NEUROD1 Intrinsically Initiates Differentiation of Induced Pluripotent Stem Cells into Neural Progenitor Cells

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Cell type specification is a delicate biological event in which every step is under tight regulation. From a molecular point of view, cell fate commitment begins with chromatin alteration, which kickstarts lineage-determining factors to initiate a series of genes required for cell specification. Several important neuronal differentiation factors have been identified from ectopic over-expression studies. However, there is scarce information on which DNA regions are modified during induced pluripotent stem cell (iPSC) to neuronal progenitor cell (NPC) differentiation, the cis regulatory factors that attach to these accessible regions, or the genes that are initially expressed. In this study, we identified the DNA accessible regions of iPSCs and NPCs via the Assay for Transposase-Accessible Chromatin sequencing (ATAC-seq). We identified which chromatin regions were modified after neuronal differentiation and found that the enhancer regions had more active histone modification changes than the promoters. Through motif enrichment analysis, we found that NEUROD1 controls iPSC differentiation to NPC by binding to the accessible regions of enhancers in cooperation with other factors such as the Hox proteins. Finally, by using Hi-C data, we categorized the genes that directly interacted with the enhancers under the control of NEUROD1 during iPSC to NPC differentiation.

Keywords: chromatin accessibility, Hi-C, induced pluripotent stem cell, NEUROD1, neuronal progenitor cell

INTRODUCTION

Human induced pluripotent stem cells (hiPSCs) have been heavily utilized as experimental platforms by various research areas studying lineage differentiation and modelling of human developmental process and diseases (Cotovio and Fernandez, 2020; Rowe and Daley, 2019; Shum et al., 2015). The need for iPSC is especially high in disease modelling given the difficulties in isolating cells from patients and obtaining the optimal number of cells for experiments. Among different cell lineages from iPSC, neuronal lineage which can be matured to functional neuron is of special clinical interest, and iPSC-derived neuronal differentiation models are already
in use in analysis of neurodegenerative disease such as Alzheimer's disease, Parkinson's disease, Huntington's disease and motor neuron diseases (Chang et al., 2020; Wu et al., 2019). Although iPSC-derived NPC model has been well studied in terms of defining cellular phenotype and neuronal function according to gene expression, the mechanism of gene regulation is still elusive (Kang et al., 2017; Zhang et al., 2013).

Gene expression is tightly regulated during cell fate commitment (Rue and Martinez Arias, 2015), and one extensively studied layer of regulation is epigenetic regulation such as histone and DNA methylation, which regulates the nuclear environments to control gene transcription (Cedar and Bergman, 2009). Other than direct modification, chromatin accessibility to nuclear macromolecules such as transcription factors (TFs) itself is another facet in gene regulation (Klemm et al., 2019). Highly accessible regions of chromatin are considered to be open and promote gene expression, whereas inaccessible regions are closed and inhibit expression (Cedar and Bergman, 2009). It is therefore important to localize the regions with cell-type specific accessibilities as they are inferred to contain genes that determine cell fate (Scott-Browne et al., 2016). Enhancers, for example, have been identified as regions that hold most chromatin accessibility changes during neuronal development (Frank et al., 2015).

The enhancer is one of the cis regulatory elements known to regulate gene expression via physical interaction with target genes from distal genomic regions (Schaffner, 2015). At the point of cell fate transition, lineage-specific enhancers initiate the first wave of gene expression in collaboration with lineage-determining TFs. First discovered and studied in Drosophila, these basic helix-loop-helix (bHLH) TFs are critical in neuronal differentiation, and have been coined proneural factors to emphasize the importance of their role (Ross et al., 2003). Similar studies in mammals initially showed that that a trio of proneural or BAM factors (BRN2 [POU3F2], ASCL1, and MYT1L) initiate changes in chromatin accessibility during the direct reprogramming of dermal fibroblasts into neuronal cells (Wapinski et al., 2013). Further research indicated that NEUROD1 is another proneural factor that promotes neuronal development in a synergistic manner with the BAM factors and is solely sufficient for direct neuronal reprogramming (Pang et al., 2011; Pataskar et al., 2016). However, following the characterization of initial TFs involved in neuronal differentiation and discovery of some target enhancer regions for BAM factors and NEUROD1 by overexpressing the proneural factors in dermal fibroblasts (Wapinski et al., 2013), much still remains unknown about which DNA regions undergo chromatin accessibility changes, where the enhancers for neuronal development are, or which factors can bind to the enhancers during stem cell differentiation.

In this study, we identified cell type-specific accessible chromatin regions of iPSCs and NPCs by Assay for Transposase-Accessible Chromatin sequencing (ATAC-seq) and also noted the DNA regions that changed during differentiation and found that the accessibility of the enhancer, but not the promoter, was related to active histone modification changes. Through motif enrichment analysis, we found that NEUROD1, Hox proteins, and ONECUT1 may control neuronal differentiation by binding to the accessible regions of the enhancer. Furthermore, we identified the initial genes that are controlled by NEUROD1 during iPSC to NPC differentiation using Hi-C sequencing. Finally, our results can be used as additional resource to aid studies of regulatory mechanism in neuronal development-related genes during iPSC to NPC differentiation by chromatin accessibility changes and TF bindings.

MATERIALS AND METHODS

Culture for iPSC cells and iPSC-derived neural progenitor cells

The use of human iPSCs was approved by the Institutional Review Board (IRB) of Yonsei University (permit No. 7001988-201802-BR-119-01E). In this study, we used pre-constructed iPSC cell lines (Cat. No. C-004-5C; Gibco, USA). We cultured iPSC cells on Matrigel (BD Biosciences, USA) with Essential 8 medium (Invitrogen, USA) coating (Chen et al., 2011; 2017; Higuchi et al., 2015). To differentiate iPSC to NPC, an embryoid body (EB) was generated through culturing of human iPSCs for 5-6 days on non-adherent petri dishes in Essential 8 (Invitrogen) with additional supplement of 5 μM dorsomorphin (DM; Sigma-Aldrich, USA) and 5 μM SB431542 (Sigma-Aldrich). EBs were then attached to the new culture dish coated with Matrigel (BD Biosciences) with neural induction medium comprised of DMEM/F12 medium (Invitrogen), 1× N2 supplement (Invitrogen), 1× nonessential amino acids (Invitrogen) for 6 days. When neural rosette formation appears in the center of the EBs, NPCs were collected by using Pasteur pipet (Jin et al., 2019; Seo et al., 2015; Shin et al., 2017).

ATAC-seq

ATAC-seq samples were prepared by described method (Buengoastro et al., 2015). We washed 50,000 cells with cold phosphate-buffered saline (PBS) and resuspended cells by cell lysis buffer (10 mM Tris-HCl [pH 7.4], 10 mM NaCl, 3 mM MgCl2, 0.1% IGEPAL CA-630) and centrifuged cells (10 min, 2,000g at 4°C). After centrifugation, the nuclei pellet were resuspended with Transposase mixture (12.5 μl 2X TD buffer, 12 μl Nuclease-free water and 0.5 μl of Tn5 per sample) in Illumina Nextera Kit (FC-121-1031; Illumina, USA) and incubated for 30 min at 37°C. Tagmented DNA fragments were purified using MinElute PCR purification kit (Qiagen, Germany) following manufacturer’s protocol. For eluted DNA samples, we executed small scale quantitative PCR (qPCR) to determine adequate PCR amplification cycles for library preparation. We checked Ct value for qPCR sample mix (5 μl 2× KAPA HiFi HotStart Ready Mix, 0.1 μl 100X SYBR green, 0.3 μl Nextera Ad1.2 [25 μM], 0.3μl Nextera Ad2.X [25 μM], 2.3 μl Nuclease-free water and 2 μl eluted DNA) and determined PCR amplification cycles with value of Ct+1. We then executed large scale PCR (25 μl 2× KAPA HiFi HotStart Ready Mix, 1.5 μl Nextera Ad1.2 [25 μM], 1.5 μl Nextera Ad2.X [25 μM], 2 μl Nuclease-free water and 20 μl eluted DNA). After PCR step, we cleaned library samples and performed size selection (200-400 bp) by AMPure XP beads (A63881: Beckman Coulter, USA) following manufacturer’s instruction.
quality of libraries was checked by Agilent Technologies 2100 Bioanalyzer (Agilent, USA) regarding concentration and size. Finally, we sequenced libraries with Illumina HiSeq 2500 with 101 bp paired-end reads.

**ATAC-seq data processing and Stage-specific peak calling**

Prior to mapping, quality control steps were executed. We assessed reads by ‘FastQC’ for read quality control and adapter removing process was done by ‘Cutadapt’ (cutadapt -a CTGTCTCTTATACACATCT -q 10 --minimum-length 36 -o sample_1_trimmed.fasqt -p sample_2_trimmed.fasqt sample_1.fasqt sample_2.fasqt). Processed reads were aligned by ‘Bowtie2’ (bowtie2 --very-sensitive -x <genomelIndexName> -X 2000 -1 sample_1_trimmed.fasqt -2 sample_2_trimmed.fasqt). We next removed duplicated reads using Picard MarkDuplicate (1.141) for elimination of technical bias. iPSC and NPC chromatin accessible peaks were determined by MACS2 with specific option (macs2 callpeak --verboese 3 --treatment sample.bam -g hs -B -q 0.05 --extsize 200 --nomodel --shift -100 --nolambda --keep-dup all -f BAMPE --outdir dir --call-summits). To identify the confident stage-specific ATAC peaks, we employed a strategy that confirming background signal of each iPSC and NPC ATAC peaks. We first listed stage-specific peaks based macs2 peak calling results by selecting peaks which was not defined in other cell types. To define cell type-specific accessible regions, we further manipulated macs2 peak calling results. For each stage-specific peak, we plotted normalized ATAC peak intensity (log2RPKM) distribution plot and defined this point as background signal. For each stage-specific peak, we employed a strategy that confirming background signal of each iPSC and NPC ATAC peaks. We first listed stage-specific peaks based macs2 peak calling results by selecting peaks which was not defined in other cell types. To define cell type-specific accessible regions, we further manipulated macs2 peak calling results. For each stage-specific peak, we plotted normalized ATAC peak intensity (log2RPKM) distribution graph of corresponding cell types and another cell types simultaneously. We observed crossing point of two distribution plot and defined this point as background signal. Finally, we defined revised stage-specific ATAC peaks. For data visualization on genome browser, we used bamCoverage tool with following options: --normalizeUsing CPM --effectiveGenomeSize 2864785220.

**Histone ChIP-seq data processing**

We utilized H3K4me3 and H3K27me3 ChIP-seq data for iPSC and NPC in Gene Expression Omnibus (GEO) (GSE156723). For read alignment, we used BWA (0.7.7) to map reads to the human genome sequence (hg19). We further removed duplicated reads using Picard MarkDuplicate (1.141). We further inspected the quality of the ChIP-seq experiment by measuring FRIP (fraction of reads in peaks) using ChIPQC (3.3). For peak calling, we used MACS2 (2.1.0) to call the significantly enriched ChIP region compare to the input IgG control “q 0.01 (FDR < 0.01%)”. For the ATAC peak intensity comparison, we generated the normalized RPKM values by counting the number of reads for 50 bp bins then dividing by the total sequencing depth.

**Motif analysis**

To identify specifically enriched motifs in ATAC peak groups, we used the HOMER algorithm with default options (Heinz et al., 2010). We analyzed the known motifs results derived from published data which are provided by HOMER algorithm.

**RNA-sequencing data processing**

We utilized RNA-seq data for iPSC and NPC in GEO (GSE156723). Sequenced reads were mapped and aligned to the hg19 reference using TopHat2 (v2.1.0) and STAR (v2.6.0a) with the support of GRCh37 reference. From the mapped reads, we executed calculation of TPM (transcripts per million) using RSEM (v1.2.31) at the gene level. For data visualization browser, bamCoverage (deepTools 3.5.0) was used with default options.

**In situ Hi-C library preparation**

In situ Hi-C was performed on with iPSC and NPC cells, each comprising two biological replicates. About 1 million cells were used for crosslinking with 1% formaldehyde. In situ Hi-C was then conducted on the samples using protocol modified from previously study (Rao et al., 2014). Briefly, crosslinked cells were lysed (10 nM Tris-HCl [pH 8.0], 10 mM EDTA) with protease inhibitor (Roche, Switzerland). After sonication (S220: Covaris, USA), the sonicated chromatin samples were incubated with H3K27ac antibody (39133: Active Motif, USA) and magnetic bead (10001D; Thermo Fisher Scientific, USA) for 4 h in 4°C with rotation. At the same time, input chromatin was stored from sonicated samples to be used as input control. After incubation, the bead complex was washed by RIPA-α (140 mM NaCl, 1 mM EDTA pH 8, 0.5 mM EGTA pH 8, 1% Triton X-100, 0.1% SDS, 0.1% sodium deoxycholate, 10 mM Tris pH 8) with 4 times. The chromatin immunoprecipitated complex was treated with RNaseA (19101: Qiagen) and executed overnight reverse-crosslinking at 68°C. The chromatin immunoprecipitated DNA was extracted using AMPure XP beads (A63881: Beckman Coulter), and libraries were prepared using NEB Next Ultra II DNA library Prep Kit (E7645; NEB, USA) following the manufacturer’s instruction. The ChIP-seq libraries were sequenced in 150 bp paired-end mode using Illumina NovaSeq 6000 platform.

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Hi-C data processing

Hi-C data processing is executed by described method in Yang et al. (2018). Raw reads were aligned to hg19 human reference genome. To focus on cis-chromosomal interactions, invalid Hi-C reads were removed by criteria which defined in previous studies: inter-chromosomal interactions, putative self-ligations, and non-restriction site specific ligations (Dixon et al., 2015; Schmitt et al., 2016). Valid cis-interactions were collected to build raw interaction frequency matrix with 5 kb resolution. A negative binomial model-based normalization pipeline removed biases of the interaction frequency matrix. Finally, distance dependent background signal was used to normalize for emphasizing biologically significant long-range chromatin interactions.

Data deposition

The NGS sequence data generated in this paper have been deposited in the GEO database, www.ncbi.nlm.nih.gov/geo (accession No. GSE158382).

RESULTS

Identification of chromatin accessibility alteration regions during iPSC to NPC differentiation

Before producing ATAC-seq data for iPSC to NPC, we confirmed our differentiation system by detecting pluripotency markers for iPSC (Oct4 and E-cadherin) and neuronal markers for NPC (Sox2 and Nestin) (Fig. 1A). To identify DNA regions that become accessible during neuronal differentiation, we performed ATAC-seq on both iPSCs and NPCs. We made biological replicates of the samples for ATAC-seq, confirmed their reproducibility, and proceeded to integrate them for further analysis (Fig. 1B). At first, we defined ATAC peaks in each cell type using MACS2 with specific options (Zhang et al., 2008). By using ChiPseeker with default settings (Yu et al., 2015), we observed that most of the ATAC peaks were located at a distance over 5 kb from the TSS, suggesting that changes in chromatin accessibility were positively linked to H3K4me3 and H3K27ac, which have roles in neuronal development and neural function. Using RNA-seq data, we confirmed that the gene expression was related to changes in chromatin accessibility (Fig. 2B). For example, the expression of ACTB and GAPDH from Group C were maintained in both iPSC and NPC samples as validated by RNA-seq analysis. Chromatin accessibility was high in the promoters of these genes in both iPSC and NPC samples (top of Fig. 2B). In the case of POU5F1 and ETS1, their expression level decreased after differentiation into NPC, and promoter chromatin accessibility consequently decreased (middle of Fig. 2B). By contrast, both expression level and promoter accessibility of NEUROG2 and HES1 increased after differentiation into NPC (bottom of Fig. 2B). We also performed gene ontology analysis with each group by DAVID (Fig. 2C). The genes of Group C were involved with cell division, cell cycle phase, and transcription initiation, which are related to general cellular function. The genes of Group I were involved with somatic stem cell population maintenance. The genes from Group N were involved with neuronal differentiation and function terms such as midbrain development, Wnt signaling pathway, and axon guidance. Taken together, we concluded that our three ATAC peak groups correctly reflect the properties of each cell type.

Chromatin accessibility changes in enhancer reflected during iPSC to NPC differentiation

To further examine the features of each ATAC peak group, we decided to look into any histone modification changes corresponding to DNA accessibility during differentiation into NPC. To do this, we utilized three histone ChIP-seq data sets: H3K4me3 (active histone marker), H3K27me3 (repressive histone marker), and H3K27ac (active histone and enhancer marker). Group I showed a high level of H3K27me3 and low levels of H3K4me3 and H3K27ac compared with Group C (Fig. 3A). By contrast, Group N showed a lower level of H3K27me3 and elevated levels of H3K4me3 and H3K27ac, suggesting that changes in chromatin accessibility were positively linked to levels of H3K4me3 and H3K27ac and negatively to H3K27me3. For further analysis, we sub-categorized the groups into either promoter or enhancer. The promoter
Fig. 1. Defining the iPSC and NPC-specific chromatin accessible regions by ATAC-seq. (A) Immunocytochemistry data of iPSC (OCT4 and E-Cadherin) and NPC (Nestin and Sox2). Scale bars = 100 μm. (B) Scatter plot of RPKM value for biological replicates of iPSC (left) and NPC (right) ATAC library. For all peaks defined by MACS2, RPKM of each cell type was calculated and compared with each other. Then we checked Pearson correlation coefficient. (C) Genomic distribution of iPSC and NPC ATAC peaks. The data was generated by ‘ChIPseeker’. (D) Genomic localization of common (left), iPSC-specific (middle) and NPC-specific (right) ATAC peaks. The center of plots indicates transcription start site (TSS). (E) Density plots of the RPKM values for iPSC (blue) and NPC (red) samples. Three graphs are presented representing ATAC-seq common (left), iPSC-specific (middle), and NPC-specific (right) ATAC peaks. RPKM was normalized using a quantile normalization method. The crossing point of iPSC and NPC plots in the iPSC-specific and NPC-specific ATAC peaks is marked with a dotted line.
Fig. 2. Cell type-specific ATAC peaks and associated genes. (A) A heatmap of hierarchical clustering for iPSC and NPC ATAC-seq signals for common (Group C) and re-defined iPSC (Group I) and NPC (Group N) ATAC peaks based on Fig. 1. Group C involved house-keeping genes, Group I involved stem cell maintenance related genes, and Group N involved neural-related genes. (B) A graphical comparison of ATAC-seq and mRNA-seq reads for representative genes associated with Group C, Group I, and Group N is presented by the WashU Genome browser. The intensity of ATAC-seq and mRNA-seq read counts was calculated by command of bamCoverage in deepTools. (C) Results of gene ontology for common (upper left), iPSC-specific (upper right), and NPC-specific (lower left) ATAC peaks associated genes. Genes located in +/- 5 kb from ATAC peak regions were associated with each ATAC peak. The P value was calculated using the Benjamini-Hochberg method in DAVID.
Fig. 3. Epigenetic modification of promoters and enhancer regions according to cell type-specific ATAC peaks. (A) Boxplots representing the differential level of H3K4me3, H3K27me3, and H3K27ac during iPSC to NPC differentiation in the common, iPSC-specific, and NPC-specific ATAC peak groups. Scale is log2(fold change). For the box-and-whisker plots, the upper and lower bounds of the boxes represent the 75th and 25th percentiles, respectively, and the horizontal lines indicate the median value. The upper and lower error bars indicate the 90th and 10th percentiles, respectively. **p < 0.01 (unpaired t-test, Mann–Whitney test, two-tailed). (B) Averaged RPKM signal intensity of H3K4me3, H3K27me3, and H3K27ac in common and cell type-specific ATAC peak groups. The graphs represent the histone modifications to promoters (upper) and enhancers (lower) of each ATAC peak group.
group was determined as the regions located within +/− 2 kb from the TSS, and the enhancer group was defined from the H3K27ac peak data with ENCODE candidate Regulatory Elements (cRE) data. We calculated the average levels of H3K4me3, H3K27me3, and H3K27ac respectively, and then profiled them in the regions of +/− 500 bp from the ATAC peak summit (Fig. 3B). During iPSC to NPC differentiation, the level of H3K4me3 in the promoter was decreased and the level of H3K27me3 was increased in the iPSC-specific peak (Group I), as expected. Surprisingly, no change was observed for H3K4me3 and H3K27me3 in Group N, even after differentiation into NPC. However, in the enhancer, the level of H3K27ac was relatively low in the iPSC-specific peak (Group I) and increased in the NPC-specific peak (Group N), corresponding with the differentiation process. Taken together, we concluded that the changes to chromatin accessibility have more effect on the activity of the enhancers rather than promoters.

**NEUROD1 binds to the accessible regions of the enhancers during differentiation into NPC**

It was reported that the cellular differentiation processes are regulated by specific TFs that initiate chromatin structure changes via cell type specific enhancers (Lupien et al., 2008). We performed TF motif enrichment analysis targeting our cell type specific ATAC peaks in the enhancer regions by HOMER analysis (Heinz et al., 2010). At first, we sought to find TFs that satisfy our criteria of P value (< 10^{-10}) and enrichment ratio (1.4) in each ATAC peak group. We clustered the TFs with -log (P value) and then found three groups: Group TF R (recurrence), Group TF I (iPSC-specific), and Group TF N (NPC-specific) (Fig. 4A). We found that the binding motifs for POU5F1 (OCT4) and SOX family members such as SOX2 and SOX9 were enriched in Group TF R. Although OCT4 and

![Fig. 4. Identification of TFs associated with cell type-specific ATAC peaks.](image-url)
SOX2 are best known to induce pluripotency and maintain stemness, some papers also suggest crucial roles in NPC development (Cimadamore et al., 2011; Episkopou, 2005; Mitchell et al., 2014). Thus, it was not surprising that OCT4 and the SOX family were included in Group TF R. However, many homeo-domain containing genes (HOXA2, HOXA9, HOXB8, HOXC9, PHOX2A, PBX3, POU3F2 [Brn2]) and NEUROD1 were included in Group TF N, which suggests that their binding motifs were exclusively enriched in NPC-specific accessible DNA regions and rarely found in any other DNA regions. Zinc finger domain genes (NR4A1, ONECUT1) and paired box genes (PAX3, PAX6) were also found in Group TF N. To further understand the TFs in Group TF N, we performed a k-means clustering analysis. The associated TFs could be further classified into five groups: Group 1 (HOX and NEUROD1 diminished), Group 2 (HOX dominant), Group 3 (NEUROD1 with PBX1 and PKNOX1), Group 4 (NEUROD1 and ONECUT1), and Group 5 (NEUROD1 dominant) (Fig. 4B). The five groups were mainly classified by the degree of NEUROD1 expression; therefore, we concluded that NEUROD1 was a major determinant of NPC differentiation.

First line of genes that are induced during differentiation from iPSC into NPC

To characterize these five groups of TFs, we sought to determine which genes were connected to them by utilizing Hi-C data to analyze the physical chromatin interactions. We made an intra-chromosomal interaction matrix with 5 kb resolution, linked enhancers to target genes, and performed gene ontology analysis (Table 1). As a result, we found that Group 1 (HOX and NEUROD1 diminished) included genes related to organ morphogenesis, cellular differentiation, and the Hippo signaling pathway. Group 2 (HOX dominant) included genes linked to neural tube development and the Wnt signaling pathway, and Group 3 (NEUROD1 with PBX1 and PKNOX1) were linked to the canonical Wnt signaling pathway and cell cycle related genes. Group 4 (NEUROD1 and ONECUT1) included genes related to neural crest cell migration, neural tube closure, and axon guidance. Lastly, Group 5 (NEUROD1 dominant) were linked to genes that promote cell proliferation, except keratinocytes, and promote the Notch signaling pathway (Table 1). Interestingly, Group 2 and Group 4 were related to neuronal development terms; however, overall the genes from Groups 1 to 5 were required for intrinsic NPC development from iPSCs.

DISCUSSION

Profiling chromatin accessibility changes during differentiation provides an insight into the regulatory mechanism of lineage-specific genes. These chromatin accessibility changes are mediated by a certain combination of TFs, so-called pioneer factors (Frank et al., 2015; Schaffner, 2015; Scott-Browne et al., 2016). The Yamanaka factors, which consist of OCT4, SOX2, NANOG, and MYC, are the most well-recognized pioneer factors that convert fibroblasts into stem cells (Takahashi and Yamanaka, 2006). Pioneer factors involved in several lineages have been discovered as well, including GATA4, MEF2C, and TBX5 which are able to induce direct reprogramming of fibroblasts into cardiomyocyte-like cells (Qian et al., 2013). Pioneer factors related to neuronal differentiation are also termed proneural factors, which mainly have BHLH structures (Baker and Brown, 2018; Bertrand et al., 2002). It is known that proneural factors, such as the BAM factors, NEUROD1, and NEUROG2, are able to bind nu-

Table 1. Target genes linked with enhancers that are associated with NPC-specific ATAC peaks

| Enhancer-TF group | P value | Genes |
|------------------|---------|-------|
| Group 1 NEUROD1 and HOX diminished | | |
| Organ morphogenesis | 1.80E-02 | GATA2, LHX3, SIX3, CDX1 |
| Cell differentiation | 1.50E-02 | DHC7, DDX41, CSF1 |
| Hippo signaling pathway | 1.90E-03 | SOX2 |
| Group 2 HOX dominant | | |
| Neural tube development | 1.60E-03 | EPHA2, TCF7 |
| Wnt signaling | 2.20E-02 | SKP1, CUL1, HHEX, MCC, SFRP2 |
| Group 3 NEUROD1 with PBX3 and PKNOX1 | | |
| Canonical Wnt signaling pathway | 2.90E-02 | NKX2-5, WNT7A |
| Cell cycle | 3.30E-02 | PIM1, APEX2, CCND1 |
| Group 4 NEUROD1 with ONECUT1 | | |
| Neural crest cell migration | 5.80E-04 | EFNB1, GBX2 |
| Neural tube closure | 9.10E-03 | CECR2, LH2, FZD3, SKI |
| Axon guidance | 3.30E-02 | WNT3A, DVL1, GDF7 |
| Group 5 NEUROD1 dominant | | |
| Positive regulation of cell proliferation | 2.00E-02 | CTBP2, MFNG, DTX4 |
| Negative regulation of keratinocyte proliferation | 3.40E-02 | EFNB2, EPP1K1, KIF1, SFN |
| Positive regulation of Notch signaling pathway | 2.70E-02 | GSX2, SLC35C2 |

We linked enhancers and genes by Hi-C interaction analysis with 5 kb resolution. Interactions with a ≥ 2-fold change above the background signal and a > 5 of normalized interaction counts were included in the analysis. P values were calculated using the Benjamini-Hochberg method in DAVID.
Neuronal Differentiation of Stem Cells and NEUROD1

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pressed in the spinal cord (Philippidou and Dasen, 2013). The HOX 1 to 5 genes are highly expressed in the hindbrain, while HOX 4 to 11 are predominantly expressed in the spinal cord (Philippidou and Dasen, 2013). The HOX 1 to 5 genes are highly expressed in the hindbrain, while HOX 4 to 11 are predominantly expressed in the spinal cord (Philippidou and Dasen, 2013). PHOX2 is known to play a role in neuronal development (Brunet and Pattyn, 2002; Dubreuil et al., 2002). PBX and PKNOX1 controls the localization each other, and PBX3 is highly expressed in the nervous system development (Ferretti et al., 2006; Rhee et al., 2004). In addition to the homeobox genes, the zinc finger proteins also play crucial roles in neuronal development (Lamar et al., 2001; Qin et al., 2009; Shimizu et al., 2010). It is known that NR4A1, a member of the nuclear receptor family of TFs, has a role in regulating neuronal development in the central nervous system, and together with ONECUT1/2, is highly expressed in the developing mouse retina (Akiyama et al., 2008; Honkaniemi and Sharp, 1999; Wu et al., 2012). Furthermore, PAX3 and PAX6 which belong to the paired box gene family, have distinctive roles in early neuronal differentiation and the developing spinal cord (Bel-Vialar et al., 2007; Terzic and Saraga-Babic, 1999). Finally, based on physical chromatin interactions with the above TFs, we were able to identify the first line of genes involved in neuronal lineage specification. Our findings carry significance—while various studies involving iPSC-derived neuronal disease models have suggested causal relationship between non-coding disease variants and defect of regulatory elements, giving rise to deregulated TF binding to neuronal cell-type specific accessible regions in neuronal diseases such as Alzheimer’s disease and schizophrenia (Forrest et al., 2017; Schwartzentuber et al., 2018; Zhang et al., 2020)—there still remains a lack of information on the downstream genes that are affected by defective regulatory elements. Cell fate specification consists of multiple steps, necessitating precise control of gene regulation (Frank et al., 2015) and we have provided a framework that is able to accurately identify genes involved in early neuronal differentiation from iPSCs to NPCs through the integrated analysis of chromatin accessibility, histone modification and chromatin spatial organization.

While the role of NEUROD1 in inducing changes to chromatin accessibility during direct reprogramming process (Matsuda et al., 2019) has been recognized, other factors that cooperate with NEUROD1 are still unknown, particularly during the intrinsic developmental process from iPSC into NPC. To the best of our knowledge, our study is the first report to fully delineate the following: 1) localization of chromatin sites that are first opened, 2) corresponding TFs that bind to these sites, and 3) initial genes under regulation of the TFs during neuronal differentiation. Although there remains a need to characterize expression dynamics of NeuroD1 during iPSC to NPC differentiation, this study sheds light on the understanding of normal early neuronal development and holds potential to be used as a resource in comparing with neuronal disease model using iPSC.

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AUTHOR CONTRIBUTIONS

W.Y.C. and Y.J.K. conceived and designed the study. W.Y.C. and A.J.L. produced and processed next generation sequencing data and W.Y.C. analyzed the data. A.N.C. performed cellular work for iPSC maintaining and differentiation. I.J. and S.W.C. contributed advice in experiment and data analysis. W.Y.C., J.H.H., and L.K.K. wrote the manuscript. L.K.K. and Y.J.K. supervised the project.

CONFLICT OF INTEREST

The authors have no potential conflicts of interest to disclose.

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