Using a ChIP-cloning technique, we identified a Zinc finger protein 804a (Zfp804a) as one of the putative Hoxc8 downstream target genes. We confirmed binding of Hoxc8 to an intronic region of Zfp804a by ChIP-PCR in F9 cells as well as in mouse embryos. Hoxc8 upregulated Zfp804a mRNA levels and augmented minimal promoter activity in vitro. In E11.5 mouse embryos, Zfp804a and Hoxc8 were coexpressed. Recent genome-wide studies identified Zfp804a (or ZNF804A in humans) as a plausible marker for schizophrenia, leading us to hypothesize that this embryogenic regulatory control might also exert influence in development of complex traits such as psychosis.

1. Introduction

Hox genes encode transcription factors containing a 61-amino acid motif that enables binding and transcriptional regulation of target gene expression [1–3]. The mammalian Hox genes have 39 members organized in four clusters (HoxA-D) each located on a different chromosome. Resulting from successive duplications during the evolution of an ancestral Hox complex, each cluster contains a series of paralogues (groups I–XIII). Each Hox gene has a conserved helix-turn-helix DNA-binding domain that recognizes and binds to specific sequence motifs (TAAT/ATTA/TTAT/ATAA) located in promoter sequences, intergenic and intronic regions. Such conserved sequence motif is also found repeatedly several times in the genome underscoring a potentially expansive regulatory landscape of Hox genes [4, 5]. Hox expression domains along the anterior-posterior (A-P) axis is characterized as overlapping, with the order and timing of expression correlating to the position of genes along a cluster. The combinatorial expression of various Hox genes constitutes the proverbial “Hox code”: the genetic program that determines cell fates, that is, morphogenesis, growth, and differentiation during embryonic development [6]. Meanwhile, in adult cells, Hox genes assume some poorly understood oncogenic roles. For example, expression of HOXC5 and HOXC8 is selectively elevated in human cervical cancer cells when compared to normal keratinocytes [7]. In human prostate adenocarcinomas, HOXC8 expression is directly correlated with the loss of tumor differentiation based on Gleason scoring [8]. Moreover, Hox genes have been ascribed with “nonconventional” roles such as postnatal neuronal circuit development [9] although data is still scant.

To gain better understanding of the mechanisms by which Hox functions during mouse embryogenesis, the identification and functional analysis of target genes are necessary. In this study, we focused our analysis on the downstream targets of Hoxc8, one of three members of the paralogous group VIII, which our group and other laboratories have extensively studied in the past years [6, 10–13]. Several molecular tools have allowed us to identify the developmental regulatory targets of Hoxc8 in a high-throughput manner. Microarray expression analysis by the
group of Frank Ruddle has identified 34 genes involved in cell adhesion, migration, metabolism, and apoptosis whose expression levels were differentially changed in mouse embryonic cells overexpressing Hoxc8 [6]. Likewise, our group identified, using proteomics, 15 genes associated with cell motility, protein homeostasis, and metabolism [14]. More recently, modified chromatin immunoprecipitation-(ChIP-) based methods have been developed, such as ChIP-cloning, ChIP Display, Differential Chromatin Scanning, and ChIP-chip and DamID chromatin profiling, permitting the screening for DNA-protein interaction in vivo at a genome-wide scale [15]. We took advantage of a ChIP-cloning gene discovery strategy [16] to search for novel downstream target genes of Hoxc8 that may be important during mouse development. We identified 12 putative target sites by ChIP-cloning and subjected these to comparative genomics analysis to identify Hoxc8-binding motifs that are conserved across the mammalian lineage leading to the identification of Zfp804a, as a strong candidate target. We further confirm the regulation of Zfp804a by ChIP-PCR, coexpression analysis and a reporter assay.

2. Materials and Methods

2.1. Animal Preparation. In order to get E11.5 embryos, ICR mice were allowed to mate at 6:00 pm, and presence of a vaginal plug in the following morning indicates the 0.5 day post coitum (dpc) time point. After 11 days, the pregnant mice were sacrificed and E11.5 embryos were taken. Maternal and extraembryonic tissues were removed.

2.2. Cell Culture and Transfection. F9 cells were cultured in DMEM media supplemented with 10% of fetal bovine serum (FBS) and 100 μg/ml penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA) in plates that were coated with 0.1% gelatin. The cells were incubated at 37 °C in a 5% CO2 environment. In 100 mm dishes, upon reaching ~50%, the cells were transfected with 4 μg of pcDNA3 (empty vector control) or pcDNA3:Hoxc8 plasmid DNA using Lipofectamine 2000 system (Invitrogen).

2.3. ChIP Assays. ChIP assays were performed on fresh E11.5 embryos with the head and internal organs removed using a ChIP assay kit (Upstate Biotechnology, Lake Placid, NY, USA) with slight modification. To enrich specificity, immunoprecipitation step was performed thrice using a Hoxc8-monoclonal antibody (MMS-266R, Covance Research products, Princeton, NJ, USA). Immunoprecipitation in the absence of the antibody served as negative control. ChIP-DNA was cloned into pBluescriptII SK(+) vector and its inserts were sequenced [17]. We reported our identification of Hoxc8 gene targets using this ChIP-cloning protocol in our previous paper [10]. In order to verify whether the identified genes are a target of Hoxc8, we PCR-amplified fragments from ChIP-DNA of E11.5 embryos and F9 cells using the primers F, 5′ggagtct-acaaccaatctc 3′ and R, 5′cagggaattttgagcttattattct 3′. PCR was carried out with a 5-minute hot start at 94 °C, followed by 33 cycles of denaturation (94 °C for 1 minute), annealing (1 minute at 55 °C), and extension (1 minute at 72 °C).

2.4. RNA Isolation and RT-PCR. Total cellular RNA was extracted with RNAzol Bee (Tel-test, Inc. Industries Inc., Texas, USA), partitioned with chloroform/isoamyl alcohol and centrifuged at 12 000 rpm at 4 °C for 15 minutes. RNA was precipitated by adding equal volume of cold isopropanol and centrifugation at 12 000 rpm at 4 °C for 15 minutes. Pellet was washed with 70% ethanol, air-dried, and resuspended in DEPC-H2O. RT-PCR was performed on 2 μg of the RNA with the following PCR conditions: 5 minutes hot start at 94 °C, followed by denaturation at 94 °C for 1 minute, annealing step at the specific temperatures for 50 seconds (β-actin, 56 °C, Hoxc8, 56 °C, and Zfp804a, 55 °C), and extension step at 72 °C for 1 minute for 28 cycles. Each reaction was terminated after a final elongation step of 72 °C for 7 minutes. The sequences of the primers were as follows: β-actin (F, 5′catgtttgagactccaacccc 3′; R, 5′gcatctctctgctctgta 3′), Hoxc8 (F, 5′cattacaca-gctcgcccttc 3′; R, 5′ttgaccgagagttacagtc 3′), and Zfp804a (F, 5′tccaggggacataaatct 3′; R, 5′ttacagttttggcgaagctg 3′).

2.5. Effector-Reporter Assay. An effector-reporter assay was done using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). Zfp804a gene fragment was cloned into the pGL3MP vector [18] to make the effector-reporter construct. For the luciferase assay, 2 × 10⁵ F9 cells/well were seeded into a 6-well plate and cultured until 50% of confluency. Four micrograms of effector (pcDNA3:Hoxc8; pcDNA3:Hoxc8 + siHoxc8) were cotransfected in the cells with 1 μg of the reporter (pGL3-MP; pGL2-MP:Zfp804a) and 25 ng of pRL-TK vectors. After 36 hours, the cells were lysed and luciferase activity was measured using the GLOMAX 20/20 luminometer (Promega).

3. Results and Discussion

Hox genes play a role as a “realisator” of body pattern formation [2, 3]. Since the discovery of Drosophila Hox genes in the early 1980s, there has been an avalanche of studies on their roles in mammalian development. However, while much is already known about Hox gene structure and its function, how it determines the precise formation of the body parts along the A-P axis is still enigmatic. In order to gain further insight into the molecular basis of how various Hox genes control tissue fate, it is necessary that their downstream genes are identified and characterized.

We obtained target genes of Hoxc8 by ChIP-cloning [10] and further demonstrated regulation of a candidate gene. ChIP-cloning approach is a modification of the ChIP assay which combines immunoprecipitation of sheared chromatin with subsequent DNA cloning and sequencing (Figure 1). In our ChIP-cloning procedures, we used E11.5 mouse embryos because Hoxc8 expression is relatively elevated at this particular stage compared to other time points [10]. After removal of the head and internal organs, the embryo was fixed with formaldehyde to cross-link DNA-protein
interactions. After the DNA complexes were pulled down by an anti-Hoxc8 monoclonal antibody, purified ChIPed DNA fragments were cloned and sequenced. Out of 146 clones, we identified 12 candidate genes from 16 sequences, most of which contained Hox-binding motifs (see Table 1). As it is logical to assume that Hox-binding will likely occur in the promoter regions of its target genes, in our experiment, all our cloned DNA fragments were derived from intronic and intergenic regions. A similar observation was reported by Birkaya et al. [19] showing that >40% of DeltaNp63-ChIP fragments were from the introns. One of the cloned regions of our candidate targets, calpain 10 (NM_011796.2), does not have a Hox-binding motif implying that if this was confirmed as true target, Hoxc8 may likely regulate the gene via indirect binding, probably in collaboration with a cofactor(s), as in the case of TGF-β signaling with Hoxc8 [20–22]. Among the candidate genes, we chose to study zinc finger protein 804a (Zfp804a; NM_175513) since this cloned intronic region contains ~8.4 Hox-binding sites per 100 bp. In subsequent in silico analysis, however, out of the 75 Hox-binding motifs present, only 4 were conserved across a wide range of mammalian species: human, chimpanzee, rhesus, orangutan, marmoset, cat, horse, dog, opossum, mouse, rat, and guinea pig.

To further demonstrate Zfp804a-Hoxc8-binding, we validated the interaction by performing ChIP-PCR on cell extracts from Hoxc8-overexpressing F9 murine embryonic teratocarcinoma cell line (in vitro) and E11.5 mouse embryos (in vivo). Located in chromosome 2, the Zfp804a gene contains 4 coding exons with a noncontiguous string of Hoxc8-binding motifs within its first intron. We designed primers to target the area where putative Hox-binding sites are abundant (Figure 2(a)). Zfp804a gene was clearly amplified from the DNA samples that had been precipitated by a commercial monoclonal (Figure 2(b)) and an in-house polyclonal antibody (data not shown) but not by an antinouse IgG. The strongest band of Zfp804a band was also detected from input lane which served as the internal control. Our results, therefore, indicate that the Hoxc8 protein stably and directly binds to the first intron of Zfp804a.

Does this binding lead to a modulation of gene expression? Given the fact that the regulation of Zfp804a is largely unknown, we utilized an unbiased effector-reporter assay by cloning the Zfp804a intronic fragment into the minimal promoter plasmid, pGL3-MP (pGL3-MP-Zfp804a). With transfection into F9 cells and assaying for dual-luciferase activity after 36 hours, enhancement of promoter activity relative to pGL3-MP was seen when Hoxc8 expression plasmid was cotransfected compared with the empty vector (pcDNA3 alone). In addition, we were able to demonstrate that the augmented promoter activity was abolished by adding Hoxc8 siRNA (Figure 2(c)). These data suggest that the intronic fragment of Zfp804a may function as an alternative promoter, and the modulation of activity of the minimal promoter [18] by such intronic sequence endorses the functionality of the Hoxc8-Zfp804a interaction.

Finally, we studied whether Hoxc8 modulates expression of Zfp804a. For this, we transiently transfected with a Hoxc8 overexpression construct F9 cells having undetectable levels of Hoxc8 and Zfp804a mRNAs. Compared with the empty vector control, the cells overexpressing Hoxc8 showed induction of Zfp804a (Figure 3(a)) indicating that, in this particular in vitro system, expression of Zfp804a was positively regulated by Hoxc8. To prove in vivo, we analyzed levels of Hoxc8 and Zfp804a mRNA transcripts along the A-P axis of E11.5 embryos. From Figure 3(b), Hoxc8 was expressed in the trunk area (regions 3 to 5) except in head whereas Zfp804a mRNA was seen generally throughout the trunk with only a residual signal in the head area (Figure 3(b)) [23]. Thus, the expression patterns on Zfp804a correlate to some extent with that of Hoxc8 in this developmental stage. Since the regulation target genes by Hox is complex and multifactorial [24, 25], we are cautious in interpreting such data since Hox regulates its target in a context-dependent manner and proves to be both a repressor and activator at the same time [26].

An interesting twist in the relationship of Hoxc8-Zfp804a regulation lies in the recent discovery that its human homologue, ZNF804A, turns out to be a marker for schizophrenia and bipolar disorders [27]. Although
| ACCN no. | Gene name | No. of Hox-binding motifs | Chromosome | Location | Molecular/cellular function |
|----------|-----------|---------------------------|------------|----------|----------------------------|
| NM_175513 | zinc finger protein 804A | 75 | 2 | intron 1 | zinc ion binding; metal ion binding |
| NM_001085495 | ADP-ribosylation factor guanine nucleotide-exchange factor 2 (brefeldinA-inhibited) | 22 | 2 | intron 1 | ARF guanyl-nucleotide exchange factor activity; binding; myosin binding; exocytosis; intracellular signaling cascade; regulation of ARF protein signal transduction |
| NW_001030820 | 118.3 kb at 5′ side: hypothetical protein | 20 | 6 | intergenic region | unknown |
| XM_920612.2 | 22 kb at 3′ side: Similar to lanin A-related sequence 1 protein isoform 2 | | | | unknown |
| NM_028533.2 | 47 kb at 5′ side: hypothetical protein LOC 73410 | 20 | 9 | intergenic region | unknown |
| NM_011916.2 | 50 kb at 3′ side: 5′-3′ exoribonuclease 1 | | | | 5′-3′ exonuclease and hydrolase activities |
| NM_080788.3 | tau tubulin kinase 2 isoform 1,2 | 16 | 2 | intron 10 | nucleotide binding; protein serine/threonine kinase activity; ATP binding; transferase activity |
| NM_153386.2 | Usher syndrome 3A homolg isoform 1,2 | 13 | 3 | intron 1 | sensory perception of sound; photoreceptor cell maintenance; equilibrioception |
| NM_172964.3 | 134 kb at 5′ side: Rho GTPase activating protein 28 | 11 | 17 | intergenic region | GTPase activator activity |
| NM_177278.3 | 221 kb at 3′ side: hypothetical protein LOC320858 | | | | unknown |
| NM_172825.2 | G protein-coupled receptor 128 | 10 | 16 | intron 4 | neuropeptide signaling pathway; G-protein coupled receptor activity |
| NM_001083919.1 | 139 kb at 5′ side: Cardiomyopathy associated 3 isoform 2 | 10 | 2 | intergenic region | unknown |
| NM_020283.2 | 459 kb at 3′ side: UDP-Gal:βGlcNAc β1,3-galatosyl transferase | | | | protein amino acid glycosylation; oligosaccharide biosynthetic process; lipid glycosylation |
| NM_013600 | mutS homolog 5 | 8 | 17 | intron 11 | nucleotide binding; DNA binding; protein binding; ATP binding; mismatched DNA binding; NF-kappaB binding |
| NM_008960.2 | phosphatase and tensin homolog | 4 | 19 | intron 5 | phosphatidylinositol-3-phosphatase activity; protein serine/threonine phosphatase activity; PDZ domain binding |
| NM_011796.2 | calpain 10 | 0 | 1 | intron 5/6 | SNARE binding; calcium-dependent cysteine-type endopeptidase activity; cytoskeletal protein binding; peptidase activity |
**Figure 2:** The mouse *Zfp804a* gene is located in chromosome 2 and has Hoxc8 responsive elements in the intron1 region. (a) *Zfp804a* gene organization. Coding regions or exons (black box) and the introns (grey box) are shown. Hoxc8-binding core sequences are shown in bold and the underlined sequences indicate primer targets for ChIP-PCR. (b) A ChIP experiment was performed using mouse E11.5 embryos and F9 cell line. The DNA-Hoxc8 complexes were immunoprecipitated by a Hoxc8-monoclonal antibody (lane 4). Input (prior to immunoprecipitation) was used for an internal control (lane 1), and mouse IgG and vehicle were used as a negative control (lane 3-4). (c) Hoxc8 activates *Zfp804a* in the effector-reporter assay. Luciferase construct is driven by a minimal promoter fused to the intronic segment of *Zfp804a* (pGL3-MP:Zfp804a; reporter) and cotransfected with a empty vector pcDNA3, pcDNA3:Hoxc8 (effector), and a siRNA for Hoxc8 in F9 cells. Thirty-six hours after transfection, luciferase activities were measured. The experiments were performed in 3 independent trials. The mean values of the luciferase activity are shown as fold change relative over that of the pcDNA3.

**ZNF804A** is uncharacterized and its functions are still unknown to date, it is interesting that fMRI images of ZNF804A SNPs carriers show altered functional coupling between the dorsolateral prefrontal cortex (DLPFC) and the hippocampus [28]. Since genetic variation in dopaminergic and glutaminergic neurotransmission affects DLPFC or hippocampus connectivity, the examination of ZNF804A and HOXC8 in these neurotransmitter cascades would be interesting in the future. During embryogenesis, Hox gene expression is activated prior to rhombomeric segmentation,
and its anterior boundaries, limited to those in the paralogue
groups I–IV, are found only in the neuroepithelia that give
rise to the hindbrain [29]. Because of this bias, no systematic
study on neurobiology of the other Hox paralogues has been
done. Therefore, the finding here is rather surprising and
very intriguing.

Recently, some HOX genes have been reported to be
expressed in adult cells, and misexpression of certain HOX
gene(s) can lead to diseases such as cancer [30]. Therefore
we examined the expression of Hoxc8 in adult mouse brain.
As shown in Figure 3(c), we were also able to verify the
expression of Hoxc8 in adult normal brain tissue, cortex, and
hippocampus by RT-PCR. Interestingly, Zfp804a was only
detected in cortex, but not in hippocampus. Consistent with
this expression patterns, Hoxc8 showed a prominent binding
to Zfp804a in cortex as well as whole brain (Figure 3(d)).

Given that Hox genes are master transcription factors at
the apex of the genetic hierarchy of developmental control, it
is tantalizing to regard that their “fetal” molecular networks
would extend influence to adulthood and more so, in such
complex systems, such as psychosis. A future challenge from
our work is to elucidate the molecular mechanisms respon-
sible for this phenomenon and the potential implications of
Hox regulatory control in human behavior.

Acknowledgments

H.J. Chung, H. Min and S.H. Kim are graduate students
supported by BK21 (Brain Korea 21) scholarship. The
authors thank Kalyani Ruthala for her technical assistance;
Drs. Ethan Patterson (Johns Hopkins University), Jinwoong
Bok, and Heejei Yoon for the helpful discussions, and
JiHyun Ma and Dong-Su Jang for their help in manuscript
preparation. This work was supported by Grants 2008-
0058561 (2010-0000155) and 2009-0070964 from NRF and
partly by 20070401-034-030 from the BioGreen21 Program,
RDA, Korea. H. J. Chung and J.-Y. Lee contributed equally to
this work.

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Figure 3: RT-PCR analysis of the Zfp804a gene expression in vitro and in vivo. Total cellular RNA was isolated (a) in vitro with F9 cells
transfected with pCDNA3 vector (control) and Hoxc8 overexpression vector and (b) in vivo, from mouse E11.5 embryos. (c) Total cellular
RNA was isolated from mouse adult whole brain, cortex, and hippocampus (males, 6 weeks old). (d) A ChIP experiment was performed
using mouse adult whole brain, cortex, and hippocampus. The DNA-Hox8 complexes were immunoprecipitated by a Hox8 monoclonal
antibody (lane 2). Input (prior to immunoprecipitation) was used for an internal control (lane 1), and mouse IgG and vehicle were used as
a negative control (lane 3-4) and DW (lane 5).
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