives including mesonephric duct and mesonephric tubule were also detected (Figure 2I).

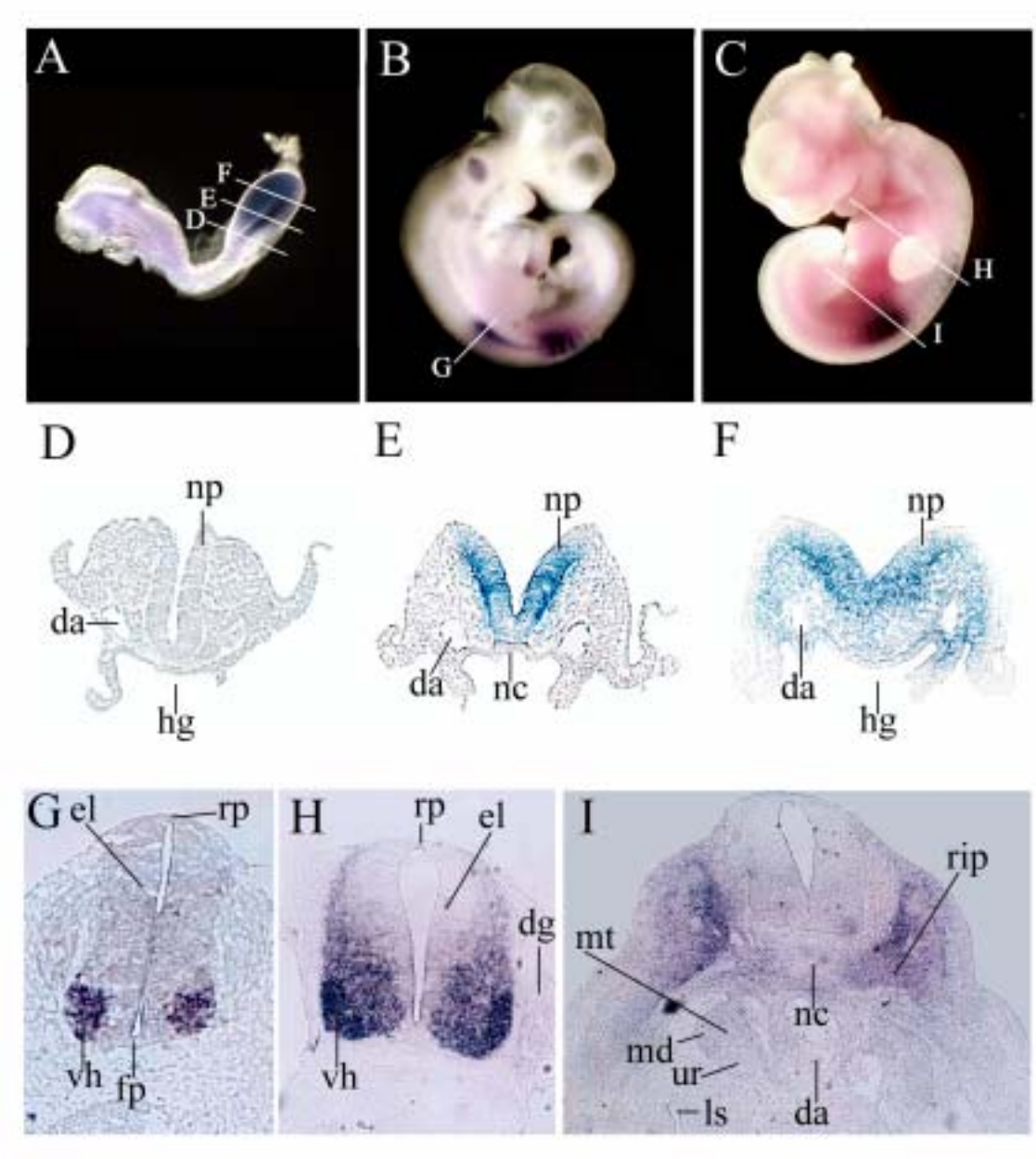


**Figure 2.** Expression of *Hoxc8* on cross sections in embryos at day 8.5 p.c. (A), 10.5 p.c. (B), 11.5 p.c. (C). The specimen in D, E, F is cut as indicated in A. Panel G shows a cross section through the neural tube of 10.5 day p.c. (B). H, I are cross sections through the 11.5 day p.c. (C). embryo. da, dorsal aorta; dg, dorsal root ganglia; el, ependymal layer; fp, floor plate; hg, hind gut; ls, lumen of stomach; md, mesonephric ducts; mt, mesonephric tubules; nc, notochord; np, neural plate; rp, roof plate; rip, rib primordium; ur, urogenital ridge; vh, ventral horn.

### 3. Nuclear localization of *Hoxc8* generation

In order to investigate the function of *Hoxc8*, cellular localization of *Hoxc8* was examined as a fusion protein with EGFP (Figure 3A,B). Direct comparison between the expression pattern of EGFP and stain pattern generated by nuclear staining dye Hoechst 33258 in the same sets (Figure 3C,D) demonstrated that virtual identity of both nuclear pattern. Nuclear localization of *Hoxc8* was confirmed and this result supports the fact that *Hoxc8* is a transcription factor to control gene regulation.

### 4. Generation of stable cell line overexpressing *Hoxc8*

Stable cell lines overexpressing *Hoxc8* in F9 teratocarcinoma embryonic stem cells were generated. Selection with geneticine after transfection was performed by RT-PCR to confirm the overexpression of *Hoxc8* in selected colonies. Overexpression of *Hoxc8* was detected easily in *Hoxc8* transfectants compared with control which showed weak expression of *Hoxc8* (Figure 4).

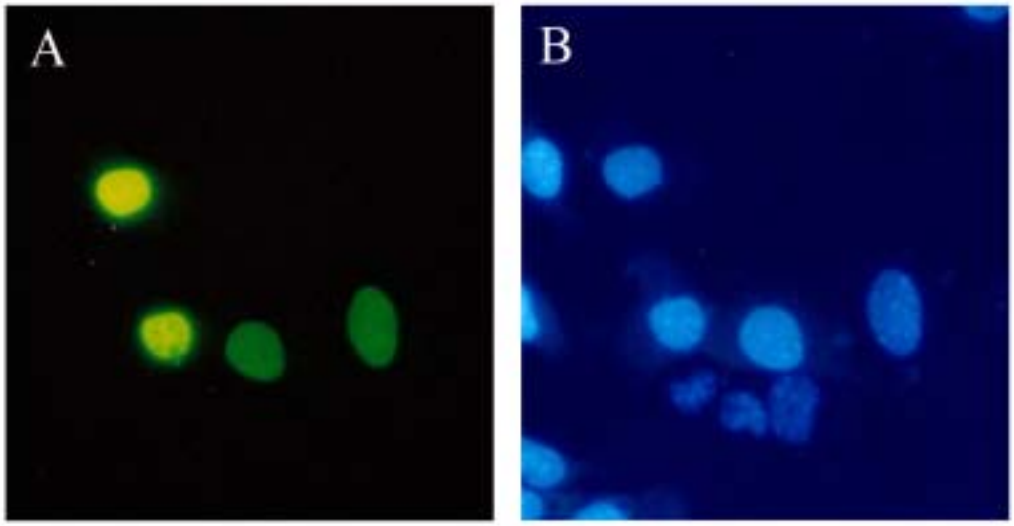


**Figure 3.** Nuclear localization of Hoxc8 in F9 cells. pEGFP:C8 transfectant (A). After transfection with pEGFP:C8, cells were stained with hoechst (B).

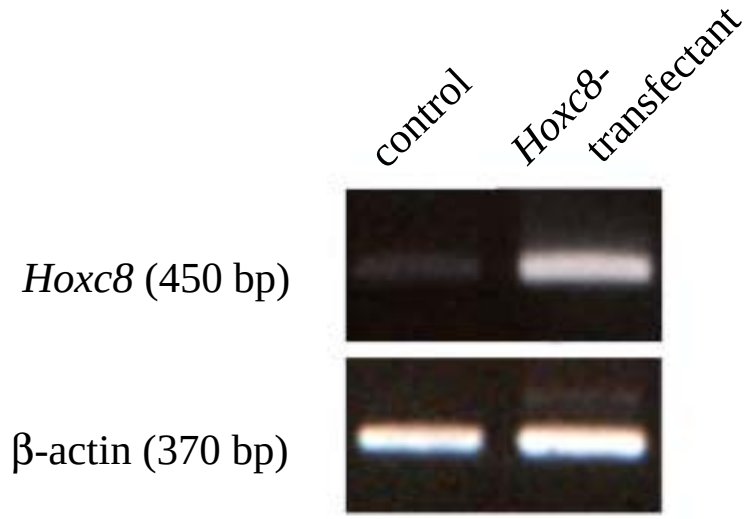


**Figure 4.** RT-PCR analysis of the *Hoxc8* expression in a control (left) and *Hoxc8* transfectant overexpressed cells (right). Cellular RNA was isolated from cultured cells and subjected to RT-PCR. After 40 cycles of amplification with *Hoxc8* primers[a)], samples were electrophoresed on a 1.0% agarose gel.

a) *Hoxc8* sense primer: 5'-CACGTCCAAGACTTCTTCCACCACGGC-3'
antisense primer: 5'-CACTTCATCCTTCGATTCTGGAACC-3'

## 5. Analysis of the plausible downstream target genes of *Hoxc8 by* using 2-DE and MALDI-TOF

To identify downstream target genes of *Hoxc8*, 2 groups of proteom samples were prepared from pcDNA (control) and pcDNA:C8 transfected cell lines. After 2-DE, CBB stain of gels was carried out to examine spots on gels. (Figure 5, 6) Proteins above 7 in pI (Isoelectric Point) value were excluded from analysis due to the insolubility of proteins above 7 in pI value. Spots showing differences more than 4 times in density, whether increased or decreased, were selected.

Table 1 shows proteins analysed by MALDI-TOF. Categorization into 4 groups based on their functions was shown in Table 2. 1) Cytoskeleton and motility; vimentin, γ-actin and tropomyosin were increased and tubulin β-5 chain whose strong expression was reported mainly in immature brain also was increased. 2) Folding, modification and degradation of protein; both Dna-k type molecular chaperon reported to be expressed in immature brain and proteosome subunit α type 5 were increased, whereas 26s proteosome regulatory subunit p27 and stress inducible protein disulfide isomerase related protein were decreased. 3) Metabolism; ATP synthase and phosphoglycerate mutase I were increased, while carbonic anhydrase II (CAII), whose deficiency was known to cause metabolic disorders of bone, kidney and brain. This is interesting considering that *Hoxc8* is involved in osteogenesis. 4) Transcription factors and general binding proteins; while histone acetyltransferase type b subunit 2 essential for initiation of basal transcription, proliferating cell nuclear antigen (PCNA) and elongation factor 1-β were increased, whereas nucleophosmin decreased.

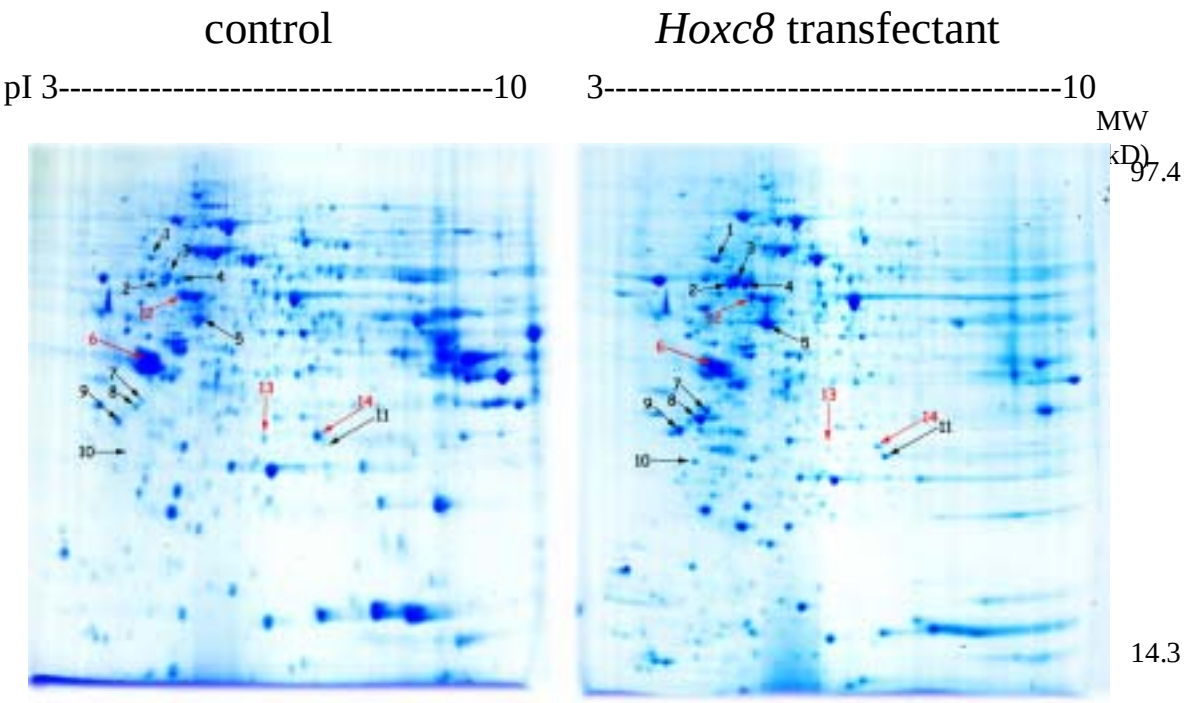


**Figure 5.** Composite gel image of CBB-stained 2-DE of a control and *Hoxc8* overexpressed cells. Red arrows indicate the decreased spots, and black arrows show those increased in intensity in the *Hoxc8* transfectant.

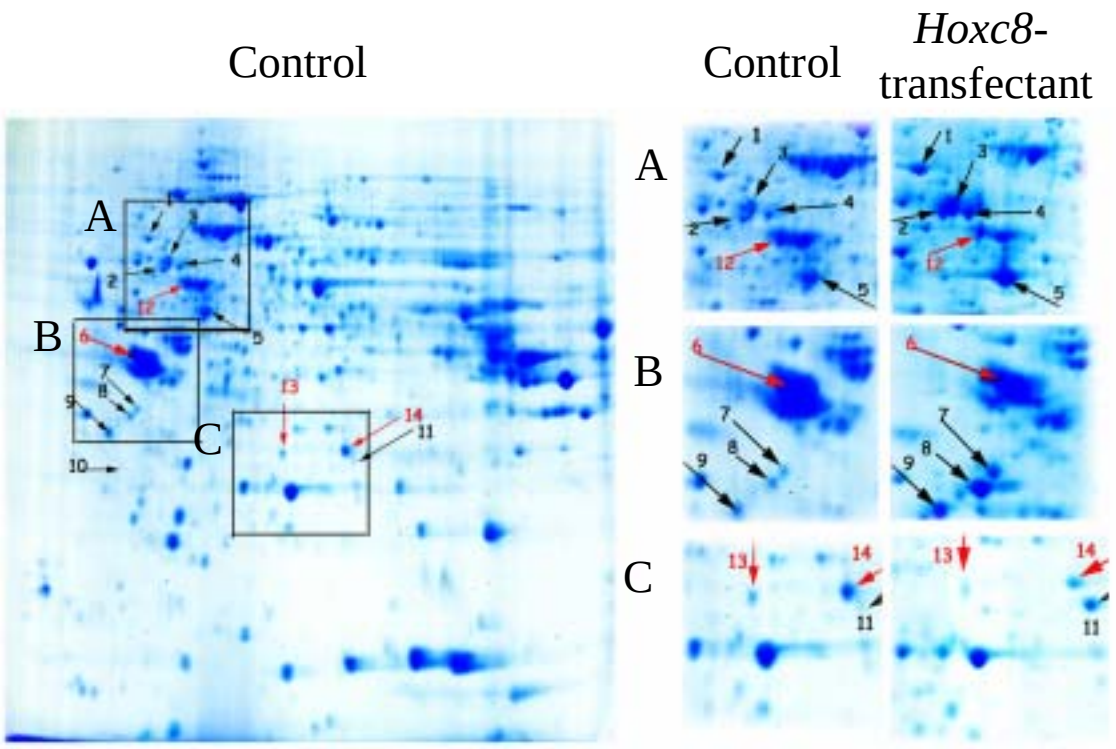


**Figure 6.** Composite image of CBB-stained 2-DE gels of pcDNA3 transfectant as a control(left panel, and A-C left) and *Hoxc8* transfectant (A-C right). The region containing the particular specific spots was enclosed with a square in the 2-DE profile of control and compared with the matched profile from *Hoxc8* transfectant (A-C). Red arrows show the decreased spots, and black arrows show those increased in intensity in the *Hoxc8* transfectant.

**Table 1**. Induced or repressed spots by *Hoxc8* in 2-DE

| Spot NO. | Protein Name | Access. # | Mol. MS(Da) | pI |
|---|---|---|---|---|
| 1 | DnaK-Type molecular chaperone precursor(GRP 78) | 109893 | 72421.7 | 5.12 |
| 2 | 1)Histone acetyltransferase type B subunit2 | 2494892 | 47790.1 | 4.89 |
| | 2)Vimentin | 281012 | 53630.1 | 5.09 |
| 3 | Tubuline beta-5 chain | 91859 | 49671.3 | 4.78 |
| 4 | ATP synthase ($H^+$transporting mitochondrial F1 complex) | 12845667 | 56301.0 | 5.19 |
| 5 | Gamma-actin | 809561 | 41019.4 | 5.56 |
| 6 | Nucleophosmin | 200011 | 32560.3 | 4.62 |
| 7 | Proliferating cell nuclear antigen | 56862 | 28749.1 | 4.57 |
| 8 | Tropomyosin | 438878 | 29006.8 | 4.75 |
| 9 | Elongation factor 1-beta(EF-1-beta) | 13124180 | 24708.9 | 4.47 |
| 10 | Proteasome subunit alpha type 5 | 12229953 | 26411.3 | 4.47 |
| 11 | Phosphoglycerate mutase 1 | 12844989 | 28703.1 | 6.68 |
| 12 | Similar to protein disulfide isomerase-related protein | 13905146 | 48100.8 | 4.99 |
| 13 | 26S Proteasome regulatory subunit P27 (Transactivating protein BRIDGE) | 12832148 | 24720.1 | 6.00 |
| 14 | Carbonic anhydrase II | 12832236 | 29032.8 | 6.49 |

**Table 2**. Function-based classification of *Hoxc8* induced or repressed proteins.

| Protein Category | Spot no. on 2D-PAGE | Induction status [a)] | Gene product |
|---|---|---|---|
| **Cytoskeleton And Motility** | 2[2)] | + | Vimentin |
| | 3 | + | Tubuline beta-5 chain |
| | 5 | + | Gamma-actin |
| | 8 | + | Tropomyosin |
| **Folding, Modification and Degradation of Protein** | 1 | + | Dnak-type molecular chaperone precursor |
| | 10 | + | Proteasome subunit alpha type 5 |
| | 12 | - | Similar to protein disulfide isomerase-related protein |
| | 13 | - | 26S Proteasome regulatory subunit P27 |
| **Metabolism** | 4 | + | ATP synthase |
| | 11 | + | Phosphoglycerate mutase 1 |
| | 14 | - | Carbonic anhydrase II |
| **Transcription Factors And General DNA Binding Proteins** | 2[1)] | + | Histone acetyltransferase type B subunit2 |
| | 6 | - | Nucleophosmin |
| | 7 | + | Proliferating cell nuclear antigen |
| | 9 | + | Elongation factor 1-beta |

a) Induced and repressed genes by *Hoxc8* are marked as + and -, respectively.

## IV. DISCUSSION

Important information concerning the functions of certain homeobox genes during development of the fruit fly *Drosophila* has been gained by comparing their distinct expression patterns in mutant and wild type embryos.[36-38] An essential outcome of these studies was that the domains of highest expression levels of a particular homeobox gene usually corresponded closely to the body regions being most severely affected when the gene was mutated.[39] Accordingly, an important step toward determining the functions of vertebrae homeobox genes is a detailed analysis of their spatial and temporal patterns of expression during development.

Here dynamic expression pattern of *Hoxc8* was described in embryos from day 7.5 to 12.5 p.c. by using whole mount *in situ* hybridization. Since previous data analyzed by *in situ* hybridization had difficulty in analyzing time dependent expression pattern,[8, 14-19] whole mount *in situ* hybridization was performed because it had advantages in examining spatiotemporal expression of *Hoxc8* and determining anterior boundary of expressions three dimensionally. Comparison between the characteristics spatially restricted expression pattern of *Hoxc8* transcripts as determined by wholemount *in situ* hybridization from 8.5 to 12.5 day p.c. embryos and antibody staining of Hoxc8 determined by whole mount immunohistochemistry from 8.5 to 1.5 day p.c. demonstrated high similarity with some differences. While antibody staining of Hoxc8 was observed first at day 8.0 p.c.,[20] mRNA of *Hoxc8* was detected at day 8.5 p.c. in this experiment. Because 8.0 day p.c. is between 7.5 and 8.5 day p.c. used in this study, 8.0 day p.c. might be closer to the starting point of *Hoxc8* expression. Furthermore, weak antibody stain at limb portion was reported,[20] whereas nothing was detected in this experiment. This might be explained with rapid RNA degradation caused by short half-life of *Hoxc8* in RNA. However, strong correspondence was shown in the establishment and maintenance of anterior boundary of expression and downregulation in the posterior region. This consistency in the spatiotemporal expression pattern suggests that *Hoxc8* expression is controlled at the level of transcription.

Our data showed that the distinct anterior boundary of *Hoxc8* expression within the neural tube established on day 10.5 p.c. and shifted cranially during subsequent stages slightly.[40-42] Forward spreading of expression has also been reported in other *Hox* genes in mouse and chick. However, it has not been elucidated whether this apparent shift of *Hoxc8* expression towards the cranial direction is due to anterior movement of *Hoxc8* expressing cells, or whether it is the consequence of *de novo* expression in more anteriorly located cells of the spinal cord. By vital dye marking, it was shown that forwardspreading of *Hoxc4* does not depend on cell migration but rather on *de novo* activation in more anterior cells. The extension of *Hoxc8* expression along the anteroposterior axis is not as extensive as that for more anteriorly expressed genes nevertheless, it also appears to be an important mechanism in the establishment of differential anterior boundaries in the neural tube and mesoderm of more posterior *Hox* genes.

*Hoxc8* was distributed over the entire dorsoventral axis of the neural fold in the unsegmented region at day 8.5 p.c.. This pattern, however, changed drastically after 10.5 p.c.. In 10.5 day p.c. embryos, the neural tube consists largely of a thick inner layer of undifferentiated neuroepithelial cells, known as the ependymal layer. During subsequent developmental stages most of the centrally located ependymal layer is replaced by the surrounding, progressively growing mantle layer, which harbors differentiating neuroblasts. The early phase of *Hoxc8* expression appears to simply demarcate a domain along the body axis, whereas the ventrolateral expression domain within the late phase is associated with the onset and progression of neuronal differentiation within the neural tube. Frozen section of embryos at day 10.5-11.5 p.c. showed that expression predominant in the ventral horn expanded in the ventral and mediolateral region of the mantle layer. A similar distribution along the dorsoventral axis of the neural tube has been observed for most genes in the *Hoxc*-cluster, including *Hoxc4-6,*[43-45] *Hoxc9*[19] and *Hoxc10-13.*[46]

In the case of mesoderm, *Hoxc8* was expressed in most paraxial and intermediate mesoderm, whereas specific distribution of *Hoxc8* was detected at day 12.5 p.c.. *Hoxc8* was detected predominantly in sclerotome cells, which are determined to become

cartilage of vertebrae and rib. *Hoxc8* was on the way to notochord and neural tube away from dermomyotome. Collectively, *Hoxc8* seemed to be related to forming vertebrae and rib. Additionally, strong stain at the rib primordium was detected. As regards rib formations, some explain that sclerotome cells will form proximal protein of the rib and central and distal portion is formed by myotome.[47] On the other hands, others suggest that entire rib originate from the sclerotome.[48] Considering that sclerotome cells which is *Hoxc8* positive were forming rib primordium only in paraxial portion of the rib, not only sclerotome but myotome seems to be involved in rib formation.

In this study, intermediate mesoderm derivatives such as mesonephric tubule and mesonephric duct were stained weakly. At day 8.0 p.c., the pronephric duct arises in the intermediate mesoderm. The cells of this duct migrate caudally and the anterior region of the duct induces the adjacent mesenchyme to form pronephros, which degenerate but the more caudal portion of the pronephric duct persist and serve as the nephric or wolffian duct. As pronephros degenerate, the middle portion of the nephric duct induces mesonephros at day 10.5 p.c., which persists through generation of metanephros.[49] After 12.5 day p.c., expression in metanephros and kidney as well as mesonephros has been reported.[8, 16] The increased level of restriction in the sclerotome expression pattern of *Hoxc8* correlates with an increased level of restriction in mesodermally derived and mesodermally induced tissues.[39] According to this study, firstly, strong expression pattern of *Hoxc8* was detected in the mesoderm of embryos at day 10.5 p.c. and secondly, expression of mesonephros was shown at 11.5 day p.c.. Furthermore, expression of *Hoxc8* in kidney has been reported. These results all together indicates that *Hoxc8* seems to be important to give cellular identity essential for differentiation of kidney including proliferation and differentiation of mesonephros and metanephros.

To examine the putative downstream target genes of *Hoxc8*, 2-DE and MALDI-TOF were performed and proteins that were induced or reduced by *Hoxc8*. Most of the proteins seemingly up or down regulated by *Hoxc8* was divided into 4 categorize; 1) Cytoskeleton and motility, 2) Folding, modification and degradation of protein, 3) metabolism, 4)

transcription and general DNA binding protein.

Among those, carbonic anhydrase II (CAII) was interesting. Deficiency of CAII produced metabolic disorder of bone, kidney and brain.[50] Reduced CAII generated osteopetrosis caused by defects in osteoclasts responsible for bone resorption.[51] Accordingly, reduced expression of CAII by *Hoxc8* is followed by osteopetrosis that might be due to defects in differentiation or malfunctions of osteoclasts. However, this data was contrast to the previous result, in which transient transfection of *Hoxc8* repressed osteoprotegrin (OPG) and induced differentiation of osteoclasts.[33] Differentiation of osteoclasts must be regulated tightly. Therefore, it is important to balance between repression and activation of osteoclast differentiation factor or related proteins at the transcription level. In the case of osteoblast, differentiation is induced by phosphorylation of R-Smads, whereas inhibited by activation of I-Smads by BMP signaling.[52] Similarly, *Hoxc8* might be also involved in both differentiation of osteoclasts and antagonistic mechanism against it.

It is also provocative that *Hoxc8* induced expression of proliferating cell nuclear antigen (PCNA) known as cyclin appearing at the G1/S boundary during the cell cycle.[53] Because of its relationship with cell proliferation, it has been used as a marker of cell proliferation.[54] Considering previous results relevant to relationship between *Hoxc8* and cell proliferation, *Hoxc8* overexpressing transgenic mouse showed that *Hoxc8* affects differentiation of cartilage in the way that leads to the accumulation of proliferating immature chondrocytes or chondrocyte precursor cells.[31] However it still remains to be investigated whether *Hoxc8* actively promote proliferation of cells or if they inhibit differentiation of chondrocytes. In both situations, *Hoxc8* in chondrogenic cells could modulate the rate of chondrocyte differentiation. Immunohistochemistry by antibody against PCNA, a marker for proliferating chondrocytes, confirmed the proliferative phenotype of *Hoxc8* transgenic mouse. Our data, which is correspondent to the previous report,[31] suggested that *Hoxc8* induce or maintain cell proliferation by activating expression of PCNA. Additionally it was also demonstrated that *HOXC8* was expressed

mainly in malignant epithelial cells.[55] Besides, *Hoxc8* overexpression is associated with the loss of tumor differentiation in human prostate cancer and may play a role in the acquisition of the invasive and metastatic phenotype of this malignancy.[55] This fact implies that *Hoxc8* also might be related to the malignancy in cancer cells. Considering PCNA and *Hoxc8* together, upregulated PCNA by *Hoxc8* is suggested to be involved in loss of tumor differentiation and lead to malignancy of cancer cells.

Among 14 protein spots, 10 were induced and 4 were repressed by *Hoxc8* overexpression. It could be partially explained by the following; small amount of endogenous *Hoxc8* was detected in F9 teratocarcinoma cells themselves. If endogenous *Hoxc8* were enough for its function as a repressor, it would be difficult to analyze genes reduced by *Hoxc8* because overexpression of enough repressors doesn't make sever differences. Reduced genes analyzed here seemed to be blocked incompletely, whether directly or indirectly by *Hoxc8*. To study genes repressed by *Hoxc8*, it would be better to block endogenous *Hoxc8* with antisense *Hoxc8*.

## V. CONCLUSION

The aim of this study was to investigate the function of *Hoxc8* during embryogenesis through two distinct experiments. Firstly, *in situ* hybridization on both whole mount and frozen section was carried out to analyze their spatiotemporal expression pattern during early embryogenesis. Secondly, 2-DE and MALDI-TOF was performed to examine *Hoxc8* target genes. The results obtained were as follows:

Expression of *Hoxc8* was first observed at 8.5 day p.c.. In the case of ectoderm-derived neural tube, the distinct anterior boundary of *Hoxc8* expression was established at the level of the $10^{th}$ somite in embryos at day 10.5 p.c.. In the other hands, *Hoxc8* was detected in the paraxial mesoderm with an anterior boundary at the $16^{th}$ somite at day 9.5 p.c. and this expression pattern was maintained through later stages. At day 12.5 p.c., forward progression of the expression pattern was observed and the expression level was reduced. When the expression pattern of *Hoxc8* transcripts was compared with that of Hoxc8 protein reported previously, *Hoxc8* expression seemed to be controlled at the level of transcription.

Frozen section of embryos at day 10.5-11.5 p.c. revealed that predominant expression in the ventral horn of the neural tube expanded in the ventral and mediolateral region of the mantle layer. This ventrolateral expression seemed to be associated with the onset and progression of neural differentiation. Moreover, *Hoxc8* was detected strongly in sclerotome cells on the way to notochord and neural tube. This result showed the possible role of *Hoxc8* in forming vertebrae and rib. Additionally mesonephros also expressed *Hoxc8*. Considering the previous report of *Hoxc8* expression in the kidney, our data imply that *Hoxc8* might be a molecule involved in the differentiation into kidney.

Downstream genes of *Hoxc8* was analyzed through proteomics and categorized as follows; 1) cytoskeleton and motility, 2) protein folding, modification and degradation,

3) metabolism, 4) transcription factors and general DNA binding proteins. Among those downstream genes, CAII was particularly interesting, since CAII deficiency cause osteopetrosis due to defects in osteoclasts. The expression level of CAII was decreased by overexpression of *Hoxc8*. Considering the fact that *Hoxc8* induced osteoclast differentiation, *Hoxc8* might be also involved in the antagonistic mechanism in osteoclast differentiation. Another interesting downstream genes was PCNA which upregulated by *Hoxc8*. Since *Hoxc8* has been reported to be involved in cell proliferation, it could be partly explained by the increased expression of PCNA.

## REFERENCE


1. De Robertis EM, Oliver G, Wright CV. Homeobox genes and the vertebrate body plan**.** Sci Am 1990 Jul;263(1):46-52

2. Kim MH, Kessel M. Homeobox genes as regulators of vertebrate development. AgBiotech News Information 1993 5:301-308

3. McGinnis W, Hart CP, Gehring WJ, Ruddle FH. Molecular cloning and chromosome mapping of a mouse DNA sequence homologous to homeotic genes of Drosophila. Cell 1984 Oct;38(3):675-80

4. Garcia-Bellido A, Ripoll P, Morata G. Developmental compartmentalization in the dorsal mesothoracic disc of Drosophila**.** Dev Biol 1976 Jan;48(1):132-47

5. Sharkey M, Graba Y, Scott MP. Hox genes in evolution: protein surfaces and paralog groups. Trends Genet 1997 Apr;13(4):145-51

6. Maconochie M, Nonchev S, Morrison A, Krumlauf R. Paralogous Hox genes: function and regulation. Annu Rev Genet 1996;30:529-56

7. Deschamps J, van den Akker E, Forlani S, De Graaff W, Oosterveen T, Roelen B *et al*. Initiation, establishment and maintenance of Hox gene expression patterns in the mouse. Int J Dev Biol 1999;43(7 Spec No):635-50

8. Gaunt SJ. Mouse homeobox gene transcripts occupy different but overlapping domains in embryonic germ layers and organs: a comparison of Hox-3.1 and Hox-1.5. Development 1988 May;103(1):135-44

9. Duboule D, Dolle P. The structural and functional organization of the murine HOX gene family resembles that of Drosophila homeotic genes. EMBO J 1989 May;8(5):1497-505

10. Graham A, Papalopulu N, Krumlauf R. The murine and Drosophila homeobox gene complexes have common features of organization and expression. Cell 1989 May 5;57(3):367-78

11. Schughart K, Kappen C, Ruddle FH. Mammalian homeobox-containing genes: genome organization, structure, expression and evolution. Br J Cancer Suppl 1988 Dec;9:9-13

12. Izpisua-Belmonte JC, Falkenstein H, Dolle P, Renucci A, Duboule D. Murine genes related to the Drosophila AbdB homeotic genes are sequentially expressed during development of the posterior part of the body. EMBO J 1991 Aug;10(8):2279-89

13. Simeone A, Acampora D, Arcioni L, Andrews PW, Boncinelli E, Mavilio F. Sequential activation of HOX2 homeobox genes by retinoic acid in human embryonal carcinoma cells. Nature 1990 Aug 23;346(6286):763-6

14. Awgulewitsch A, Utset MF, Hart CP, McGinnis W, Ruddle FH. Spatial restriction in expression of a mouse homoeo box locus within the central nervous system. Nature 1986 Mar 27-Apr 2;320(6060):328-35

15. Utset MF, Awgulewitsch A, Ruddle FH, McGinnis W. Region-specific expression of two mouse homeo box genes. Science 1987 Mar 13;235(4794):1379-82

16. Breier G, Dressler GR, Gruss P. Primary structure and developmental expression pattern of Hox 3.1, a member of the murine Hox 3 homeobox gene cluster. EMBO J 1988 May;7(5):1329-36

17. Holland PW, Hogan BL. Spatially restricted patterns of expression of the homeobox-containing gene Hox 2.1. during mouse embryogenesis. Development 1988 Jan;102(1):159-74

18. Le Mouellic H, Condamine H, Brulet P. Pattern of transcription of the homeo gene Hox-3.1 in the mouse embryo. Genes Dev 1988 Jan;2(1):125-35

19. Erselius JR, Goulding MD, Gruss P. Structure and expression pattern of the murine Hox-3.2 gene. Development 1990 Oct;110(2):629-42

20. Belting HG, Shashikant CS, Ruddle FH. Multiple phases of expression and regulation of mouse Hoxc8 during early embryogenesis. J Exp Zool 1998 Sep-Oct 1;282 (1-2):196-222

21. Gehring WJ, Hiromi Y. Homeotic genes and the homeobox. Annu Rev Genet 1986;20:147-73

22. McGinnis W, Krumlauf R. Homeobox genes and axial patterning. Cell 1992 Jan 24;68(2):283-302

23. Levine M, Hoey T. Homeobox proteins as sequence-specific transcription factors. Cell 1988 Nov 18;55(4):537-40

24. Raman V, Tamori A, Vali M, Zeller K, Korz D, Sukumar S. HOXA5 regulates expression of the progesterone receptor. J Biol Chem 2000 Aug 25;275(34):26551-5
25. Jave-Suarez LF, Winter H, Langbein L, Rogers MA, Schweizer J. HOXC13 is involved in the regulation of human hair keratin gene expression. J Biol Chem 2002 Feb 1;277(5):3718-26
26. La Celle PT, Polakowska RR. Human homeobox HOXA7 regulates keratinocyte transglutaminase type 1 and inhibits differentiation. J Biol Chem 2001 Aug 31;276(35):32844-53
27. Muragaki Y, Mundlos S, Upton J, Olsen BR. Altered growth and branching patterns in synpolydactyly caused by mutations in HOXD13. Science 1996 Apr 26;272(5261):548-51
28. Boncinelli E. Homeobox genes and disease. Curr Opin Genet Dev 1997 Jun;7(3):331-7
29. Dolle P, Dierich A, LeMeur M, Schimmang T, Schuhbaur B, Chambon P *et al*. Disruption of the Hoxd-13 gene induces localized heterochrony leading to mice with neotenic limbs. Cell 1993 Nov 5;75(3):431-41
30. Mortlock DP, Innis JW. Mutation of HOXA13 in hand-foot-genital syndrome. Nat Genet 1997 Feb;15(2):179-80
31. Le Mouellic H, Lallemand Y, Brulet P. Homeosis in the mouse induced by a null mutation in the Hox-3.1 gene. Cell 1992 Apr 17;69(2):251-64
32. Yueh YG, Gardner DP, Kappen C. Evidence for regulation of cartilage differentiation by the homeobox gene Hoxc-8. Proc Natl Acad Sci U S A 1998 Aug 18;95(17):9956-61
33. Wan M, Shi X, Feng X, Cao X. Transcriptional mechanisms of bone morphogenetic protein-induced osteoprotegrin gene expression. J Biol Chem 2001 Mar 30;276(13):10119-25
34. Shi X, Yang X, Chen D, Chang Z, Cao X. Smad1 interacts with homeobox DNA-binding proteins in bone morphogenetic protein signaling. J Biol Chem 1999 May 7;274(19):13711-7
35. Yang X, Ji X, Shi X, Cao X. Smad1 domains interacting with Hoxc-8 induce osteoblast differentiation. J Biol Chem 2000 Jan 14;275(2):1065-72
36. Gehring WJ. The molecular basis of development. Sci Am 1985 Oct;253(4):153-62

37. Department of Genetics, Cambridge, UK. The molecular basis for metameric pattern in the Drosophila embryo. Akam M. Development 1987 Sep;101(1):1-22
38. Scott MP, Carroll SB. The segmentation and homeotic gene network in early Drosophila development. Cell 1987 Dec 4;51(5):689-98
39. Awgulewitsch A, Jacobs D. Differential expression of Hox 3.1 protein in subregions of the embryonic and adult spinal cord. Development 1990 Mar;108(3):411-20
40. Wilkinson DG, Bhatt S, Cook M, Boncinelli E, Krumlauf R. Segmental expression of Hox-2 homoeobox-containing genes in the developing mouse hindbrain. Nature 1989 Oct 5;341(6241):405-9
41. Deschamps J, Wijgerde M.. Two phases in the establishment of HOX expression domains. Dev Biol 1993 Apr;156(2):473-80
42. Gaunt SJ, Strachan L. Forward spreading in the establishment of a vertebrate Hox expression boundary: the expression domain separates into anterior and posterior zones, and the spread occurs across implanted glass barriers. Dev Dyn 1994 Mar;199(3):229-40
43. Geada AM, Gaunt SJ, Azzawi M, Shimeld SM, Pearce J, Sharpe PT. Sequence and embryonic expression of the murine Hox-3.5 gene. Development 1992 Oct;116(2):497-506
44. Gaunt SJ, Coletta PL, Pravtcheva D, Sharpe PT. Mouse Hox-3.4: homeobox sequence and embryonic expression patterns compared with other members of the Hox gene network. Development 1990 Jun;109(2):329-39
45. Oliver G, Wright CV, Hardwicke J, De Robertis EM. Differential antero-posterior expression of two proteins encoded by a homeobox gene in Xenopus and mouse embryos. EMBO J 1988 Oct;7(10):3199-209
46. Peterson RL, Papenbrock T, Davda MM, Awgulewitsch A. The murine Hoxc cluster contains five neighboring AbdB-related Hox genes that show unique spatially coordinated expression in posterior embryonic subregions. Mech Dev 1994 Sep;47(3):253-60
47. Hall BK, Miyake T. Divide, accumulate, differentiate: cell condensation in skeletal development revisited. Int J Dev Biol 1995 Dec;39(6):881-93
48. Huang R, Zhi Q, Schmidt C, Wilting J, Brand-Saberi B, Christ B. Sclerotomal origin of the ribs. Development 2000 Feb;127(3):527-32

49. Gilbert SF. Developmental biology. 6th ed. Massachusetts: Sinauer associates inc publisher; 2000.
50. Ocal G, Berberoglu M, Adiyaman P, Cetinkaya E, Ekim M, Aycan Z, Evliyaoglu O. Osteopetrosis, renal tubular acidosis without urinary concentration abnormality, cerebral calcification and severe mental retardation in three Turkish brothers. J Pediatr Endocrinol Metab 2001 Nov-Dec;14(9):1671-7
51. Lowe C, Yoneda T, Boyce BF, Chen H, Mundy GR, Soriano P. Osteopetrosis in Src-deficient mice is due to an autonomous defect of osteoclasts. Proc Natl Acad Sci U S A 1993 May 15;90(10):4485-9
52. Bai S, Cao X. A nuclear antagonistic mechanism of inhibitory Smads in transforming growth factor-beta signaling. J Biol Chem 2002 Feb 8;277(6):4176-82
53. Matsumoto K, Moriuchi T, Koji T, Nakane PK. Molecular cloning of cDNA coding for rat proliferating cell nuclear antigen (PCNA)/cyclin. EMBO J 1987 Mar;6(3):637-42
54. Wu WY, Xu Q, Shi LC, Zhang WB. Inhibitory effects of Curcuma aromatica oil on proliferation of hepatoma in mice. World J Gastroenterol 2000 Apr;6(2):216-219
55. Waltregny D, Alami Y, Clausse N, de Leval J, Castronovo V. Overexpression of the homeobox gene HOXC8 in human prostate cancer correlates with loss of tumor differentiation. Prostate 2002 Feb 15;50(3):162-9

## 국문요약

# 발생 중인 생쥐 배자에서 Hoxc8 의 발현 양상과 Hoxc8 에 의해 조절되는 유전자 분석

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권 윤 정

혹스 유전자는 시공간 특이적인 발현양상을 보이며 중뇌에서 꼬리까지 전후축을 따라 발현한다. 각 부위는 각기 다른 그룹의 혹스 유전자가 발현하며 이러한 혹스 유전자의 발현양상이 배자의 각기 다른 부위 형성에 기여한다. 본 실험에서는 *Hoxc8* 의 발현양상을 알아봄으로써 그것이 초기 배자에서 언제 어느 부위에 영향을 미치는 지 알아보고자 하였다. 또한 분자적 수준에서 그 기능을 밝히기 위해 프로테옴 분석 기법을 이용하여 그것에 의해 조절되는 유전자의 총체적 분석을 시도하였다.

*Hoxc8 의* 발현양상은 *in situ* hybridization 을 통해 많이 연구되었으며 공통적으로 중배엽 유래 조직과 경흉부의 신경관에서 발현하는 것으로 보고되었으나, 대부분 조직을 절단하여 평면적으로 분석한 것으로서 배자의 발달 시기별로 *Hoxc8* 의 발현 구획을 비교하는 데는 어려움이 있었다. 본 실험에서는 whole mount *in situ* hybridization 을 통해 발생 7.5 일부터 12.5 일까지의 발현을 삼차원적으로 관찰하였다. *Hoxc8* 은 발생 8.5 일에서 처음 발현하여 외배엽과 중배엽에서 각기 그 발현이 조절되는 양상을 보였다. 외배엽의 경우 전방 경계는 10.5 일에서 10 번째 체절로 설정되었고 중배엽의 경우는 9.5 일에 16 에서 20 번째 체절 위치에서 강하게 발현하였다. 이런 발현 양상은 발생 11.5 일까지 유지되었으며 12.5 일에는 발현이 약해지면서 발현부위도 머리 쪽으로 이동했다. 이 번 결과는 항체를 사용하여 *Hoxc8* 의 발현을 관찰한 기존의 결과와 유사한 것으로 초기 장배형성시기

에 배자는 전사 수준에서 *Hoxc8*의 발현이 조절되는 것으로 생각된다.

다음으로 조직절단을 통해 *Hoxc8* 이 발현된 부위와 양상을 살펴보았다. 신경관에서 *Hoxc8* 은 주로 벤트럴 혼에서 발현하며 점차 에펜디멀 레이어주위로 퍼지는데 이는 신경세포 분화의 진행과 관련 있는 것으로 생각된다. 또, 체절에서 분화한 경절에서의 발현은 경절이 신경관을 감싸고 갈비뼈를 형성할 부분에 위치하는 것으로 보아 *Hoxc8* 이 척추와 갈비뼈 분화에 기여할 것으로 생각된다. 한편, 중신에서 발현한 것은 후에 신장에서도 발현이 관찰되었다는 기존의 결과와 함께 생각해 볼 때 *Hoxc8* 이 신장으로의 분화에 관여할 가능성이 있는 것으로 보여진다.

이와 더불어, *Hoxc8*에 의해 조절되는 유전자를 분석하였다. *Hoxc8*이 과발현됨에 따라 발현 양의 차이를 보인 단백질들은 1) 세포골격과 운동성, 2) 단백질의 변형과 분해, 3) 대사작용, 4) DNA 조절 인자와 전사인자에 걸쳐 다양하게 나타났다. *Hoxc8*이 과발현되면 탄산탈수효소 II가 감소하는데 이는 골파괴세포의 장애를 일으킨다. *Hoxc8* 이 골파괴세포의 분화를 유도한다는 기존의 결과와 비교해 볼 때, 이 번 결과에 의하면 *Hoxc8*은 과발현되면서 스스로를 조절하는 기능도 하는 것으로 유추된다. Proliferation cell nuclear antigen (PCNA)는 *Hoxc8* 의 발현이 증가함에 따라 함께 증가하였는데 *Hoxc8* 이 세포의 분화를 억제하고 증식 수준에 머물게 한다는 기존의 결과들과 관련 지어 볼 때 *Hoxc8* 은 PCNA의 발현을 증가 시킴으로써 세포증식을 유지하는 것으로 생각된다.

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핵심되는말 : Hoxc8, *in situ* hybridization, 전방경계, 중신, 2-DE, 조절되는 유전자, 탈탄산효소, PCNA