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Single-cell transcriptomics analysis showing functional heterogeneity in decidual stromal cells during labor

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ABSTRACT

To investigate the heterogeneity of decidual stromal cells (DSCs) and their functional alterations during delivery, we conducted single-cell RNA sequencing analysis to characterize the transcriptomic profiles of DSCs before and after labor onset. According to their transcriptomic profiles, DSCs (6382 cells) were clustered into five subgroups with different functions. Similar to stromal cells, cells in cluster 1 were involved in cell substrate adhesion. On the other hand, cells in clusters 2 and 3 were enriched in signal transduction-related genes. Labor onset led to significant alterations in many pathways, including the activator protein 1 pathway (all clusters), as well as in the response to lipopolysaccharide (clusters 1–3). The downregulated genes were involved in coagulation, ATP synthesis, and oxygen homeostasis, possibly reflecting the oxygen and energy balance during delivery. Our findings highlight that peripartum DSCs are heterogeneous and play multiple roles in labor.

Significance of this study

What is already known about this subject?
- The decidua is essential for pregnancy maintenance.
- Decidual stromal cells are key decidual tissue components.
- Decidual stromal cells (DSCs) regulate the function of the maternal–fetal interface during pregnancy.

What are the new findings?
- DSCs are heterogeneous at the transcriptomic level.
- DSCs form five clusters based on their single-cell RNA profiles.
- The five clusters are predicted to have different functions during pregnancy.
- Labor onset had different effects on the various DSC subsets.

How might these results change the focus of research or clinical practice?
- DSC heterogeneity may have important implications for labor, which may help to understand the onset of labor more accurately.

INTRODUCTION

Decidual tissue plays a crucial role in embryo implantation and delivery.[1 2] The initial stages of delivery, including uterine activation, are the most critical aspect of labor. Recent evidence suggests that activation of uterine smooth muscles results from physiological alterations due to labor onset.[3] Therefore, changes in decidual tissue adjacent to the myometrium may contribute to initial uterine activation.

In the initial stages of labor, multiple inflammatory mediators, including prostaglandins and cytokines, are released at the maternal–fetal interface.[4 5] Given that decidual tissue is a multicomponent tissue, the inflammatory reaction of the decidua inevitably involves various cell types, including decidual T cells and natural killer cells.[6 7] With the exception of decidual stromal cells (DSCs), these immune cell types are also present in tissues other than the decidua.

DSCs are derived from endometrial cells, and decidualization during embryo implantation gives rise to this unique cell type in pregnancy.[8 9] After decidualization, DSCs acquire secretory properties and play an important role in the maintenance of pregnancy by progesterone.[10] Decidualization and progesterone withdrawal after labor cause changes in DSCs. Characterization of the alterations in DSCs during delivery may provide further insight into the unique role of the decidua in parturition.

Although the critical role of DSCs about progesterone has become evident, it remains unclear whether DSCs contribute to the termination of pregnancy (ie, the onset of labor). The heterogeneity of the decidua further complicates the characterization of its role in parturition. Studies of women in the early stages of pregnancy have confirmed that the decidua and placenta are highly heterogeneous tissues.[11–13] DSCs may maintain their heterogeneity during the perinatal period until delivery. To

characterize the heterogeneity of DSCs and elucidate their role in delivery, we performed single-cell RNA sequencing of peripartum DSCs.

MATERIALS AND METHODS

Clinical information

Similar to previous studies,[14 15] all pregnant women (in the non-labor group) requested cesarean section and had one of the following conditions: macrosomia, fetal distress, or breech presentation. None of the study subjects exhibited maternal abnormalities (eg, diabetes, pre-eclampsia, hypertension). After delivery, none of the women exhibited chorioamnionitis or other abnormal conditions at the maternal–fetal interface. All of the six singleton pregnant women gave birth between 37 and 40 weeks. Three had vaginal births (labor onset) and the remaining three had caesarean sections (before labor).

Tissue isolation and single-cell sequencing

Single-cell suspensions were obtained as previously described.[11–13] Decidua were collected and washed in phosphate-buffered saline (Genview). After delivery, the decidua was obtained from the full-term placenta by scraping, as previously described.[16] Subsequently, tissues were dissociated and digested using collagenase (Sigma-Aldrich) dissolved in phosphate-buffered saline. Single-cell suspensions were obtained by passing the dissociated cells through a 40 µm cell strainer (Falcon).

Single-cell cDNA library preparation and sequencing

A single-cell RNA sequencing library was constructed using the Single Cell 5' Library and Gel Bead Kit (10×Genomics) following the manufacturer's instructions and previously described protocols.[17] Sequencing was conducted by Capitalbio Technology Corporation (Beijing) using an Illumina NovaSeq 6000 Sequencing System (at least 100 000 reads per cell).

The sequencing data were analyzed and visualized using Cell Ranger V.2.0.1 and Cell Browser V.2.0.0 (10×Genomics). T-distributed stochastic neighbor embedding and principal component analysis were performed using the R t-distributed stochastic neighbor embedding package of R software.

Gene ontology and pathway analyses

We used the gene list obtained from the RNA sequencing analysis and Metascape to conduct gene ontology and

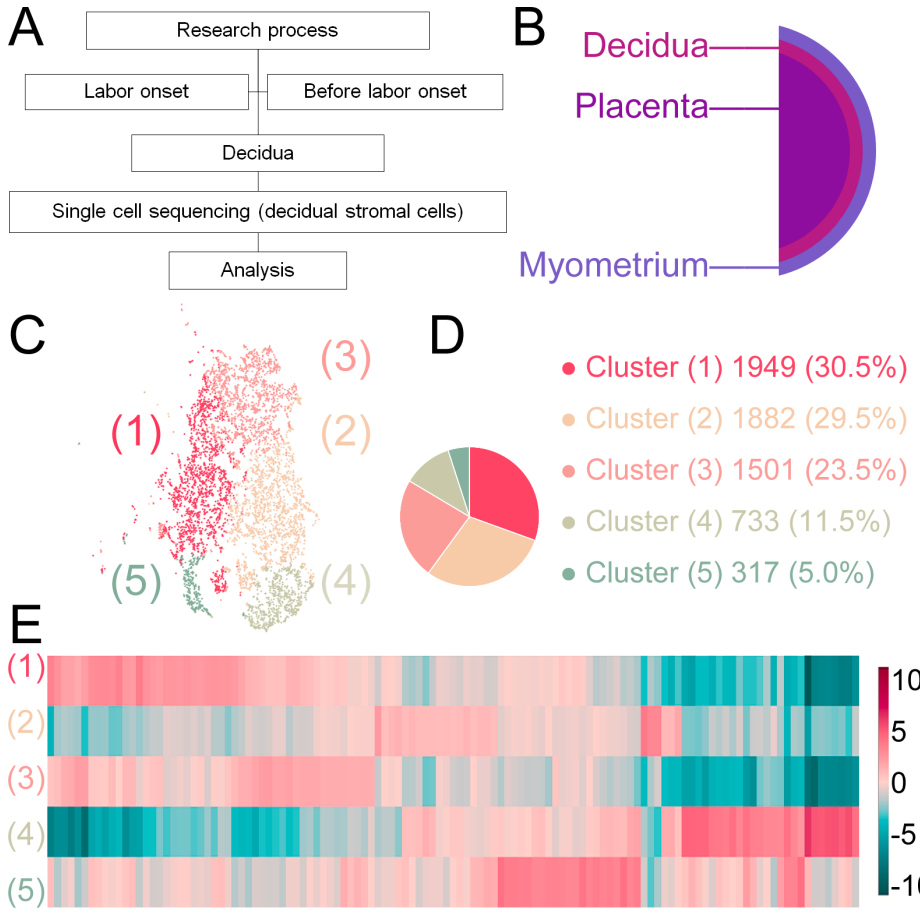

Figure 1 Heterogeneity of DSCs. (A) Schematic illustration of the study design and procedures. (B) Position of the decidua at the maternal–fetal interface. (C) T-distributed stochastic neighbor embedding diagram showing the distribution of DSC subgroups before and after labor onset. DSCs formed five clusters based on their transcriptomic profiles. The different clusters are shown in different colors. The numbers represent the cluster numbers. (D) Pie chart comparing the number of cells in each cluster. (E) Heat map showing the differences in gene expression of the cells in the five clusters. DSC, decidual stromal cell.

Table 1 Main functions of each cluster

Cluster gene ontology	Count	%	Log10 (P)
Cluster 1			
Actin filament-based process	54	14.75	−19.98
Focal adhesion	29	7.92	−19.47
Cell-substrate adhesion	36	9.84	−18.77
Blood vessel development	49	13.39	−16.58
Cluster 2			
Interleukin-10 signaling	3	27.27	−5.95
Second messenger-mediated signaling	3	27.27	−3.04
Response to toxic substance	3	27.27	−2.81
Cluster 3			
Activator protein one pathway	7	17.95	−10.74
Skeletal muscle cell differentiation	5	12.82	−7.06
Interferon alpha/beta signaling	4	10.26	−5.33
Cluster 4			
Respiratory electron transport	15	3.76	−9.93
Regulated exocytosis	41	10.28	−9.86
PID integrin 3 pathway	10	2.51	−8.8
Steroid metabolic process	23	5.76	−8.05
Cluster 5			
Extracellular matrix organization	41	9.98	−20.68
Cell morphogenesis involved in differentiation	40	9.73	−9.58
Cell junction organization	24	5.84	−9.4
Transmembrane receptor protein tyrosine kinase signaling pathway	38	9.25	−8.81
Tissue morphogenesis	36	8.76	−8.67

Log10 (P) is the p value in log base 10.

pathway analyses.[18] The analyses included enrichment analyses for pathways, biological processes, reactome gene sets, and canonical pathways. Based on the accumulative hypergeometric distribution, the p values were calculated.

RESULTS

Heterogeneity of DSCs

The decidua is located at the center of the maternal–fetal interface, adjacent to the myometrium and placenta. This unique position of the decidua reflects its complex organization (figure 1A,B). Decidualization during pregnancy entails the development of DSCs, which are characterized by high expression levels of *IGFBP1*; hence, *IGFBP1* is widely used as a DSC marker.[12 13] After screening cells for *IGFBP1*, we identified and analyzed a total of 6382 DSCs (online supplemental figures S1 and S2). Five subgroups (clusters) of cells were identified based on differences in gene expression profiles; all clusters were numbered according to the number of cells that comprised them (figure 1C,D).

The five clusters exhibited significant transcriptomic differences (figure 1E). We conducted functional analyses of the differentially expressed genes in each cluster (online supplemental table S1) and noted profound differences in the functional characteristics of the different DSC clusters (table 1). According to the results of functional analyses, the functions of cells in cluster 1 (eg, cell-substrate adhesion) were consistent with those of stromal cells. In contrast, cells in clusters 2 and 3 were enriched in signal transduction-related processes. These findings highlight the transcriptomic and functional diversity of DSCs.

Each cluster has a different role in labor

In this study, we collected samples of DSCs in two states. According to the distribution of t-distributed stochastic neighbor embedding diagrams, differences in the transcriptomic profiles of cells in some clusters were enhanced after labor. Additionally, the number of cells in each cluster changed after labor. In general, the number of cells in each cluster was higher before delivery. Before labor onset, cluster one had the highest number of cells, and cluster five had the lowest number of cells. After delivery, cluster two had the highest number of cells among all clusters (figure 2A–D).

Interestingly, the transcriptomic profiles and functional characteristics of the cells in each cluster were altered after labor. Cluster 1 exhibited 57 upregulated genes after labor, while cluster 2 had 69 genes, cluster 3 had 42 genes, cluster 4 had 108 genes, and cluster 5 had seven genes. These findings suggest that the cells in cluster 5 may have a less important role in labor than those in the other clusters. These results also suggest that, in addition to the functional heterogeneity of DSCs, DSCs may have different roles in the onset of labor (figure 2E,F; online supplemental table S2).

Similarly, the numbers of downregulated genes in the various clusters differed after labor. These transcriptomic changes indicated that the cells in clusters 2 and 4 had a more critical role in labor. Functional analyses revealed that the downregulated genes were involved in coagulation, ATP synthesis, and regulation of oxygen levels, possibly reflecting the oxygen and energy balance during labor (figure 2G,H; online supplemental table S3).

DISCUSSION

As a part of the maternal–fetal interface, the decidua is important in embryo implantation and pregnancy maintenance. Single-cell RNA sequencing of the decidua collected from women in early pregnancy revealed the high heterogeneity of this tissue.[11–13]The DSCs showed differences in decidual prolactin expression levels,[13] reflecting adaptation to microenvironmental changes. Thus, the heterogeneity of DSCs may begin early during pregnancy, possibly in the first trimester.

In the first trimester, the onset of decidualization is associated with enhanced proliferation, differentiation, and angiogenesis in stromal cells.[19] DSCs support the integrity of the endometrium and regulate the function of dendritic cells by secreting macrophage inhibitory cytokine-1, thereby supporting early pregnancy.[10 20] During the perinatal period, the increase in decidual prostaglandin levels promotes delivery, and prostaglandin E2 secretion by DSCs is regulated by the *ALK3* signaling pathway.[10 21]The evidence suggests functional heterogeneity of DSCs. To obtain further insight into this heterogeneity, we conducted single-cell RNA sequencing analyses of peripartum DSCs. The inflammation-related pathways interleukin-10 signaling (cluster 2) and activator protein one signaling (cluster 3) were significantly enriched in different DSC clusters (table 1), suggesting high heterogeneity in the inflammatory states of DSCs.[22–24]Aberrant type I interferon (including

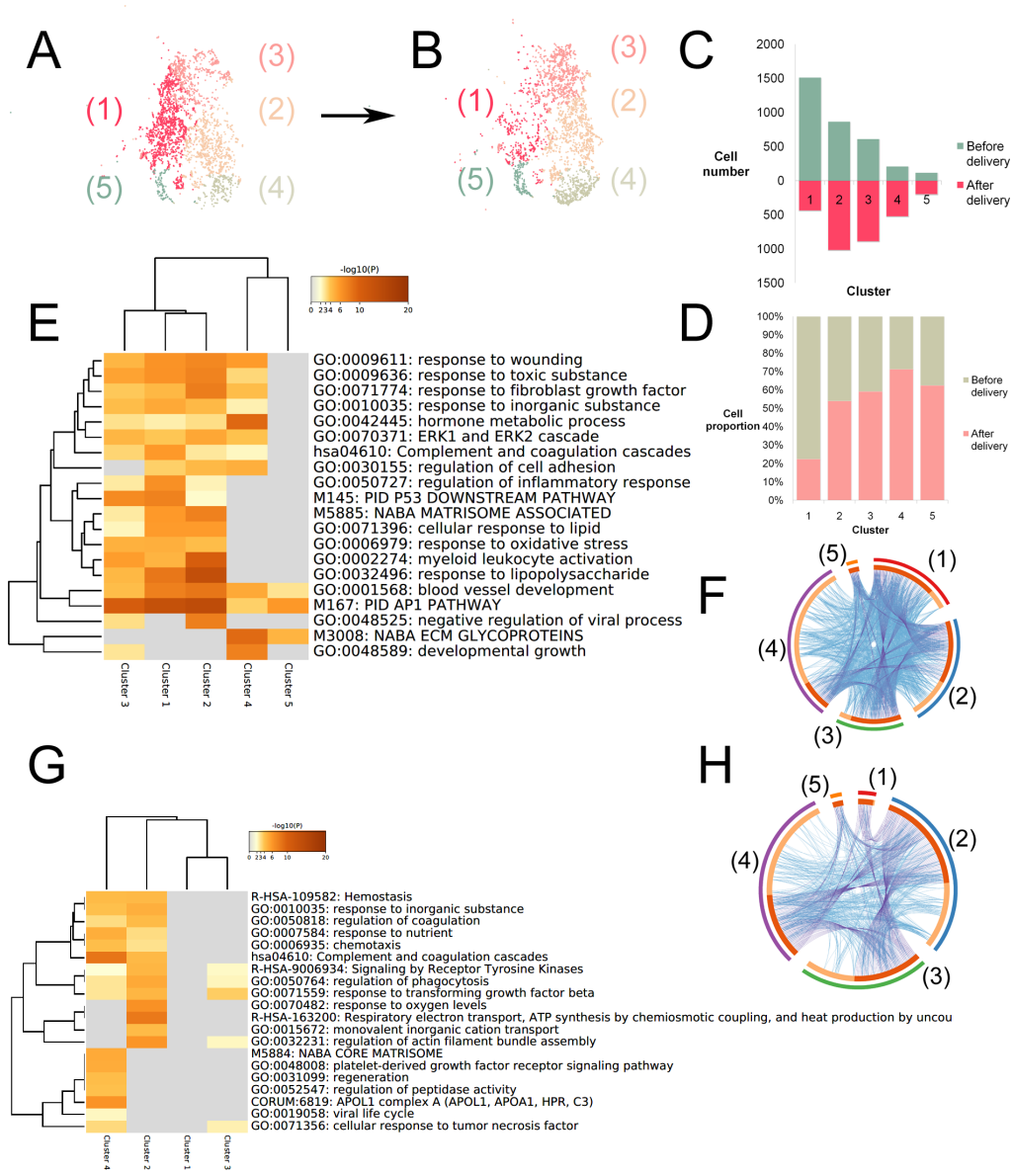

Figure 2 Changes in DSC subgroups after labor. (A) T-distributed stochastic neighbor embedding diagram showing the distribution of DSC subgroups before labor onset. (B) T-distributed stochastic neighbor embedding diagram showing the distribution of DSC subgroups after labor onset. (C) Comparison of the number of cells in each cluster before and after delivery. (D) Comparison of the proportion of cells in each cluster before and after delivery. (E) Heat map of enriched terms in upregulated genes in DSC subgroups after delivery. Log10 (P) is the p value in log base 10. (F) overlap of upregulated genes in DSCs in the different clusters. blue curves link genes that belong to the same enriched ontology term. the inner circle represents gene Lists, where hits are arranged along the Arc. genes that hit multiple Lists are colored in dark orange, whereas unique genes are shown in light orange. (G) heat map of enriched terms across downregulated genes of DSC subgroups after delivery. Log10 (P) is the p value in log base 10. (H) Overlap of downregulated genes in DSC in the different clusters. Blue curves link genes that belong to the same enriched ontology term. The inner circle represents gene lists, where hits are arranged along the arc. Genes that hit multiple lists are dark orange in color, whereas unique genes are shown in light orange. DSC, decidual stromal cell. ECM, extracellular matrix.

interferon-alpha/beta) signaling has been associated with miscarriage.[25] Interferon-related pathways were found to be enriched in cluster 3, suggesting the potential role of DSCs in this cluster in the regulation of immune homeostasis during pregnancy. Furthermore, the receptor protein tyrosine kinase signaling pathway was found to be significantly enriched in cluster 5. Protein tyrosine kinase can activate the intracellular signaling pathways that regulate cell proliferation, metabolism, and differentiation[26 27]; all of these cellular processes are pivotal to vascular growth of the placenta. These findings confirm previous results indicating DSC functional heterogeneity. Further investigation is required to understand the cross-talk (direct or indirect) between these DSC functions and their role in labor onset.

In this study, we also analyzed changes in DSC gene expression and function during labor. Intriguingly, these

differences varied markedly among the different DSC subgroups. Notably, the number of DSCs in cluster 1 decreased after labor, whereas the numbers in the other clusters increased. Functional analyses revealed the critical role of DSCs in cluster 1 in cell-substrate adhesion; the decrease in the number of cells in this cluster may be linked to the onset of labor. However, the potential influence of sampling and population differences on these findings should be further assessed.

After labor, all DSC clusters were enriched in activator protein 1 pathway-related genes. This pathway has previously been shown to be active in uterine smooth muscle cells, suggesting shared functions between DSCs and uterine smooth muscle cells on the maternal side of the maternal–fetal interface.[22 28] In DSC clusters 1–3, the upregulation of genes involved in the response to lipopolysaccharides suggested that, in these DSC subgroups, labor may be perceived as an inflammatory response.[29–31]Genes related to hormone metabolism were upregulated in DSC clusters 1–4. Hormonal changes can be observed during delivery; these changes include withdrawal of progesterone and changes in estrogen levels.[32 33]Therefore, these findings suggest that certain DSC subsets may regulate progesterone withdrawal and estrogen levels.

To comprehensively evaluate the transcriptomic profiles and functions of peripartum DSCs, we also assessed the genes downregulated in these subgroups after labor. Only the DSCs in cluster 2 were found to be involved in the regulation of oxygen levels. While oxygen levels can regulate fetal membrane biology before delivery, low oxygen levels after delivery do not affect the fetal membrane.[34] In the present study, we focused on the decidua and found that only DSC cluster 2 was involved in the regulation of oxygen levels. Moreover, DSCs in cluster 2 were implicated in ATP synthesis. After the onset of labor, myometrial smooth muscle cells located close to DSCs generate ATP and lactic acid, both of which exert potent anti-inflammatory effects through GPR81.[35]Thus, our results highlight the crucial role of inflammatory responses in certain DSC subsets in the onset of labor.

Our study has some limitations. We focused on the heterogeneity of DSCs only at the single-cell transcriptomic level; future comprehensive studies are required to further elucidate the functional heterogeneity of DSCs. The decidua is a complex and heterogeneous tissue. In this study, we analyzed DSCs from full-term delivery samples. However, the transcriptional profiles of these DSCs may be affected by the tissular (decidual) environment and other cell types in the decidua, such as decidual T cells. Additionally, we did not collect samples from women with maternal diseases. Nevertheless, it is possible that the transcriptional profiles and functions of DSCs are influenced by the fetal conditions. Therefore, this potential impact should be considered.

In summary, we evaluated the transcriptional heterogeneity of DSCs at the single-cell level. We identified various DSC subsets with different functions during labor and demonstrated the complex roles of DSCs in labor. Our results provide further insight into the biological processes preceding uterine smooth muscle activation and alterations in the decidual tissue during pregnancy.

Contributors Conceptualization: JH and WZ; data curation: JH, QP and JL; formal analysis: CP, YZ and JN; funding acquisition: WZ; investigation: QP and LL; methodology: JH, QP and YX; project administration: WZ; resources: YX, WW, CP, YZ, RL, LH, TL, XL and XZ; supervision: WZ; writing the original draft: JH; writing, review and editing; WZ. All authors read and approved the final manuscript.

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Competing interests None declared.

Patient consent for publication Not required.

Ethics approval Informed consent was obtained from each subject prior to research start. The study protocol was approved by the medical ethics committee of the Xiangya Hospital Central South University (2018081027) and Changsha Hospital for Maternal and Child Health Care Ethics Committee (2018810).

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