

# The chromatin accessibility landscape in the dental pulp of mouse molars and incisors

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The dental pulp is a promising source of progenitor cells for regenerative medicine. The natural function of dental pulp is to produce odontoblasts to generate reparative dentin. Stem cells within the pulp tissue originate from the migrating neural crest cells and possess mesenchymal stem cell properties with the ability to differentiate into multiple lineages. To elucidate the transcriptional control mechanisms underlying cell fate determination, we compared the transcriptome and chromatin accessibility in primary dental pulp tissue derived from 5–6-day-old mice. Using RNA sequencing and assay for transposase-accessible chromatin using sequencing (ATAC-seq), we correlated gene expression with chromatin accessibility. We found that the majority of ATAC-seq peaks were concentrated at genes associated with development and cell differentiation. Most of these genes were highly expressed in the mouse dental pulp. Surprisingly, we uncovered a group of genes encoding master transcription factors that were not expressed in the dental pulp but retained open chromatin states. Within this group, we identified key developmental genes important for specification of the neural crest, adipocyte, neural, myoblast, osteoblast and hepatocyte lineages. Collectively, our results uncover a complex relationship between gene expression and the chromatin accessibility landscape in the mouse dental pulp.

**Keywords:** dental pulp, ATAC-seq, chromatin; gene body, promoter

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**Abbreviations:** ATAC-seq, assay for transposase-accessible chromatin using sequencing; MSCs, dental pulp-derived mesenchymal stem cells; RNA-seq, RNA sequencing

## INTRODUCTION

Over the past few years, dental pulp-derived mesenchymal stem cells (MSCs) have emerged as strong candidates for tissue engineering and regenerative medicine (Masuda *et al.*, 2021; Augustine *et al.*, 2021; Gan *et al.*, 2020; Bonaventura *et al.*, 2020). MSCs are capable of differentiating into multiple lineages such as osteogenic, angiogenic, myogenic, and chondrogenic cells (Nagata *et al.*, 2021; Luo *et al.*, 2018; Potdar *et al.*, 2015). However,

we know very little about the molecular mechanisms that determine specification of MSCs along these lineages. The regulation of MSCs during cell fate determination is a complex process and can be delineated at the level of chromatin accessibility. A growing body of evidence indicates that the higher-order chromatin architecture is a critical component of gene regulation and cell fate specification (Harada *et al.*, 2017; Guo *et al.*, 2017; Hernandez-Hernandez *et al.*, 2020). Genome-wide chromatin accessibility can be measured based on the assay for transposase-accessible chromatin using sequencing (ATAC-seq), a powerful method for profiling open chromatin regions and delineating regulatory sequences in the mammalian genome (Marinov *et al.*, 2021).

Chromatin accessibility deduced from ATAC-seq is often used with RNA sequencing (RNA-seq) to correlate *cis*-acting regulatory regions such as enhancers and promoters with gene expression (Nair *et al.*, 2021). Both approaches have become increasingly powerful for investigating distinct differences in the regulatory wirings among closely related cell types or tissues (Miao *et al.*, 2021). For example, using ATAC-seq and RNA-seq, Arenas-Mena and others reported evidence for the existence of a gene regulatory hierarchy during sea urchin embryogenesis (Arenas-Mena *et al.*, 2021). In another study, chromatin accessibility and transcriptome analyses provided a more refined understanding of differences in the transcriptional programs between embryonic and extraembryonic tissues (Zhang *et al.*, 2021a).

We reasoned that employing ATAC-seq could provide insights into the chromatin states and associated regulatory regions of key developmental genes in the mouse dental pulp. Using ATAC-seq and RNA-seq, we analyzed chromatin accessibility and gene expression of master regulators of cell lineage and fate determination in the mouse dental pulp tissue.

## MATERIALS AND METHODS

### Primary dental pulp isolation and genome-wide sequencing

Primary pulp from mouse incisors and molars was prepared from 5-6-day-old mice according to previously described procedures (Balic *et al.*, 2010). ATAC-seq and RNA-seq services were performed by Active Motif (Carlsbad, CA). RNA isolation was performed using the RNAeasy Mini/Midi kit (Qiagen, Germantown, MD). RNA-seq was performed using an Illumina NextSeq 500

to generate 42-nt paired-end sequences. For ATAC-seq, the paired-end 42 bp sequencing reads generated by Illumina NextSeq 500 sequencing were mapped to the genome using the Burrows-Wheeler Aligner algorithm with default settings.

### Computational analyses

Alignment information for each read was stored in the BAM format. Genomic regions with high levels of transposition/tagging events were determined using the MACS2 peak-calling algorithm (Zhang *et al.*, 2008). Since both reads (tags) from paired-end sequencing represent transposition events, both reads were used for peak-calling but were treated as single, independent reads. To identify the density of the transposition events along the genome, the genome was divided into 32 bp bins and the number of fragments in each bin was determined. For this purpose, the reads were extended to 200 bp, which is close to the average length of the sequenced library inserts. In the default analysis, the tag number of all samples was reduced (by random sampling) to the number of tags present in the smallest sample. To compare peak metrics between two or more samples, overlapping intervals were grouped into “merged regions”, which were defined by the start coordinate of the most upstream interval and the end coordinate of the most downstream interval (= union of overlapping intervals). In locations where only one sample had an interval, this interval defined the merged region. After defining the intervals and merged regions, their genomic locations along with their proximities to gene annotations and other genomic features were determined and presented in Excel spreadsheets.

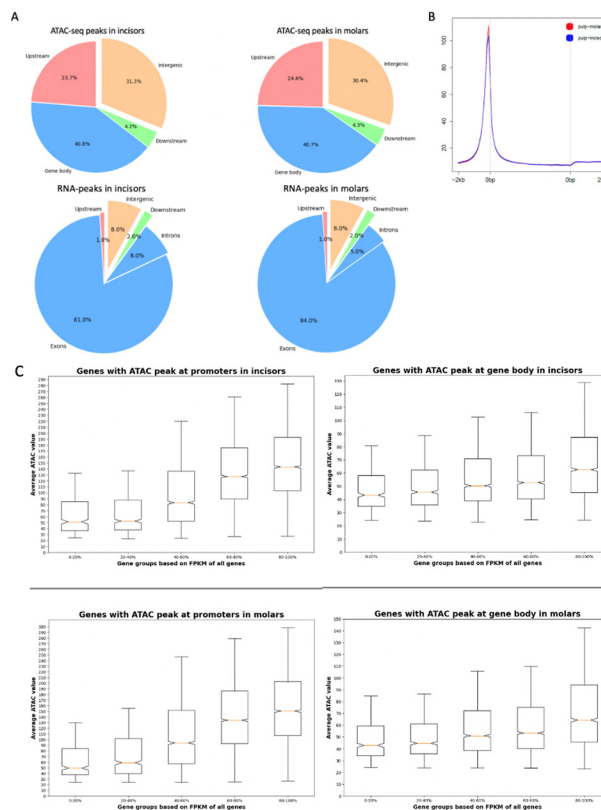
To clarify the relationship between ATAC-seq enrichment and RNA expression, we used a previously published approach (Wang *et al.*, 2016) where genes are separated into five groups (10–20%, 20–40%, 40–60%, 60–80% and 80–100%) based on their mRNA expression levels. Box plots of average ATAC-seq peak values were generated for each group of genes in promoter and gene bodies for studying both incisors and molars.

The RNA-seq fragments were mapped to the reference genome mm10 using the STAR aligner (Dobin *et al.*, 2013). The fragment assignment step was carried out to count the number of fragments overlapping the genomic sequence. Only the read pairs that had both ends aligned at the same chromosome and the same DNA strand were considered for subsequent analysis. The feature count (FPKM assignment to genes) was performed using the Subread package (Liao *et al.*, 2014). Gene annotations were originally from the NCBI RefSeq database and then adapted by merging the overlapping exons from the same gene to form a set of disjoint exons for each gene. After obtaining the same table containing the fragment (or read) of genes, differential analysis was performed using DESeq2 (Love *et al.*, 2014).

## RESULTS

### Distribution of the ATAC-seq peaks in the mouse dental pulp genome

We evaluated the chromatin accessibility landscape in the mouse dental pulp from incisors and molars by ATAC-seq. Our findings demonstrated a high signal-to-noise ratio in the ATAC-seq signals in the pulp genome. We observed that ATAC-seq reads were elevated in the gene bodies (40.8% in the incisor and 40.7% in the mo-



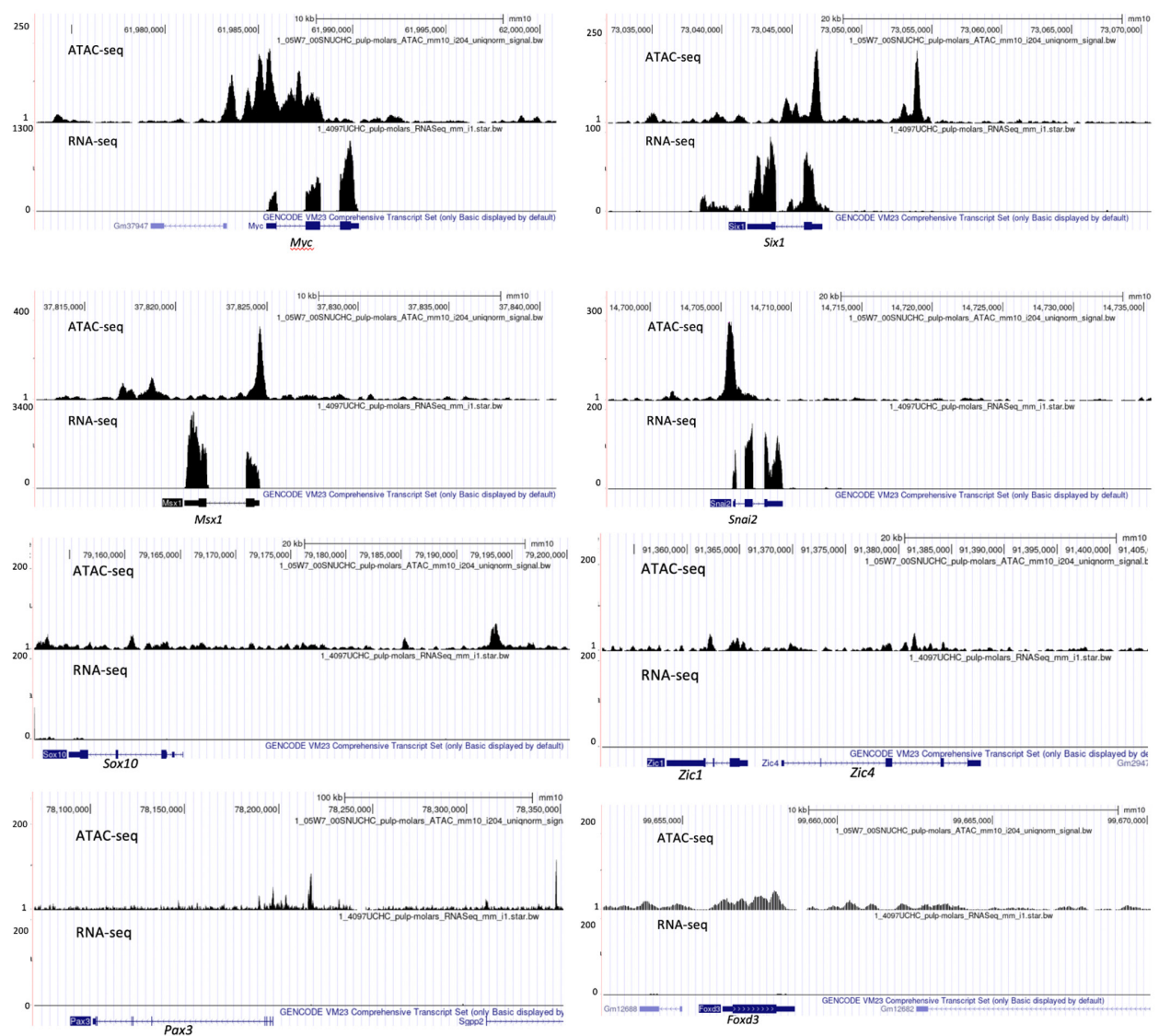
**Figure 1. Global distribution of the ATAC-seq peaks in the mouse dental pulp genome.**

(A) ATAC-seq peaks are primarily enriched in the intergenic regions and gene bodies of both the incisor and molar genomes. This ATAC-seq distribution pattern is similar to the RNA-seq reads. (B) The ATAC-seq signal is enriched at the TSS of the pulp genome. (C) The relationship between ATAC-seq enrichment and gene expression. Genes were separated into five groups according to their RNA expression levels (from low to high: 0–20%, 20–40%, 40–60%, 60–80% and 80–100%) and ATAC-seq peaks were mapped along the promoter and gene body regions. In the dental pulp genome, strong ATAC-seq signal was mapped to the promoters and gene bodies of highly expressed genes (i.e., the 80–100% group).

lar genomes) (Fig. 1A), and were also enriched in the intergenic regions (31.3% in the incisor and 30.4% in the molar genomes). Within the upstream regulatory regions, ATAC-seq peaks were enriched in the incisor and molar genomes (23.7% and 24.6%, respectively). A relatively small level of ATAC-seq reads was also mapped in the downstream regulatory regions (4.2% in the incisor and 4.3% in the molar genomes). This ATAC-seq distribution pattern is similar to the RNA-seq reads. RNA-seq peaks were enriched in exons of the incisor and molar genomes (81% and 84%, respectively). As a common feature, a strong ATAC-seq signal was centered in the vicinity of gene transcription start sites (TSSs), which is consistent with other studies (Fig. 1B).

### The ATAC-seq signal at promoters and gene bodies is correlated with RNA expression

To analyze the correlation between gene expression and the ATAC-seq signals at the promoter and gene body regions, we used a previously published approach (Wang *et al.*, 2016). Briefly, we separated genes into five groups according to their expression levels (from low to high: 0–20%, 20–40%, 40–60%, 60–80% and 80–100%)



**Figure 2. Chromatin accessibility varies across the core master regulators of neural crest development.**

Key developmental genes involved in neural crest specification, i.e., *Six1*, *Snai2*, *Myc* and *Msx1*, were well expressed in the mouse dental pulp and retained the open chromatin state. *Sox10*, *Pax3*, *Zic1*, *Zic4* and *Foxd3* were not expressed in the dental pulp. ATAC-seq analysis revealed that the chromatin in the vicinity of these genes was either in a closed or a partially accessible state. The x-axis represents genomic coordinates; the y-axis represents the ATAC-seq and RNA-seq normalized read counts.

and plotted ATAC-seq signals across the gene body regions and promoters of the corresponding genes. We measured the RNA expression profiles using transcripts per million. We found a strong correlation between the ATAC-seq peaks and RNA expression (Fig. 1C). The average ATAC-seq signal was elevated in the promoter regions and in the corresponding genes that showed the highest expression levels (80–100% and 60–80%). We observed a similar pattern of chromatin accessibility and gene expression in both the incisor and molar dental pulp genomes. In summary, our data indicated that the open chromatin landscape correlates well with RNA expression in the mouse pulp tissues.

### Chromatin accessibility varies across the genome of transcription factors associated with neural crest development

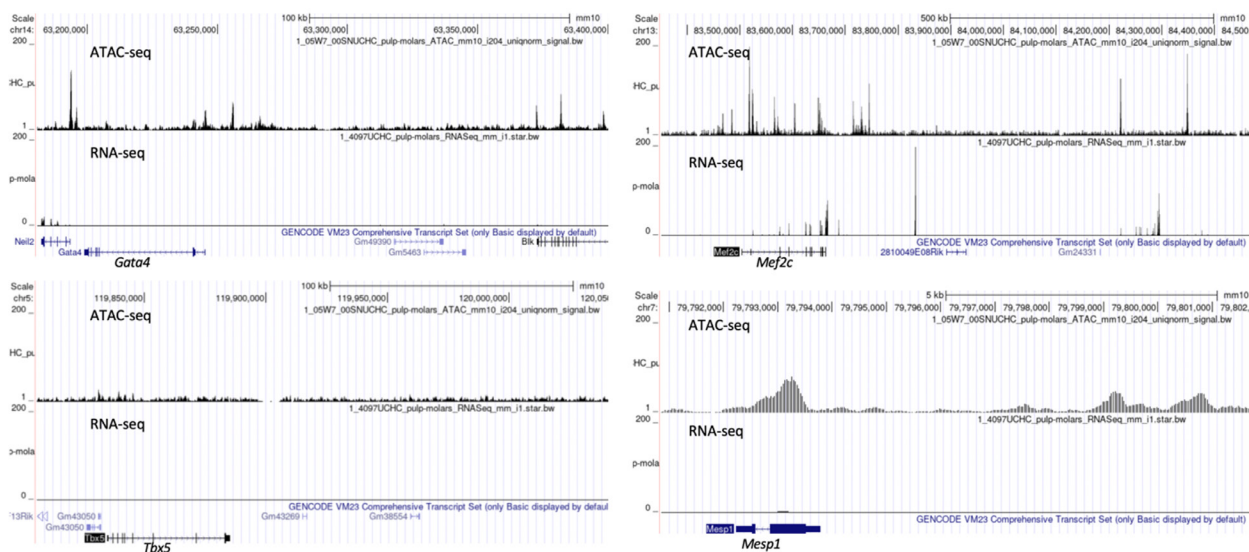
The dental pulp derives from the neural crest during embryonic development (Miletich & Sharpe, 2004; Yu *et al.*, 2018). The neural crest regulatory program is

composed of a set of master transcription factors (TFs) and several signaling pathways (Simoes-Costa & Bonner, 2015). We found that many genes involved in neural crest specification and development were expressed in the mouse dental pulp. Among these well-expressed genes, *Myc*, *Msx1*, *Six1* and *Snai2* retained an open chromatin structure (Fig. 2). However, a number of genes involved in neural crest specification were not expressed in the dental pulp. Among these genes were *Sox10*, *Pax3*, *Zic1*, *Zic4* and *Foxd3*. ATAC-seq analysis revealed that the chromatin in the vicinity of these genes was either closed (*Pax3*) or retained a partially accessible state (*Sox10*, *Zic1*, *Zic4* and *Foxd3*) (Fig. 2).

### ATAC-seq peaks at genes associated with lineage differentiation

MSCs retain the capability of differentiating into different types of cells including myogenic, adipocyte, hepatocyte and neuronal lineages. Therefore, we first analyzed genes involved in myogenic differentiation, specifically *Gata4*, *Tbx5*,





**Figure 3. Chromatin accessibility of myogenic genes in the mouse dental pulp.**

Among genes involved in myogenic differentiation, only *Mef2c* was expressed in the dental pulp; for this gene, we found strong ATAC-seq peaks along the gene body region. *Gata4*, *Tbx5* and *Mesp1* were not expressed in the dental pulp, although we detected strong ATAC-seq signal in the promoter region of *Gata4* and the gene body of *Mesp1*. No ATAC-seq reads were detected in the promoter or gene body regions of *Tbx5*.

*Mef2c* and *Mesp1* (Fig. 3). Of these genes, only *Mef2c* was expressed in the dental pulp, and we found high ATAC-seq peaks along the gene body region. *Gata4*, *Tbx5* and *Mesp1* were not expressed in the dental pulp, however, we detected strong ATAC-seq signals in the promoter region of *Gata4* and within the gene body regions of *Mesp1*. By contrast, we detected no ATAC-seq signals in the promoter or gene body regions of *Tbx5*, indicating a closed chromatin state. Second, we explored the chromatin accessibility and RNA expression of genes associated with adipocyte differentiation (i.e., *Miefl*, *Pparg*, *Egr2* and *Cebpg*) (Fig. 4). We found chromatin accessibility in the promoter and gene body regions of *Pparg*, *Egr2* and *Cebpg*. The RNA-seq analysis was consistent with the ATAC-seq results, revealing strong expression of all three genes in the dental pulp. For *Miefl*, we failed to detect transcriptional activity. We did not detect ATAC-seq peaks in the gene body region, indicating that the chromatin was closed; however, strong ATAC-seq signal was present in the promoter of *Miefl* (Fig. 4). Third, we analyzed the core regulators of hepatocyte differentiation. The promoter and gene body regions of *Cebph*, *Hbex* and *Gata2* displayed open chromatin features. All three genes were well expressed in the dental pulp. We did not detect significant expression of *Foxa1*, although the chromatin along the gene body and promoter region was modestly accessible (Fig. 4). Fourth, we interrogated genes involved in neuronal differentiation. According to RNA-seq analysis, *Neurod1*, *Ascl1*, *Nkx2-1* and *Olig2* were not expressed in the dental pulp (Fig. 5). However, we detected strong ATAC-seq reads at the promoter and gene body regions of *Ascl1*, *Nkx2-1* and *Olig2*, indicating accessible chromatin states in the vicinity of these genes. We also found a strong ATAC-seq peak in the gene body of *Neurod1*, although we failed to detect ATAC-seq signals in the promoter region.

#### Chromatin accessibility of pluripotency genes in the mouse dental pulp

The dental pulp tissue is a valuable source of MSCs that are capable of differentiating into various types of cells (Potdar *et al.*, 2015). We analyzed the RNA expres-

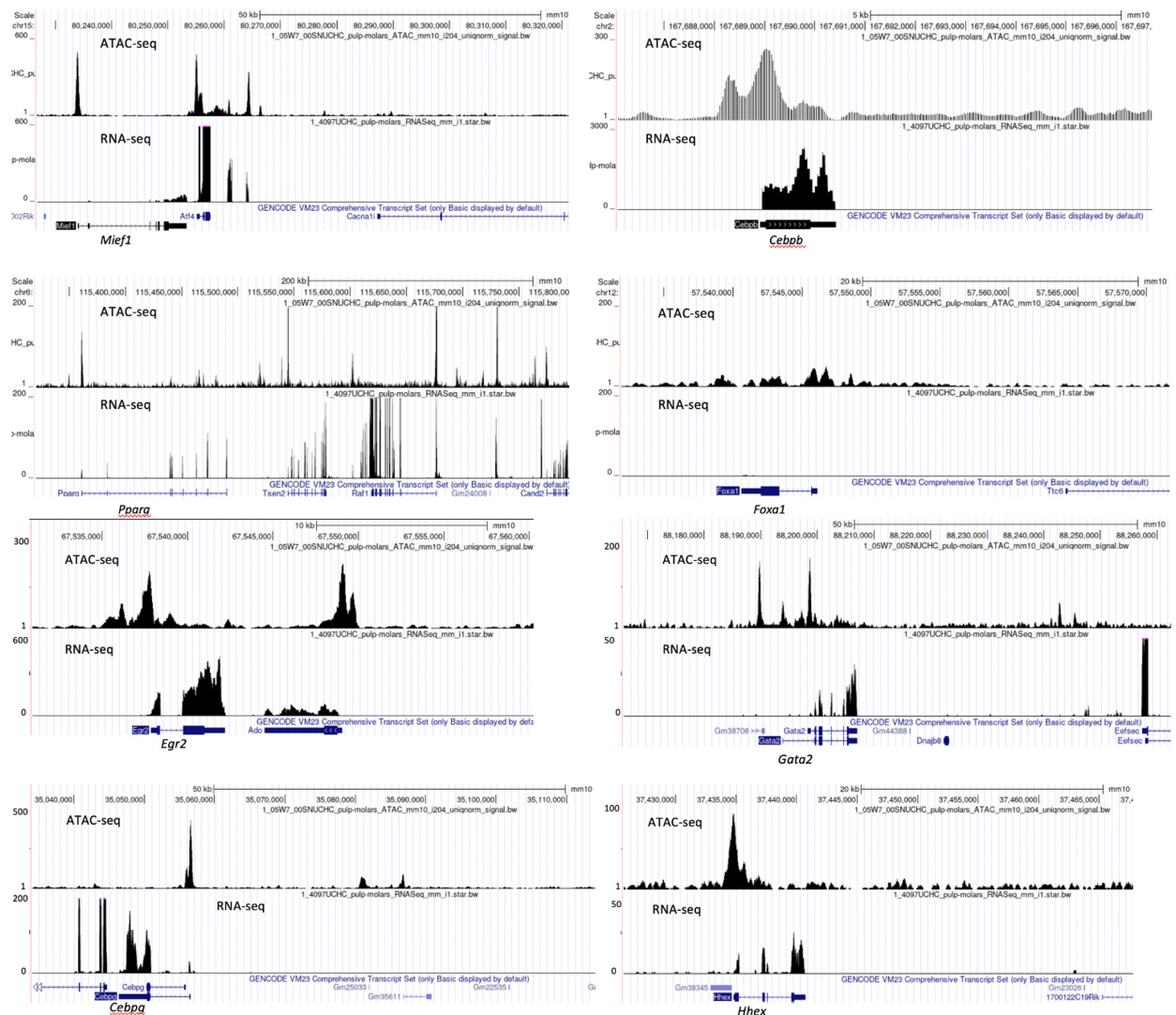
sion profiles and chromatin landscapes of the core pluripotency genes *Nanog*, *Sox2*, *Pou5f1* (*Oct4*), *Maf*, *Sall4* and *Klf4* (Fig. 6). Our analysis indicated that, of these genes, only *Klf4* and *Maf* were strongly expressed in the dental pulp and retained an open chromatin state. For the other pluripotency genes (*Nanog*, *Sox2* and *Pou5f1*), we either failed to detect RNA expression or found very weak expression (*Sall4*). Surprisingly, the ATAC-seq peaks were enriched in the promoter of *Sall4* and were enriched along the entire gene body region of *Sox2* (Fig. 6). We detected relatively weak ATAC-seq signals in the gene body of *Pou5f1*. At the same time, chromatin of *Nanog* remained in a closed state, although we noticed a strong ATAC-seq peak approximately 5kb upstream of *Nanog*, which could represent a repressive chromatin domain.

Overall, our findings for both mouse molars and incisors indicate that a number of master regulator genes associated with lineage specification or pluripotency are expressed in the dental pulp and maintain open chromatin states or are not expressed but still retain chromatin accessibility.

#### DISCUSSION

In summary, our ATAC-seq analysis demonstrated that the vast majority of key developmental genes maintain an open chromatin state in the mouse dental pulp. Cell fate commitment and differentiation involve highly coordinated events in which cell-type-specific genes are activated while others are repressed (Hernandez-Hernandez *et al.*, 2020). Our study suggests that the differentiation of pulp-derived MSCs into different cell types most likely depends on the chromatin landscape of lineage-specific genes encoding master TFs. We found that a large number of regulatory genes encoding TFs are not expressed in the dental pulp but still retain open chromatin (Fig. 7).

Chromatin accessibility is a hallmark feature of active or repressed states of individual genes. We showed that the majority of master regulatory genes in dental pulp



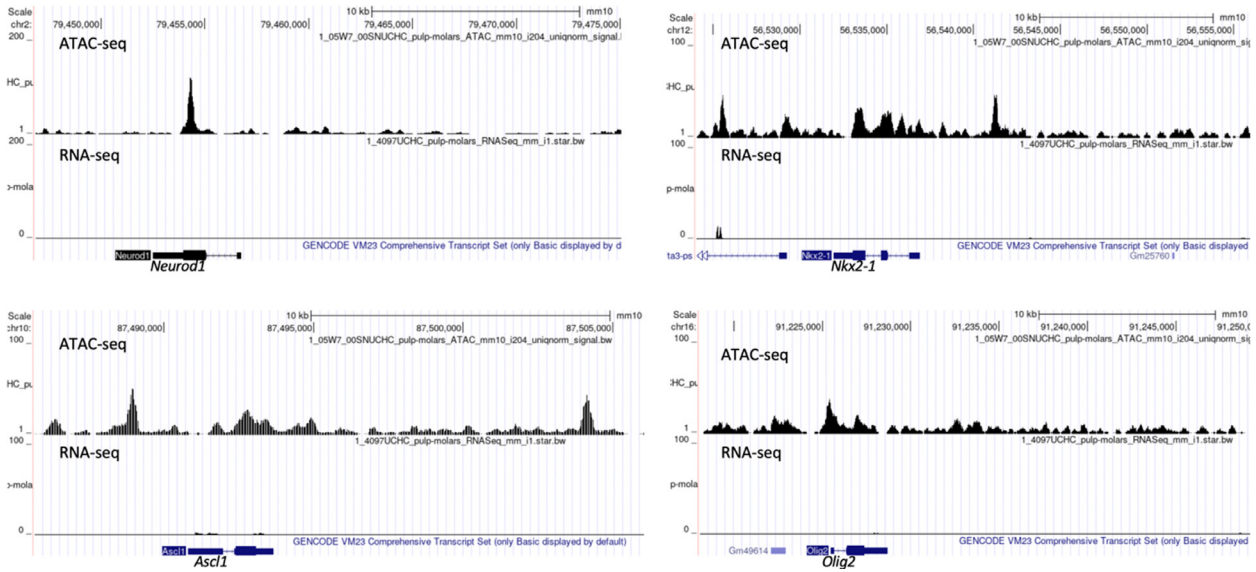
**Figure 4. Chromatin accessibility of adipocyte and hepatocyte lineage genes.**

We found chromatin accessibility in the promoter and gene body region of the adipocyte gene *Pparg*. The RNA-seq analysis indicated that *Pparg* was expressed in the dental pulp. *Mief1* was not expressed in the dental pulp. ATAC-seq reads were not detected in the gene body region of *Mief1*, although a strong ATAC-seq signal was found in the gene promoter. Among genes associated with adipocyte differentiation, we found chromatin accessibility in the promoter and gene body regions of *Egr2* and *Cebpb*. The RNA-seq analysis indicated that both genes were well expressed in the dental pulp. *Cebpb* associated with hepatocyte differentiation displayed open chromatin features in the promoter and gene body regions. *Cebpb* was well expressed in the dental pulp. Another regulator of hepatocytes, *Foxa1*, was not expressed in the dental pulp, although the chromatin along the gene body and promoter regions was modestly open. Core regulators of hepatocyte differentiation, including *Hhex* and *Gata2*, displayed open chromatin features in the promoter and gene body regions. Both genes were well expressed in the dental pulp.

maintain repressed chromatin states. Genome-wide gene silencing is initiated by the trimethylation of histone H3 at lysine 27 (H3K27me3) catalyzed by polycomb repressive complex (PRC2), whereas gene activation is dependent on trimethylation of histone H3 at lysine 4 (H3K4me3) mediated by the Set1/COMPASS complex (Piunti & Shilatifard, 2016). Hence, we believe that some of the ATAC-seq peaks could be associated with repressed chromatin enriched in H3K27me3, whereas the eviction of PRC2 from the regulatory loci may result in gene activation. Recently, Zhang *et al.* reported that during liver regeneration, H3K27me3 is depleted from promoters of genes involved in proliferation, thus facilitating their vigorous expression (Zhang *et al.*, 2021b).

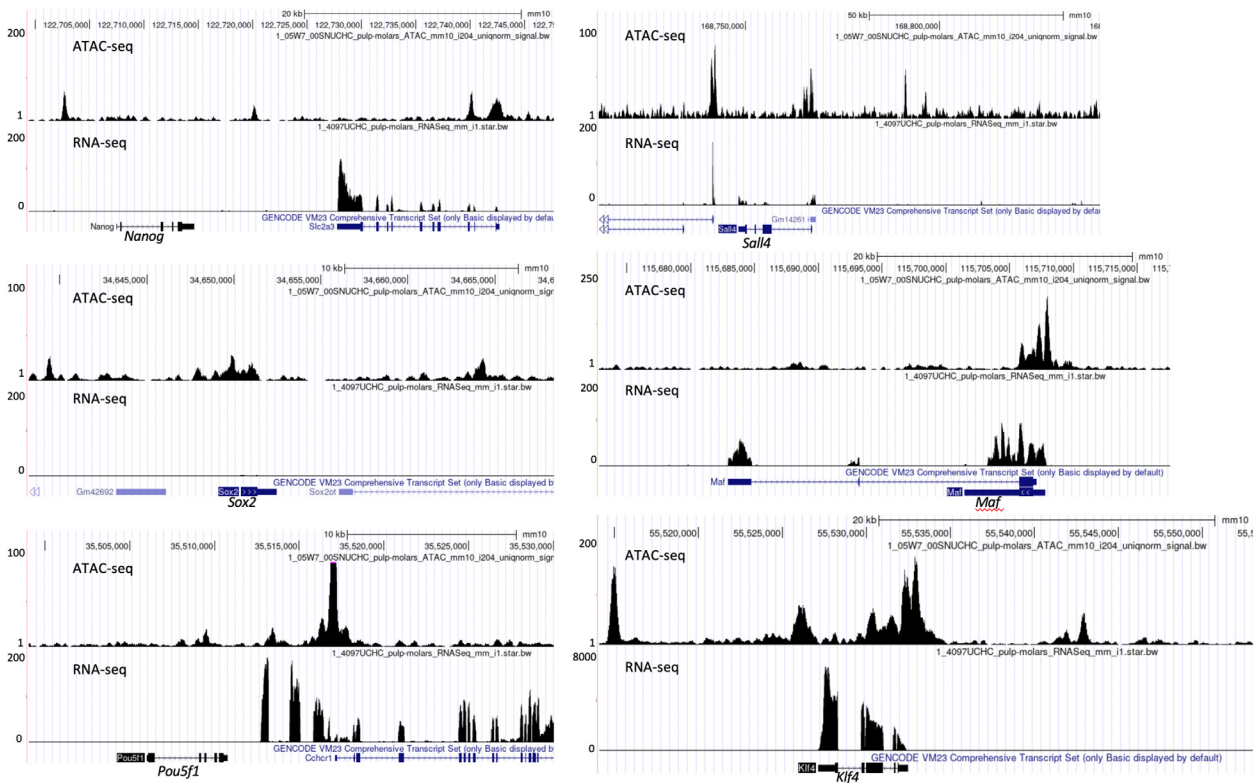
H3K4me3 and H3K27me3 define bivalent domains, which are enriched in promoters of key developmental genes in different stem/progenitor cells (Klein & Hainer, 2020). Specifically, bivalent chromatin domains

are formed at developmental genes that are poised for rapid activation. MSCs of dental pulp originate from the neural crest cells, a transient multipotent cell population derived from the dorsal neural tube (Masuda *et al.*, 2021). Distinct TFs control neural crest differentiation during craniofacial and dental development (Prasad *et al.*, 2019; Balic & Thesleff, 2015). Our findings suggest that the dental pulp genome retains memories of past events. Therefore, open chromatin was likely to have been engaged in a regulatory role during previous developmental stages. It is also possible that some of the differentiating MSCs possess distinct chromatin accessibility states and genes in their vicinity are poised for later activation. Therefore, although the majority of genes encoding pluripotency TFs and regulators of neural crest differentiation are not expressed in dental pulp, chromatin accessibility at the genome-wide level may be explained by epigenetic memory mediated by PRC2 and Set1/



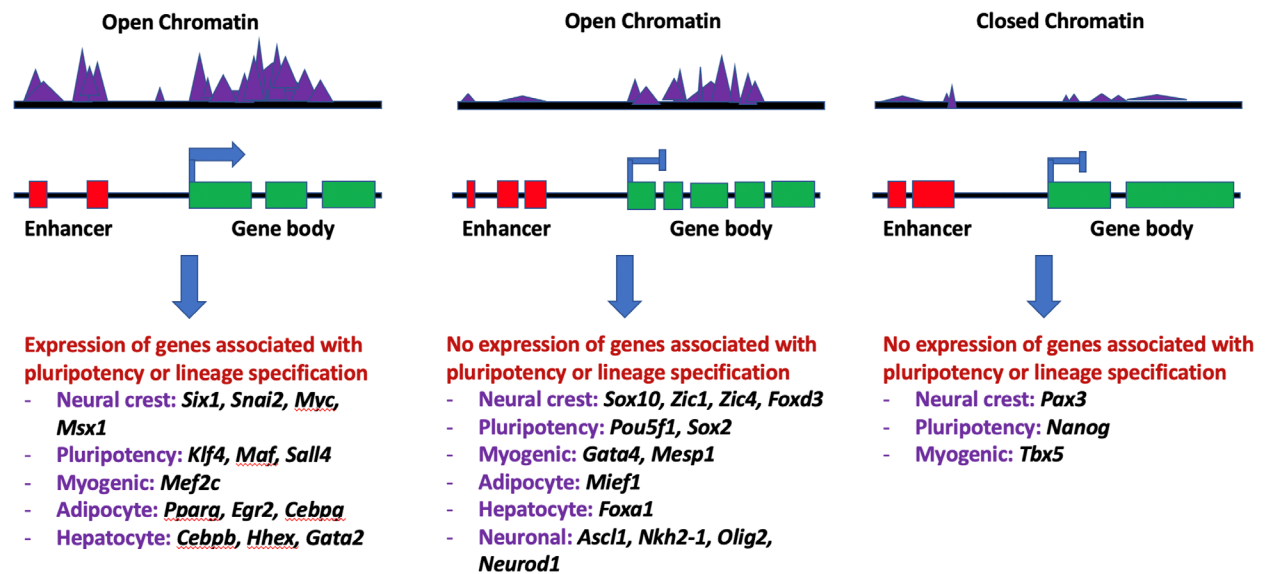
**Figure 5. Chromatin accessibility of neurogenic genes in the mouse dental pulp.**

RNA-seq analysis demonstrated that *Neurod1*, *Ascl1*, *Nkx2-1* and *Olig2*, which are involved in neuronal differentiation, were not expressed in the dental pulp. However, we detected strong ATAC-seq reads at the promoter and gene body regions of *Ascl1*, *Nkx2-1* and *Olig2*, indicating accessible chromatin states in the vicinity of these genes. We also found a strong ATAC-seq peak in the gene body region of *Neurod1*, although we failed to detect ATAC-seq signals in the promoter region.



**Figure 6. RNA expression and chromatin accessibility analysis of core pluripotency genes revealed that only *Maf* and *Klf4* were strongly expressed and retained open chromatin states in the dental pulp.** Although other pluripotency genes were not expressed in the dental pulp, ATAC-seq peaks were enriched in the gene body or promoter regions of *Pou5f1* and *Sall4* or were enriched along the entire gene body region of *Sox2*. Chromatin of only *Nanog* remained in a closed state.





**Figure 7.** A large number of regulatory genes encoding TFs are not expressed in the dental pulp but still retain open chromatin. Among these genes are those encoding pluripotency TFs as well as master regulators of neural crest, myogenic, adipocyte, hepatocyte and neuronal differentiation. A relatively small number of genes such as *Pax3* (neural crest), *Nanog* (pluripotency) and *Tbx5* (myogenic) are not expressed and retain closed chromatin. Among genes that are expressed and maintain open chromatin states are *Six1*, *Snai2*, *Myc* and *Msx1* (neural crest), *Klf4*, *Maf* and *Sall4* (pluripotency), *Mef2c* (myogenic), *Pparg*, *Egr2* and *Cebpa* (adipocytes), and *Cebpb*, *Hhex* and *Gata2* (hepatocytes). Among genes that are not expressed but still maintain open chromatin states are *Sox10*, *Zic1*, *Zic4* and *Foxd3* (neural crest), *Pou5f1* and *Sox2* (pluripotency), *Gata4* and *Mesp1* (myogenic), *Mief1* (adipocytes), *Foxa1* (hepatocytes) and *Ascl1*, *Nkh2-1*, *Olig2*, *Neurod1* (neuronal).

COMPASS complexes. For example, the inheritance of repressed chromatin domains for DNA replication ensures the stability of H3K27me<sub>3</sub>-mediated gene silencing across cell generations (Hugues *et al.*, 2020). This epigenetic mechanism has a crucial role in chromatin-based safeguarding of cell identity.

The mouse dental pulp is highly heterogeneous and composed of several different cell types including ameloblasts, odontoblasts, pericytes, immune cells and blood cells (Nikoloudaki *et al.*, 2020; Yianni & Sharpe, 2019; Farges *et al.*, 2015). Developmental master TFs are selectively expressed among the various cell types; therefore, our bulk ATAC-seq approach representing the total number of reads in the tissue is not sufficiently sensitive to detect cell-type-specific peaks. In the future, using a single-cell ATAC-seq strategy, we will be able to distinguish the chromatin accessibility patterns within distinct cell populations.

Our study supports a nonlinear relationship between gene expression and chromatin accessibility in mouse dental pulp. Moreover, our work suggests a multi-level control mechanism for MSCs in the pulp tissue, which likely depends on the intricate combinatorial genome-wide principles of gene expression. Collectively, we provided the first glimpse of the chromatin accessibility landscape in the mouse dental pulp, laying the foundation for future research on the genomic mechanisms orchestrating lineage specification of the pulp-derived MSCs.

#### Author Contribution

D.B. conceived and designed the project and prepared the manuscript. A.V. isolated the mouse dental pulp. D.B., B.E. and P.J. performed the formal analysis. M.M. and D.G. were involved in writing and editing. All authors read and approved the manuscript.

#### Competing interests

The authors declare that they have no competing financial interests.

#### Availability of data

The data that support the findings of this study are available on request from the corresponding author.

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