Integrated single-cell analyses decode the developmental landscape of the human fetal spine

Highlights
- scRNA-seq and scATAC-seq analyses reveal the developmental landscape of the fetal spine.
- Chondrocytes may originate from EPYC+HAPLN1+ fibroblasts with stem cell characteristics.
- Neurons may originate from neuroendocrine cells with regulation by MEIS2.

Yu et al., iScience 25, 104679
July 15, 2022 © 2022 The Authors.
https://doi.org/10.1016/j.isci.2022.104679

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Integrated single-cell analyses decode the developmental landscape of the human fetal spine

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SUMMARY
The spine has essential roles in supporting body weight, and passing the neural elements between the body and the brain. In this study, we used integrated single-cell RNA sequencing and single-cell transposase-accessible chromatin sequencing analyses to reveal the cellular heterogeneity, lineage, and transcriptional regulatory network of the developing human spine. We found that EPYC + HAPLN1+ fibroblasts with stem cell characteristics could differentiate into chondrocytes by highly expressing the chondrogenic markers SOX9 and MATN4. Neurons could originate from neuroendocrine cells, and MEIS2 may be an essential transcription factor that promotes spinal neural progenitor cells to selectively differentiate into neurons during early gestation. Furthermore, the interaction of NRP2_SEMA3C and CD74_APP between macrophages and neurons may be essential for spinal cord development. Our integrated map provides a blueprint for understanding human spine development in the early and midgestational stages at single-cell resolution and offers a tool for investigating related diseases.

INTRODUCTION
The spine facilitates neurotransmission and protects the body from trauma and excessive strain that may occur during physical movement (Galbusera and Wilke, 2018). Defects in the early stages of spine development may lead to neural tube defects and vertebral and rib deformities (Weiss and Moramarco, 2016). The human spine originates during the third and fourth weeks of gestation, when the blastula develops into the gastrula and is composed of the ectoderm, mesoderm, and endoderm (Slack, 2005). Previous studies on spine development have provided insights into the differentiation and development of mesodermal progenitor cells and neuroectodermal progenitor cells (Galbusera and Bassani, 2019) (Kaplan et al., 2005). However, detailed knowledge of the cell types and their developmental features in the early and midgestational stages is still lacking, especially regarding the heterogeneity of fibroblasts, chondrocytes, and neurons and their immune environment. In this study, we used integrated single-cell RNA sequencing (scRNA-seq) and single-cell transposase-accessible chromatin sequencing (scATAC-seq) to systematically dissect the cellular features and molecular regulation of the human fetal spine and reveal the landscape of transcriptomic regulation during the early stages of fetal spine development.

RESULTS
Integrated cellular landscape of the human fetal spine at the single-cell level
To investigate the developmental trajectory of fetal spines, we performed scRNA-seq and scATAC-seq of spine samples from aborted fetal tissues between 8 and 17 gestational weeks (Figures 1A and S1A, and Table S1). With scRNA-seq, we captured 16,746 cells and identified 26 cell types, including hematopoietic lineage cells, progenitor cells, neurocytes, osteoblasts, and stromal cells (Figures 1B, S1B, and S1C, Tables S2–S4). With scATAC-seq, we captured 4,848 cells and identified 18 cell types (Figures 1C and S1D, Tables S5 and S6). Overall, the cell types identified by scRNA-seq and scATAC-seq were consistent, except for eight cell types occurring at low percentages. This is possibly due to the lower number of cells captured by scATAC-seq (Tables S4 and S6).

In the fourth week of gestation, the neural tube consisting of neuroectodermal progenitor cells develops (Galbusera and Bassani, 2019), and annulus fibrosus derived from mesoderm cells appears, leading to...
vertebral bodies, ligaments, and endplates (Lawson and Harfe, 2017) (Nolting et al., 1998). Consistently, we observed that neurocytes and fibroblasts all existed in the eighth gestational week. Fibroblasts are the dominant cell type of fetal spines, aligning well with their role in osteogenesis promotion (Zhang et al., 2017b). The nucleus pulposus develops from the notochord as early as the third and fourth weeks of gestation (Alkhatib et al., 2018), and every future vertebra will begin to ossify between the ninth and fourteenth weeks (Szpinda et al., 2013). Our results show that chondrocytes appeared in the 10th gestational week at a
| Clusters | GO terms                                      | Key genes                                              |
|----------|----------------------------------------------|--------------------------------------------------------|
| 1        | Collagen fibril organization                  | LUM, LAMA2, COL1A1, COL1A2, SPAR2                    |
| 2        | Connective tissue development                 |                                                       |
| 3        | Extracellular matrix organization             |                                                       |
| 4        | Bone development                              |                                                       |
| 5        | Reproductive structure development            | FOXC1, SPARC, COL6A1, HAS2, LOX, MMP16, ANXA2, COL1A1 |
| 6        | Reproductive system development               |                                                       |
| 7        | Chondrocyte differentiation                   | MATN4, SOX9, COL2A1                                   |
| 8        | Reproductive structure development            |                                                       |
| 9        | Endocrine system development                 | NR5A1, CITED2, CDKN1C, INSR                           |
| 10       | Carilage development                          |                                                       |
| 11       | Extracellular matrix organization             |                                                       |
| 12       | Chromosome segregation                        |                                                       |
| 13       | Mitotic nuclear division                      | NUSAP1, MKI67, TOP2A, SMC4, CENPF                    |
| 14       | Nuclear chromosome segregation                |                                                       |
| 15       | Mitotic nuclear division                      | FBXO5, NUSAP1, CENPK, CDC4, SMC4, TTE, MAD2L1, KIF2C  |
| 16       | Organelle fusion                              |                                                       |
| 17       | Ossification                                  | ID3, ID2, PBX1, FOXC1                                 |
| 18       | Embryonic organ development                   |                                                       |
| 19       | Mesenchyme development                        | BMP4, SXX1                                           |
| 20       | Muscle cell differentiation                    | EDN3, GDNF, HAND2                                    |
| 21       | Muscle development                            | MYF5, MSTN, PA7, KLHL41                              |

A. Heatmap showing expression patterns of different clusters.

B. UMAP plot for cell types, highlighting clusters.

C. Scatter plot showing cell type distribution with UMAP.

D. Expression plot for fibroblast cells.

E. Peaks plot for fibroblast cells showing HOXA10 motif and TUBB.

F. Immunofluorescence images showing DAPI + FN1 + COL1A1.
very high proportion in the 10th and 11th gestational weeks (Figure 1D), which correlates well with previously published literature (Szpinda et al., 2013). In week 17, hematopoietic lineage cells became dominant, consistent with the literature that hematopoietic cells are generated and propagate during the 11th and 20th weeks of gestation, respectively (Holt and Jones, 2000) (Figure 1D). Notably, macrophages, a type of hematopoietic cell, were already detected in the eighth gestational week. This result may indicate that early-stage macrophages migrate from organs such as the liver instead of from the bone marrow (Popescu et al., 2019).

Fibroblast heterogeneity and developmental tracking

To elucidate fibroblast cellular and functional heterogeneity, we performed unsupervised clustering and identified 13 subclusters of cells (Figures 2A, S2A, and S2C). As a result, cells in cluster 4 enriched for HIST1H1A and with minimal detection of COL2A1 (Fib_4) were defined as HIST1H1A+ COL2A1– fibroblasts (Figure S2C). Similarly, cells in cluster 12 were defined as EPYC + HAPLN1+ fibroblasts (Fib_12) because they highly expressed both EPYC and HAPLN1 (Figure S2C). By gene set variation analysis (GSVA) of differentially expressed genes among the clusters, we found that NEB + PAX7+ fibroblasts (Fib_9) were enriched for genes involved in muscle organ development (Figure 2C). PAX1+HOX7+ fibroblasts (Fib_2) and EPYC + HAPLN1+ fibroblasts (Fib_12) were enriched for genes related to the chondrocyte differentiation pathway, such as SOX9 and COL2A1 (Figure 2A). HIST1H1A + COL2A1– fibroblasts (Fib_4) were likely active in nuclear chromosome segregation and enriched for FBXO5 and NUSAP1 (Figure 2A). These results confirm that different subtypes of spinal fibroblasts might have specific roles in forming bone and protecting the spinal cord (Zhang et al., 2017b).

To gain deeper insights into the fibroblast development process, we performed trajectory analysis with the R package slingshot. Because fibroblasts are believed to develop from mesodermal progenitor cells (MPC) (Galbusera and Bassani, 2019), we revealed the potential differentiation directions between mesodermal progenitor cells and fibroblast subpopulations (Figure 2B). In detail, MPC may differentiate into HIST1H1A + COL2A1– fibroblasts (Fib_4). And the trajectory, along with HIST1H1A + COL2A1– fibroblasts (Fib_4), extended further into other subclusters with multiple developmental branches. Meanwhile, IFI44L + OGN + fibroblasts (Fib_7), LAMA2+CYP1B1+ fibroblasts (Fib_10), EPYC + HAPLN1+ fibroblasts (Fib_12), and DLK1+APOA1+ fibroblasts (Fib_13) are located at the end of the trajectory. Thus, we speculated that HIST1H1A + COL2A1– fibroblasts (Fib_4) have a higher differentiation potential than IFI44L + OGN + fibroblasts (Fib_7), LAMA2+CYP1B1+ fibroblasts (Fib_10), EPYC + HAPLN1+ fibroblasts (Fib_12), and DLK1+APOA1+ fibroblasts (Fib_13). To validate this hypothesis, we performed SLICE analysis to identify differentiation states based on single-cell entropy (scEntropy) (Guo et al., 2017). SLICE analysis showed that HIST1H1A + COL2A1– fibroblasts (Fib_4) had a high entropy score, while clusters predicted to be at the end of the trajectory, such as EPYC + HAPLN1+ fibroblasts (Fib_12) and DLK1+APOA1+ fibroblasts (Fib_13), had low entropy scores (Figure 2C), which fits well with the trajectory results. Notably, the developmental cycle trajectory showed that spinal fibroblasts finished their differentiation as early as the eighth week (Figure 2B).

To further explore genes that drive HIST1H1A + COL2A1– fibroblasts (Fib_4) to differentiate into other fibroblast subclusters, we overlapped genes in fibroblasts with an altered expression along the pseudotime and the one enriched in the eighth week, an important stage for fibroblast development. Consequently, TUBB enriched in GW8 was highly expressed in HIST1H1A + COL2A1– fibroblasts (Fib_4) (Figure 2D). As known, TUBB in fibroblasts encodes β-tubulin and is broadly expressed in the developing CNS (Sierra et al., 2020). Our result suggested that TUBB may be an essential gene for driving HIST1H1A + COL2A1– fibroblast (Fib_4) development in fetal spine. Interestingly, we also found an accessible
transcription start site (TSS) region in TUBB that can bind to the transcription factor HOXA10 by scATAC-seq analysis (Figure 2E). Previous studies indicated that transcription factors in the HOX family play an essential role in differentiation. As shown in our study, both HOXA10 and its target gene TUBB were highly expressed in HIST1H1A + COL2A1– fibroblasts (Figure 2E). This result indicates that TUBB may be a critical gene to promote the differentiation of HIST1H1A + COL2A1– fibroblasts (Fib_12) to UBE2C + DLGAP5+ fibroblasts (Fib_5) by upregulating its expression through the enriched transcription factor HOXA10, thus contributing to the heterogeneity of fibroblasts and their diverse biological functions. Through immunofluorescence staining of FN1 and COL1A1, we verified the existence of fibroblasts in the eighth week and observed that fibroblasts were distributed around the spinal cord, which confirmed the heterogeneity and protective function of fibroblasts (Figures 2F, S5A, and S5B).

**Cellular heterogeneity and developmental tracking of chondrocytes**

Chondrocytes are critical units of the spinal column and help support body weight. We identified 12 subclusters of chondrocytes (Figures 3A and S2B, Tables S7 and S8). Most of the cells in the GW10 cycle were grouped into TAOK1 + HBB– chondrocytes (Chond_1) and ASPN + MKI67– chondrocytes (Chond_5) with high expression levels of TAOK1 and ASPN, respectively (Figures 3B, 3C, S2B, and S2D). The majority of cells in the GW11 cycle were divided into HSPA1A + CXCL3– chondrocytes (Chond_2) and MT-TV + LGALS1– chondrocytes (Chond_3) (Figures 3B, 3C, S2B, and S2D, Tables S9 and S10). With further functional analysis, we found that the chondrocyte differentiation pathway was highly enriched in ASPN + MKI67– chondrocytes (Chond_5) and was characterized by high expression levels of COL12A1 and COL6A1 (Figure 3A). Genes involved in ossification and bone mineralization as well as endochondral bone morphogenesis were enriched in MEF2C + COMP + chondrocytes (Chond_7) (Figures 3A and S2D). These results display the heterogeneity of chondrocytes.

Chondrocytes, as well as fibroblasts, develop from mesodermal progenitor cells (Galbusera and Bassani, 2019). Because fibroblasts can differentiate into chondrocytes (Hoshi et al., 1997), we performed trajectory analysis to study the relationship between mesodermal progenitor cells, fibroblast subclusters, and chondrocyte subclusters. EPYC + HAPLN1+ fibroblasts (Fib_12) seem to be critical cells that can differentiate into chondrocytes (Figure 3B). Moreover, the genes MATN4, ACAN, COL2A1, COL11A1, and SOX9, which are involved in chondrocyte differentiation, are enriched in EPYC + HAPLN1+ fibroblasts (Dehne et al., 2010; Komori, 2019) (Figure 2A). This result suggests that EPYC + HAPLN1+ fibroblasts have stem cell characteristics. ASPN + MKI67– chondrocytes (Chond_5) appeared at the start of the chondrocyte subcluster differentiation trajectory after the eighth week of pregnancy (Figures 3B and 3C). Besides, MT-TV + LGALS1– chondrocytes (Chond_3) and MEF2C + COMP + chondrocytes (Chond_7) located at the end of the trajectory were from the GW11 cycle (Figures 3B and 3C). The distribution of different clusters correlates well with our developmental trajectory result (Figures 1D, 3B, and 3C). SLICE analysis also suggested that ASPN + MKI67– chondrocytes (Chond_5) have a higher differentiation potential than MT-TV + LGALS1– chondrocytes (Chond_3) (Figure 3D). Additionally, EPYC + HAPLN1+ fibroblasts (Fib_12) that may develop into chondrocytes showed a lower cell number than most other fibroblast subpopulations in the eighth week. And ASPN + MKI67– chondrocytes (Chond_5) that may originate from Fib_12 showed higher cell numbers than most chondrocyte subpopulations in the 10th week (Tables S7–S10). By multiplexed immunofluorescence staining of EPYC and HAPLN1, we verify the existence of EPYC + HAPLN1+ fibroblasts (Fib_12). Further co-localization of EPYC, HAPLN1, SOX9, and MANT4 illustrates that EPYC + HAPLN1+ fibroblasts (Fib_12) may differ into chondrocytes by highly expressing SOX9 and MANT4 (Figures 3E and S5C). Notably, we hardly captured chondrocytes in the eighth week by scRNA-seq analysis, while chondrocytes by immunostaining Aggrecan and MATN3 with the 8-week-old spine (Figures 3E and S5D). The possible reason may be that the spine used for immunostaining was older than the one used for scRNA-seq analysis due to sample heterogeneity. Besides, a low percentage of chondrocytes in 8-week-old spines may result in no chondrocytes detected by scRNA-seq experiments.
Neurocyte developmental tracking and the potential of neuroendocrine cells to differentiate into neurons

There are five types of neurocytes (Figure 1B). To understand their relationship during development, we performed pseudotime analysis and observed three developmental trajectories that originated from neuroectodermal progenitor cells, including two reported trajectories (Kaplan et al., 2005) (Cai et al., 2017). In detail, the development from neuroectodermal progenitor cells to neural progenitor cells and then to neurons (Kaplan et al., 2005) mainly occurs during the eighth and 11th gestational weeks (Figures S3A–S3C). The developmental trajectory of neuroectodermal progenitor cells to Schwann cells (Cai et al., 2017) proceeded through the eighth to 17th gestational weeks (Figures S3D–S3F). As described in the literature, almost all endocrine cells are derived from the endoderm and neuroectoderm (Gut et al., 2015). Our results confirm that spinal neuroendocrine cells are derived from neuroectodermal progenitor cells at an early stage of pregnancy. Interestingly, we also observed that neuroendocrine cells could differentiate into neurons based on developmental trajectory analysis (Figures 4A–4C).

To further interpret this potentially new trajectory of “neuroectodermal progenitor cells to neuroendocrine cells to neurons”, we found that SOX3, TH, and GPM6A from neuroectodermal progenitor cells, neuroendocrine cells, and neurons, respectively, seem to play a vital role in promoting the differentiation of neuroectodermal progenitor cells to neurons, which is also in agreement with the literature (Michibata et al., 2009; Wang et al., 2006) (Figure 4C). SOX3 is a conserved transcription factor labeling specific stages of human embryonic stem-cell-derived and murine neonatal and adult neural progenitors and was involved in neural stem cell maintenance (Wang et al., 2006). In total, we found 27 upregulated SOX3 target genes in the three developmental trajectories and found 14 genes, including TUBA1C, SLC2A1, and RGMA, which were conserved in neuroectodermal progenitor cell differentiation (Figure 4D). HOXB-AS3 and LBR upregulated by SOX3 were involved in only the trajectory of “neuroectodermal progenitor cells to neuroendocrine cells to neurons”, which suggested that they may be the essential genes to promote the selective differentiation of neuroectodermal progenitor cells to neuroendocrine cells (Solovei et al., 2013) (Figure 4D). Furthermore, TH in neuroendocrine cells may be a synergistic gene that promotes the final differentiation of neuroectodermal progenitor cells into neurons through its upregulation by the enriched transcription factor Isl1 (ISL1) (Liang et al., 2011) (Figure 4E). It has been reported that ISL1 played a central role in sensory neuron development linking sensory and spinal gene regulatory programs (Sun et al., 2008). Our Cicero analysis also showed high activity of the regulatory sequence of TH. Additionally, GPM6A may be an essential target gene that helps neuroendocrine cells differentiate into neurons via its upregulation by the enriched transcription factor PBX3 (Figure 4F). PBX3 was known to increase the DNA-binding/transcriptional activity of HOX proteins and regulate genes involved in development (Li et al., 2014). Moreover, our scATAC-seq data also confirmed the accessibility of the PBX3-binding site (Figure 4F). Interestingly, differentiation of neurons derived from neural progenitor cells may be driven by ENO2, which was highly active based on the Cicero analysis results and involved in neural differentiation (Isgro et al., 2015) (Figures 4G and S3G).

Neuronal heterogeneity and their differentiation regulatory network in the developing human fetal spine

To study neuronal heterogeneity, we performed unsupervised clustering of 984 neurons and identified nine subclusters (Figure 5A). Through GSVA, we observed functional diversity among the nine clusters. PMEL + PAPPA2+ neurons (Neu_7), NR2F1 + NR2F2+ neurons (Neu_8), and LIX1 + SLIT3+ neurons (Neu_9) displayed increased expression of genes involved in neuron projection and neuron fate specification. As well known, defects in NR2F1 are a cause of Bosch-Boonstra optic atrophy syndrome (BBOAS) (Walsh et al., 2020), indicating NR2F1 + NR2F2+ neurons (Neu_8) played a vital role in neuronal development. MAB21L2 + TAOK1+ neurons (Neu_1) and ASS1 + HES6+ neurons (Neu_6) participate in catabolic
took the terminal position (Figure S3J). The distribution of neurons from the center of the tissue to the gestational week were MNs. Moreover, SLICE analysis showed low differentiation potential of stages (e9.5 and e10.5) (Delile et al., 2019). Thus, we speculated that most of the neurons in the eighth differentiation stage (8–17 weeks) (Figure 5B). A previous study performed scRNA-seq on mouse spinal cord e8 weeks of gestation, while the other neuron subclusters were from neuroendocrine cells at the late differentiation stage (e9.5 and e10.5) (Delile et al., 2019). Thus, we speculated that most of the neurons in the eighth gestational stage week were MNs. Moreover, SLICE analysis showed low differentiation potential of MA821L2 + TAOK1+ neurons (Neu_1), which validated the developmental trajectory result that Neu_1 took the terminal position (Figure S3J). The distribution of neurons from the center of the tissue to the surrounding area for signal conduction was confirmed by immunofluorescence analysis of NEFM and CD56 in spine sections (Figure 5F).

Neurons may originate from neuroendocrine cells as well as neural progenitor cells. Based on developmental trajectory analysis of neural cells, we found that PMEL + PAPPA2+ neurons (Neu_7), NR2F1 + NR2F2+ neurons (Neu_8), and LIX1 + SLIT3+ neurons (Neu_9) were from neural progenitor cells in the eighth gestational stage (8–17 weeks) (Figure 5B). A previous study performed scRNA-seq on mouse spinal cord and displayed motor neurons (MNs) were the most prominent class of neurons at early developmental stages (e9.5 and e10.5) (Delile et al., 2019). Thus, we speculated that most of the neurons in the eighth gestational stage week were MNs. Moreover, SLICE analysis showed low differentiation potential of MA821L2 + TAOK1+ neurons (Neu_1), which validated the developmental trajectory result that Neu_1 took the terminal position (Figure S3J). The distribution of neurons from the center of the tissue to the surrounding area for signal conduction was confirmed by immunofluorescence analysis of NEFM and CD56 in spine sections (Figure 5F).

MEIS2 is a prerequisite for neuronal differentiation through nuclear accumulation in the subventricular zone (SVZ) olfactory bulb neurogenic system (Kolb et al., 2018). Based on our results, MEIS2 is upregulated during the development of neuroectodermal progenitor cells into neuronal cells and is significantly higher in PMEL + PAPPA2+ neurons, NR2F1 + NR2F2+ neurons, and LIX1 + SLIT3+ neurons. In contrast, we did not observe significant enrichment of MEIS2 in AS51 + HE56+ neurons, NEUROD6 + NEUROD6+ neurons, MA821L2 + TAOK1+ neurons, NPPA + CST3+ neurons, PCP4 + BCSA1+ neurons, or TMEM233 + PIEZO2+ neurons (Figures 5C and 5D). Therefore, we speculated that MEIS2 might be an essential transcription factor that drives spinal neural progenitor cells to selectively differentiate into neurons. It could also lead to the formation of PMEL + PAPPA2+ neurons, NR2F1 + NR2F2+ neurons, and LIX1 + SLIT3+ neurons in the early gestational stage, which regulate the differentiation of dopaminergic neurons, as mentioned above. Additionally, our scATAC-seq data show that the binding sites for MEIS2 were accessible. Furthermore, 35 target genes in LIX1 + SLIT3+ neurons that could be regulated by MEIS2 were significantly upregulated, as shown by scRNA-seq analysis. For example, the target genes ANXA2, ME1, and UCHL1 have been reported to play a role in neuronal differentiation (Naéiff et al., 1996; Neuman et al., 1993; Wang et al., 2013) (Gu et al., 2018). We found that ANXA2, ME1, and UCHL1 were upregulated by MEIS2 in LIX1 + SLIT3+ neurons, indicating that ANXA2, ME1, and UCHL1 likely contribute to the formation of PMEL + PAPPA2+ neurons in the spine after binding to MEIS2 (Figure 5D). Consistent with our result, immunofluorescence analysis showed co-localization of CD56, ME1, and UCHL1, indicating that ME1 and UCHL1 correlated highly with early neuron development (Figure 5F).

Development of the immune microenvironment and intercellular communication in the human fetal spine

The immune microenvironment during spine development is complex. In our study, we found that immune cells became dominant at 17 weeks of gestation. Specifically, granulocyte-monocyte progenitor cells, precursor B cells, and monocytes consist of 27.54%, 20.46%, and 17.62% of the total cells, respectively (Table S4). Notably, granulocyte-monocyte progenitor cells are the most dominant cell population, consistent with the results of a previous study in mice in which granulocyte-monocyte progenitor cells promoted immune cell differentiation and proliferation (Hérault et al., 2017). As mentioned above, we observed processes, including protein localization and oxygen transport. Notably, TAOK1 was associated with neuronal maturation (van Woerden et al., 2021), suggesting that MA821L2 + TAOK1+ neurons (Neu_1) may be essential for neuronal maturation. NPPA + CST3+ neurons (Neu_2), NEUROD6 + LH3X1+ neurons (Neu_5), PCP4 + BCSA1+ neurons (Neu_3), and TMEM233 + PIEZO2+ neurons (Neu_4) were involved in both neuron differentiation and embryonic organ morphogenesis (Figures 5A, S3H, and S3I). Consistently, NEUROD6 was reported to induce neuron terminal differentiation in the neonatal period of mice (Zhao et al., 2021), confirming the possible role of NEUROD6 in embryonic spinal cord morphogenesis.

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macrophages as early as the eighth gestational week (Figure 1B). Its cell number first decreased gradually as the embryo developed and then propagated at 17 weeks, indicating that the immune microenvironment is formed in early gestation with circulating immune cells.

Because nonimmune cells that communicate with immune cells may be necessary to exert biological activity, we analyzed the cell-cell interaction via Cellphone DB (Efremova et al., 2020). As a result, we obtained 66 receptor-ligand pairs that may play a role in the five main differentiation trajectories mentioned above (Figures S4A and S4B). The SPP1_CD44 interaction showed high intensity in the 11th week and 17th week of gestation and was found in all three neuroectodermal progenitor differentiation processes, suggesting its role as a typical marker of neuroectodermal progenitor differentiation (Wang et al., 2020) (Figures 5E, S4D, and S4G). Both EPHA3_EFNA4 and LGR4_RSPO3 interactions, which were related to the formation of muscle stem cells in mice (Alonso-Martin et al., 2016) and differentiation of hASCs (Zhang et al., 2017a), respectively, were only observed between immune cells and fibroblast subclusters (Figure 5E). These interactions may be a crucial part of fibroblast differentiation. Moreover, our immunofluorescence analysis of S100A9 and COLIA1 on spinal sections confirmed the potential interaction of granulocyte-monocyte progenitor cells with fibroblasts (Figure 5F). This interaction may be an essential part of fibroblast differentiation. In addition, as the embryo developed and then propagated at 17 weeks, indicating that the immune microenvironment is formed in early gestation with circulating immune cells.

In the neuroectodermal progenitor cell differentiation process, SEMA3C enriched in NR2F1 + NR2F2+ neurons (Neu_8) interacted with NRP2 in macrophages (Figure 5E), which contributed to developmental cell growth (Figure 5A). Because NRP2_SEMA3C can promote dendritic cell migration (Sanyas et al., 2012) (Curreli et al., 2016), we speculate that this interaction could drive circulating macrophages to induce spinal neuron growth in the early gestational stage. Meanwhile, the immunofluorescence analysis of CD68, CD163, NEFM, CD56, SEMA3C, and NRP2 displayed co-existence of SEMA3C and NRP2, indicating the possible role of macrophage to promote early neuron growth through NRP2_SEMA3C interaction (Figure 5F). The MIF_CD74 interaction shows increased intensity, while that of CD200_CD200R1 shows weak intensity during the gestational stage (Figure S4C and S4E). Both interactions may be essential for macrophages to stimulate spinal neuron development (Figures 5E, S4C, and S4E). This result is consistent with the literature reporting that macrophages are necessary to establish the neurovascular network during CNS development (Chen et al., 2017) and that both MIF_CD74 and CD200_CD200R1 interactions are involved in the neuron developmental process (Hernangómez et al., 2012; Su et al., 2017). From the 8th week to the 17th week, the CD74_APP interaction between macrophages and neurons also increased, and it was involved in only the development of the “neuroectodermal progenitor cell-neuroepithelial cell-neuron” trajectory (Figures 5E, S4C, and S4F), which has been reported to be associated with the neuroinflammatory response (Castillo et al., 2017). Thus, the interaction between CD74 and APP may be a way for macrophages to drive neuron subpopulation development and diversification. Our immunofluorescence analysis confirmed the co-localization of CD68, NeuN, CD74, and APP on spinal sections, suggesting the potential interaction of CD74 with APP (Figure 5F). In the process of the “neuroectodermal progenitor cell-Schwann cell” trajectory, the SELP_CD24 interaction seems to play a role in the activation of Schwann cells (Soltész et al., 2019), and CADM1_NECTIN3 may contribute to the attachment of Schwann cells to megakaryocytes (Furuno et al., 2012). Interestingly, the interaction of CMKLR1_RARRES2, which is well known to play key roles in immune environment formation (Pachynski et al., 2019), was also observed in our study (Figure 5E). These findings emphasized the importance of immune cells in the genesis and differentiation of neurocytes in the spine.

**DISCUSSION**

In this study, we integrated scRNA-seq and scATAC-seq to decode the gene expression and regulatory landscape of human fetal spine development at single-cell resolution at different developmental stages. The results of the two methods were consistent and revealed similar developmental features of the central spinal cells. In detail, fibroblasts and chondrocytes, the main components involved in spinal cord protection and body weight support, were the dominant cell types in the early gestational stage and contained highly heterogeneous subpopulations. Among 13 fibroblast clusters, HIST1H1A + COL2A1– fibroblasts enriched for TUBB showed a high differentiation potential, suggesting that TUBB is a pivotal gene for promoting HIST1H1A + COL2A1– fibroblast differentiation and helping construct the diverse stromal environment through its upregulation induced by the enriched transcription factor HOXA10. Meanwhile, EPYC + HAPLN1+ fibroblasts may be the seed cells for chondrocyte formation, which highly expressed...
chondrogenic genes of MATN4 and SOX9. Because previous reports performed scRNA-seq on adult spinal tissues, the mesenchymal stem cell subgroup (MSC-GJA1) that may differentiate into osteoblast and the heterogeneity of chondrocytes was revealed (Gan et al., 2021; Yang et al., 2021). To be complementary, our results of scRNA-seq on fetal spine showed the potential key subcluster to form chondrocytes, providing new insight on chondrocyte regeneration. Moreover, a recent study analyzed pig embryos and concluded that HOXA10, EPYC, and HAPLN1 were critical for bone formation (Li et al., 2021), confirming that chondrocytes may originate from EPYC + HAPLN1+ fibroblasts.

As a vital part of the CNS for signal transmission in the spine, neurocytes are also abundant in the early period. We found a new developmental trajectory separate from two previously reported trajectories and speculated that SOX3, TH, and GPM6A are the key factors driving this developmental trajectory. Surprisingly, neuron subclusters with the two kinds of origins showed separated trajectories at different periods. In line with the previous report that TAOK1 was associated with neuronal maturation (van Woerden et al., 2021), we observed that MAB21L2 + TAOK1+ neurons (Neu_1) took the terminal position along the developmental trajectory. MEIS2 may be an essential target to promote the early differentiation of neurons originating directly from neural progenitor cells. Based on cell-cell interaction analysis, we confirmed the importance of macrophages during spine development. Additionally, we found that NRP2_SEMA3C may be a driving force attracting macrophages to the spine and that CD74_APP may be the means by which macrophages promote neuronal subcluster development and exert heterogeneity. In future studies, work including searching EPYC + HAPLN1+ fibroblasts (Fib_12) in the mouse embryos by scRNA-seq can be done to verify the origin of chondrocytes. It will be interesting to verify this hypothesis and explore the relationship between cellular interactions and the transcriptional networks that contribute to spine development.

Limitations of the study

Our work provides a blueprint for understanding human spinal development in the early and midgestational stages at single-cell resolution. Owing to limited access of fetal spine, the number of donors at each age was less than three. Given the small sample size and heterogeneity, the percentage of cells may fluctuate erratically in samples of similar age. Therefore, the difference in developmental time/lineage may be in biological replicate. But in our case, the percentages of fibroblasts, neurocytes, and chondrocytes ranging from 8 to 17 gestational weeks were consistent with previous literature (Zhang et al., 2017b)(Szpinda et al., 2013), indicating the robustness of the scRNA-seq method and the reference value of cellular heterogeneity and developmental lineage by scRNA-seq analysis. Because spines ranging from 8 to 11 gestational weeks were cut into pieces and digested into single cells directly, the spatial distribution of cells remains unclear. In the future, performing spatial transcriptomics experiments will help map cells to their distributions, validate cellular communication, and explain cellular function. Furthermore, scRNA-seq analysis of mouse embryonic spines is critical to validate our scRNA-seq results on fetal spines. Additionally, Cre/loxP-based lineage tracing experiments can be performed to verify that chondrocytes are derived from EPYC + HAPLN1+ fibroblasts (Fib_12) if funding allows.

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

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Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.104679.

ACKNOWLEDGMENTS
This work was Supported by Science and Technology Planning Project of Guangdong Province, China (No. 2021A1515111071 and Guangdong Provincial Clinical Research Center for Birth Defects, No. 2017B 020209001).

AUTHOR CONTRIBUTIONS
Y.D. and Y.Y. conceived the study. M.O. and D.T. designed the experiments. Samples were collected and isolated by D.T., C.L., W.S., W.H., and Z.Z., and the single-cell experiments were performed by F.H. Immunohistochemistry was performed and interpreted by H.Y., and M.O., X.Z., and C.L. generated scRNA-seq datasets and performed the computational analysis. H.Y., M.O., H.W., D.T., and Y.L. interpreted the data. H.Y., M.O., and F.H. wrote the manuscript. Y.D. and W.D. reviewed and improved the manuscript. All authors read and accepted the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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## STAR★METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Anti-NeuN           | Abcam  | #ab177487  |
| Anti-Neurofilament-M| CST    | #A10011-60 |
| Anti-CD163          | Abcam  | #2838      |
| Anti-CDS6           | JieHao | #ab182422  |
| Anti-S100A9         | Abcam  | #CRM-0351  |
| Anti-COL1A          | CST    | #ab92507   |
| Anti-Fibronectin    | CST    | #72026     |
| Anti-CD15           | MXB    | #26836 ab182422 |
| Anti-Aggreccan      | Abcam  | #MAB-0779  |
| Anti-MATN3          | Abcam  | #ab186414  |
| Anti-CD74           | CST    | #ab238893  |
| Anti-APP            | CST    | #77274     |
| Anti-Neuropilin-2   | CST    | #29765     |
| Anti-HAPLN1         | Abcam  | #3366      |
| Anti-SEMA3C         | R&D    | #181997    |
| Anti-EPYC           | Novus  | #MAB1728-SP|
| Anti-MATN4          | Novus  | #NBP2-33445|
| Anti-ME1            | Novus  | #NBP1-77012|
| Anti-UCHL1          | CST    | #H00004199-M03 |
| Anti-DAPI           | Thermo Fisher | #13179 |
| **Biological samples** |    |            |
| Healthy Fetal spine | Shenzhen People’s Hospital | n/a |
| **Chemicals, peptides, and recombinant proteins** | | |
| Buffer EB           | QIAGEN | 19086      |
| VAHTS DNA clean Beads| vazyme| N411-02    |
| 1XTE Buffer         | Sangon Biotech | BS48106-0500 |
| Trypan blue         | Bio-RAD | 1450013    |
| RNase,DNase,RNA and DNA Away | Beyotime | R0127      |
| 1XPBS PH7.4         | HyClone/ | SH30256.01 |
| HBSS                | thermo | 14025076   |
| NF water            | BeyoPure Ultrapure Water | ST876      |
| Pure ethanol        | sigma  | E7023-1L   |
| sCellLive™ Tissue Preservation Solution | Singleron Biotechnologies | n/a |
| sCellLive™ Tissue Dissociation Solution | Singleron Biotechnologies | n/a |
| GEXSCOPE® red blood cell lysis buffer | Singleron Biotechnologies | n/a |
| **Critical commercial assays** | | |
| Singleron GEXSCOPE Single Cell RNAseq Library Kit | Singleron Biotechnologies | 1100011 |
| Chromium Single Cell ATAC Reagent Kits User Guide | 10x genomics | CG000168 Rev. B |

(Continued on next page)
Continued

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Yong Dai (daiyong22@aliyun.com).

Materials availability
This study did not generate new unique reagents.

Data and code availability
The datasets/code generated during this study are available at both the Genome Sequence Archive of the National Genomics Data Center: HRA002609 (https://ngdc.cnbc.ac.cn/gsa/ and China National GeneBank (CNGB): CNP0001545. http://db.cnbg.org/cnsa/project/CNP0001545_1de9a3b6/reviewlink/).

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

We obtained six developmental fetal spine tissue samples from six donors in Shenzhen People’s Hospital. The age of fetuses ranges from 53 to 119 days (Table S1). All donors signed informed consent. This work was approved by the ethics committee of Shenzhen People’s Hospital (LL-KY-2019591).

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Deposited data      | This paper | Genome Sequence Archive of the National Genomics Data Center: HRA002609. https://ngdc.cnbc.ac.cn/gsa/ and China National GeneBank (CNGB): CNP0001545. http://db.cnbg.org/cnsa/project/CNP0001545_1de9a3b6/reviewlink/ |
| Software and algorithms | CeleScope v1.9.0 | https://github.com/singleron-RD/CeleScope |
|                     | STAR version 020201 | http://code.google.com/p/rna-star/ |
|                     | featureCounts version 1.6.2 | http://subread.sourceforge.net |
|                     | Seurat v3.1.2 | https://satijalab.org/seurat/archive/v3.2/pbmc3kTutorial.html |
|                     | Monocle Monocle2 | http://monocle-bio.sourceforge.net/ |
|                     | Slingshot 1.4.0 | https://github.com/kstreet13/slingshot |
|                     | clusterProfiler 3.10.1 | http://bioconductor.org/packages/release/bioc/html/clusterProfiler.html |
|                     | STRINGdb 1.22.0 | http://bioconductor.org/packages/release/bioc/html/STRINGdb.html |
|                     | CellphoneDB | https://github.com/Teichlab/cellphonedb |
|                     | SLICE 0.99.0 | https://github.com/xu-lab/SLICE |
|                     | SnapATAC version 1.0.0 | https://github.com/r3fang/SnapATAC |
|                     | MACS2 version 2.1.4 | https://software.broadinstitute.org/software/igv/ |
|                     | Homer v4.11 | https://github.com/r3fang/SnapATAC |
|                     | Cicero version 1.4.0 | https://github.com/r3fang/SnapTools |
METHOD DETAILS
Sample collection and processing
The Ethics Committee approved the human embryo collection and research protocols of Shenzhen People’s Hospital (LL-KY-2019591). The six developmental fetal spine tissue samples from six donors ranging from 8 to 17 gestational weeks were collected after the donors signed an informed consent document in strict observance of the legal and institutional ethical regulations for collection of specimens from elective pregnancy termination at Shenzhen People’s Hospital. All of the protocols in this study complied with the Interim Measures for the Administration of Human Genetic Resources administered by the Ministry of Science and Technology of China. The tissue samples were processed immediately after isolation (Table S1). Each spine was washed with cold and sterilized PBS twice. After dissection into pieces less than 0.6 cm³, the spine segments were washed with cold and sterilized PBS twice again and transferred to a 50-ml conical tube containing GEXSCOPE Tissue Preservation Solution (Singleron), followed by shipping to the Singleron laboratory on an ice pack.

Tissue dissociation and single-cell suspension preparation
Fresh tissue samples were washed three times with Hanks’ balanced salt solution (HBSS) and shred into 1-2 mm pieces. The tissue pieces were then digested in GEXSCOPE Tissue Dissociation Solution (Singleron Biotechnologies) at 37°C for 15 minutes. A 40-micron sterile strainer (Falcon) was used to separate the cells from cell debris and other impurities. The filtered cells were centrifuged, and the cell pellets were resuspended in PBS (HyClone). GEXSCOPE Red Blood Cell Lysis Buffer (Singleron Biotechnologies) was added to the cell suspension to remove the red blood cells. The mixture was then centrifuged and resuspended in PBS. Cells were counted with a TC20 Automated Cell Counter (Bio-Rad), and the final concentration of the cell suspension was 1×10⁵ cells/ml.

scRNA-seq
Single-cell suspensions with a concentration of 1×10⁵ cells/ml in PBS were prepared. Single-cell suspensions were then loaded onto microfluidic devices, and scRNA-seq libraries were constructed according to the Singleron GEXSCOPETM protocol by the GEXSCOPETM Single-Cell RNA Library Kit (Singleron Biotechnologies). Individual libraries were diluted to 4 nM and pooled for sequencing. Pools were sequenced on the Illumina HiSeq X platform with 150 bp paired-end reads.

scATAC-seq
ScATAC-seq libraries were prepared according to the Chromium Single Cell ATAC Reagent Kits User Guide (10x Genomics; CG000168 Rev. B). Briefly, the transposition reaction was prepared by mixing the desired number of nuclei with ATAC Buffer (10X Genomics) and ATAC Enzyme (10X Genomics) before incubation for 60 min at 37°C. Indexed sequencing libraries were obtained by mixing the amplification product with Sample Index PCR Mix (10X Genomics) and Chromium i7 Sample Index (10x Genomics). Finally, libraries were loaded on an Illumina HiSeq X with 50 bp paired-end reads.

scRNA-seq quantification and statistical analysis
Gene quantification was performed by featureCounts (version 1.6.2) after aligning the raw reads to the reference genome GRCh38 with Ensembl (version 92) gene annotation software by STAR (version 020201). For high-quality data, cells with 200-5000 genes, less than 30000 UMI, and a 50% mitochondrial gene expression were selected for the following analysis.

The secondary scRNA-seq data analysis was processed by Seurat (v3.1.2). We used the 2000 highly variable genes to perform principal component analysis (PCA) and used the top 20 principal components for the following cell clustering analysis. Cell clustering was performed by the FindClusters function with a resolution set to 0.8. Finally, we obtained 26 clusters. The FindMarkers function in Seurat with the default parameters was applied to generate differential gene lists of each cluster. The corresponding cell types of the clusters were annotated manually when inspecting the differentially expressed genes. We set the resolution to 1.2 for subclustering fibroblasts, chondrocytes, and neurons. The DoHeatmap function was applied to display the chosen marker genes of each cluster with a heatmap.
**Trajectory analysis**

According to the five crucial developmental trajectories reported in other studies, we performed Monocle analysis of mesodermal progenitor cells and neuroectodermal progenitor cells. The trajectory was constructed by first choosing the top 2000 highly variable genes found by Seurat and reducing the dimensions by DDRTree. To screen out genes with highly variable expression by pseudotime, the intersection between the 2000 differentially expressed genes found above and the gene set generated by the function differentialGeneTest with qval < 0.001 was selected. These selected differentially expressed genes were clustered into 5 clusters by the function plot_pseudotime_heatmap followed by pathway enrichment analysis.

Furthermore, the R package slingshot (1.4.0) was used to model developmental trajectories after subclustering the neurons because of its ability to handle many branching events arbitrarily. SingleCellExperiment objects were created by a single-cell expression matrix and used as input for slingshot. Based on biological knowledge, neuroectodermal progenitor cells were set as the start of the trajectory when performing the slingshot and getLineages functions.

**Pathway enrichment analysis**

For GSVA pathway enrichment analysis, the average gene expression of each cell type was used as input data. We selected GO gene sets, which were composed of the molecular function (MF), biological process (BP), and cellular component (CC) categories, as the reference, and the top 30 significant pathways were visualized by a heatmap.

Additionally, we performed GO and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses with the clusterProfiler (3.10.1) R package. We regarded pathways with padj < 0.05 as significantly enriched.

According to the protein interaction database STRINGdb (1.22.0), the interactions among differential genes were displayed.

**Cell interaction analysis**

To study interactions among different cell types or subclusters, CellphoneDB was used to analyze each sample. We set the threshold of cells expressing a gene within each cluster to 0.1 and the iterations to 1000. The interaction with p < 0.05 was regarded as significant. The interaction between immune cells was displayed by the circlize (0.4.10) R package.

**Single-cell entropy analysis**

SLICE (0.99.0) was used to calculate the entropy and stemness based on single-cell expression data. After removing genes starting with ‘ERCC’ and ribosomal genes, we created a SLICE object and performed a bootstrap calculation of scEntropy to obtain entropy information by the getEntropy function.

**scATAC-seq data filter**

The scATAC-seq data were aligned to the GRCh38 genome by cellranger-atac (1.2.0). Adapters, low-quality sequences, and repeat sequences were removed, followed by quantification.

**Cluster and annotation**

The dimension reduction in scATAC-seq data was performed by a diffusion map implemented by SnapATAC software (version 1.0.0). The scATAC-seq data cluster was achieved based on a graph and displayed by Uniform Manifold Approximation and Projection (UMAP). Functional FindTransferAnchors and TransferData in Seurat were used to annotate scATAC clusters based on scRNA-seq data and the predicted results. Those with a score > 0.5 were selected.

After annotation, MACS2 (version 2.1.4) was used for peak calling and to merge peaks of each cluster to generate the cell X peak matrix. The qval threshold of MACS2 was set to 0.05.

SnapATAC was applied to select cell clusters with close distances for comparison and to test the coaccessibility significance by exactTest to obtain each cell type’s specific peaks. We obtained motifs by using the
script findMotifsGenome.pl of Homer (v4.11) based on specific peaks, and the script annotatePeaks.pl of Homer completed the peak annotation.

**Gene activity score and coaccessibility analysis**

The coaccessibility of the peaks was analyzed by the Cicero (version 1.4.0) R package function run_cicero. cellDataSet was created by the cellXpeak sparse matrix, the coaccessibility score was calculated by run_cicero, and results with a coaccessibility score > 0 were selected. Furthermore, Cicero could predict the gene activity and obtain a gene activity score by the function build_gene_activity_matrix based on the relationship among peaks and the location relationship between a peak and gene.

**Multiplexed immunofluorescence**

Multiplexed immunofluorescence was performed by staining 4-μm-thick formalin-fixed, paraffin-embedded whole tissue sections with standard, primary antibodies sequentially followed by a TSA 7-color kit (D110071-50T, Yuanxibio). Then, the samples were stained with DAPI. For example, deparaffinized slides were incubated with an anti-NeuN antibody (#ab177487, Abcam) for 30 minutes and then treated with an anti-rabbit/mouse horseradish peroxidase-conjugated (HRP) secondary antibody (#A10011-60, Yuanxibio) for 10 minutes. Then, the staining was developed for observation for 10 minutes using TSA 520 per the manufacturer’s instructions. Slides were washed in TBST buffer and then transferred to preheated citrate solution (90°C) before being heat-treated in a microwave set at 20% of the maximum power for 15 minutes. Slides were cooled in the same solution to room temperature. Between all steps, the slides were washed with Tris buffer. The same process was repeated for the following antibodies/fluorescent dyes in order: anti-Neurofilament-M (#2838, CST), anti-CD163 (#ab182422, Abcam), anti-CD56 (#CRM-0351, JieHao), anti-S100A9 (#ab92507, Abcam), anti-COL1A1 (#72026, CST), anti-Fibronectin (#26836, CST), anti-CD15 (#MAB-0779, MXB), anti-Aggregcan (#ab186414, Abcam), anti-MATN3 (#ab238893, Abcam), anti-CD74 (#77274, CST), anti-APP (#29765, CST), anti-Neuropilin-2 (#3366, CST), anti-HAPLN1 (#181997, Abcam), anti-SEMA3C (#MAB1728-SP, R&D), anti-EPYC (#NBP2-33445, Novus), anti-MATN4 (#NBP1-77012, Novus), anti-ME1 (#H00004199-M03, Novus), and UCHL1 (#13179, CST). Each slide was then treated with two drops of DAPI (D1306; Thermo Fisher), washed in distilled water, and covered with a coverslip. Slides were air-dried, and pictures were taken with the Panoramic MIDI tissue imaging system (3DHISTECH). A list of primary antibodies including manufacturer and used dilutions was provided in Table S11. Images were analyzed using Indica Halo software.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

To identify differentially expressed genes (DEGs), we used the Seurat FindMarkers function based on Wilcoxon rank sum test with default parameters, and selected the genes expressed in more than 10% of the cells in both of the compared groups of cells and with an average log(Fold Change) value greater than 0.25 as DEGs. Adjusted p value was calculated by Bonferroni Correction and the value 0.05 was used as the criterion to evaluate the statistical significance.

The statistic significance of differential accessible regions (DARs) was identified using exactTest. Significance was set to pvalue < 0.05 and logFC > 0.