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Single-cell transcriptomics reveals the heterogeneity of the decidual endothelial cells that participate in labor onset

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Abstract. – OBJECTIVE: To investigate the heterogeneity of decidual endothelial cells and their changes during delivery.

PATIENTS AND METHODS: Single-cell RNA sequencing was used to characterize the transcriptomes of decidual endothelial cells before and after the onset of labor.

RESULTS: Decidual endothelial cells (9748 cells) were divided into five subgroups with different functions according to differences in the transcriptome. The functions of cluster 5 were enriched in vascular development and response to growth factors. After the onset of labor, the activities of each cluster were different, including the interleukin 17 pathway and regulation of ERK1 and ERK2 cascade. The downregulated genes were related to scavenger receptor (cluster 5), which may reflect the process of endothelial activation. In terms of genetic changes, cluster 5 may be more actively involved in labor than the other clusters.

CONCLUSIONS: Peripartum decidual endothelial cells are heterogeneous and participate in labor to varying degrees. One of the five subtypes of endothelial cells may be more actively involved in labor onset. Our findings may enable the assessment of decidual endothelial cells and labor onset.

Key Words:
Decidua, Endothelial cell, Labor onset, Delivery, Single cell sequencing, Transcriptomics.

Introduction

Decidua is located at the center of the maternal-fetal interface and is in close contact with the myometrium and placenta, which is important for implantation and delivery[1-3]. Because myometrium activation results from labor onset, changes in decidua could reveal the state of the myometrium before activation[4]. As a multi-component tissue, evaluation of the role of decidua in labor may require information on its heterogeneity. Single-cell sequencing technology could solve the problem of heterogeneity. Indeed, single-cell sequencing research on the placenta and decidua in early pregnancy indicated the existence of decidual heterogeneity[5-7].

From implantation and pregnancy initiation, blood vessel networks are established in decidua to support blood circulation between the fetus and mother, which may involve vascular cell disruption[8]. With development of the placenta and the increase in the need for blood, considerable blood exchange occurs at the maternal-fetal interface (decidua) during delivery. This exchange is mediated by a network constructed by endothelial cells (ECs). At the end of pregnancy (labor onset), specific changes may be observed in ECs to support delivery. Decidual ECs are reportedly associated with premature delivery, implicating them in labor[9,10]. In the perinatal period, these ECs may maintain their heterogeneity until delivery and participate in labor.

Therefore, studying the heterogeneity of ECs in the perinatal period would provide insight into the changes of, and reasons for, labor onset. To this end, we performed single-cell RNA sequencing of peripartum decidual ECs. The results show the heterogeneity of ECs and their changes during labor, providing insight into the role of decidua in labor onset.

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Patients and Methods

Clinical Information

Six women with singleton pregnancies were recruited. They had no pregnancy comorbidities and delivered between 37 and 40 weeks. Three had vaginal births (after labor onset) and the remaining three had caesarean sections (before labor onset). All patients were diagnosed in Xiangya Hospital Central South University or Changsha Hospital for Maternal and Child Health Care between October 1st, 2018 and Jan 1st, 2019. All subjects gave their written informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the 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).

Tissue Isolation and Single-Cell Sequencing

Single-cell suspensions were generated as described previously[5-7]. Decidua was washed in phosphate-buffered saline (PBS, Genview, El Monte, CA, USA). Subsequently, a single-cell suspension was produced after dissociation, digestion (collagenase, Sigma-Aldrich, St. Louis, MO, USA; PBS, Genview, El Monte, CA, USA) and filtration (Falcon 40 μm Cell Strainer, Corning, Durham, NC, USA).

Single-cell cDNA Library Preparation and Sequencing

According to the manufacturer's instructions and a prior report[11], a single-cell 5' Library Gel Bead Kit was used to construct single-cell RNA-seq libraries. A Illumina Novaseq 6000 sequencer (Illumina, San Diego, CA, USA) with a depth of at least 100,000 reads per cell was used for sequencing, which was performed by Capitalbio Technology Corporation (Beijing, China).

The data were analyzed by Cell Ranger 2.0.1 (10×Genomics, Pleasanton, CA, USA). Principal component analysis and t-distributed stochastic neighbor embedding (t-SNE) were performed using the R t-SNE package in R software. Cell Browser 2.0.0 (10×Genomics, Pleasanton, CA, USA) was used for presentation.

Gene Ontology (GO) and Pathway Analysis

Metascape was used for GO and pathway analyses, which included pathways, GO biological processes, and canonical pathways[12]. The *p*-values were calculated based on the accumulative hypergeometric distribution.

Results

Heterogeneity of ECs

All ECs, identified by high expression of LYVE1, CCL21, and COLEC12 (markers of EC), were included in the analyses[6,7]. After screening, a total of 9748 ECs was analyzed. Five subgroups (clusters) were identified based on differences in gene expression profiles. All clusters were numbered according to cell number (Figure 1).

The five clusters had different transcriptome characteristics. For example, ACKR1, SPARCL1, and IFITM1 were highly expressed in cluster 5. We conducted functional analyses of the differentially expressed genes in each cluster (**Supplementary Table I**) and found that the functions of the five subgroups of ECs were different (Table I).

The functions of cluster 1 were enriched in response to inorganic substance and wound healing. The functions of clusters 3, 4, and 5 were consistent with those of classical ECs, such as roles in cell adhesion, cell migration, blood vessel development, and extracellular matrix organization. In addition, the functions of cluster 5 included response to growth factor, cytokine-mediated signaling pathway, and response to oxygen levels. Similarly, the functions of clusters 2 and 5 were related to oxygen levels, suggesting their relevance (Table I).

Each Cluster Participates in Labor to Varying Degrees

Our sample included ECs in two states, and we compared them separately. Based on the distribution of t-SNE diagrams, the difference in the internal transcriptome of some clusters may have become larger after labor. At the same time, the number and proportion of cells also changed (Figure 2A-2D).

Importantly, after labor, the activity of each cluster was not consistent, and the upregulated genes were different. Clusters 1-5 showed specific upregulation of 53, 37, 20, 38, and 69 genes, respectively (**Supplementary Table II**), suggesting that cluster 3 may be a relatively inactive subgroup. The upregulated genes may indicate the functions of the clusters, including interleukin 17 pathway, cytokine-mediated signaling pathway, and response to peptide. Among them, cluster 5

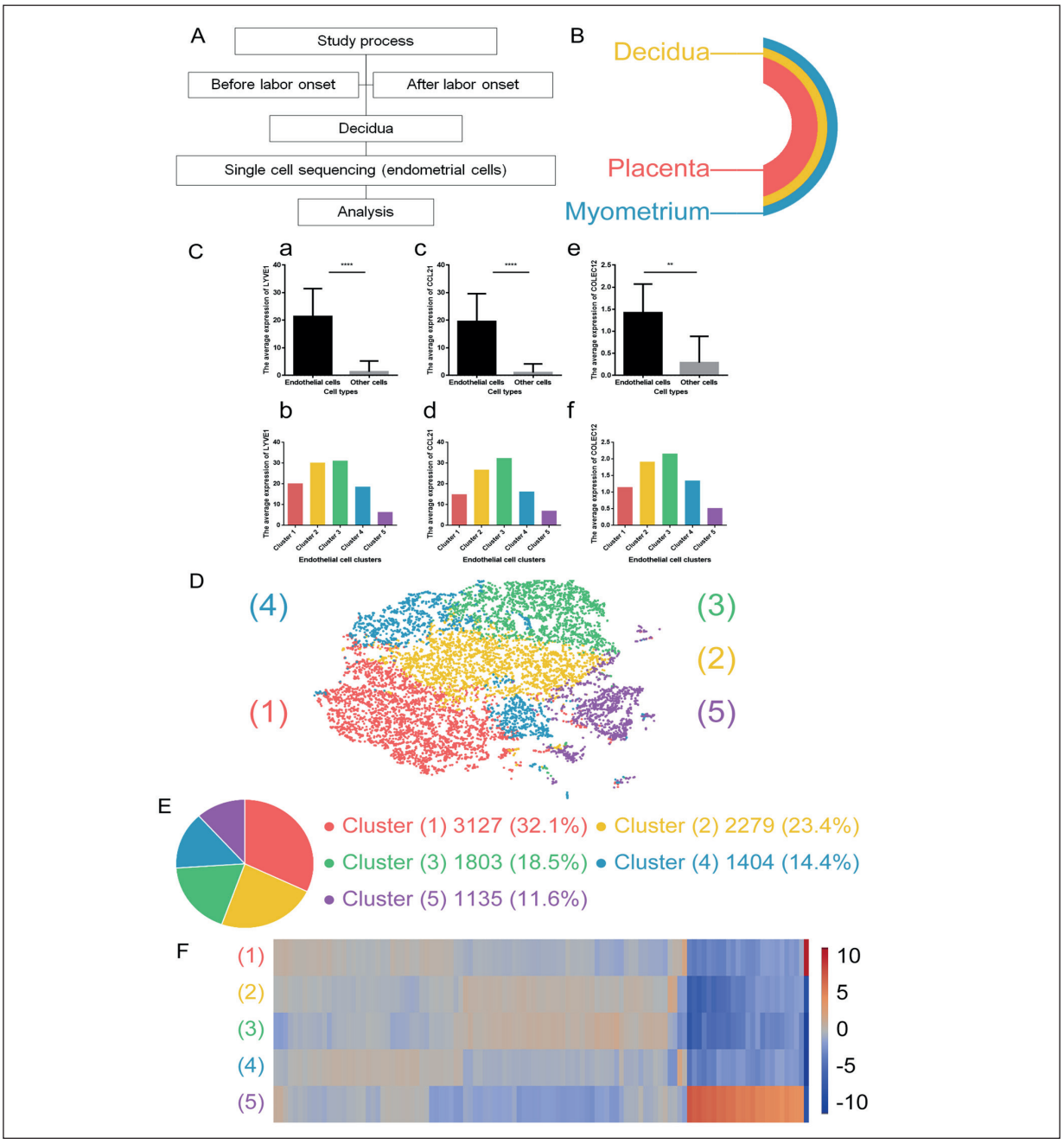

Figure 1. Heterogeneity of decidual endothelial cells. **A,** The process of the study. **B,** The position of decidua. **C,** Marker of decidual endothelial cells. (a) LYVE1 expression in decidual endothelial cells and other cells of decidua; the difference was significant. (b) LYVE1 expression in decidual endothelial cells (five clusters). (c) CCL21 expression in decidual endothelial cells and other cells of decidua; the difference was significant. (d) CCL21 expression in decidual endothelial cells (five clusters). (e) COLEC12 expression in decidual endothelial cells and other cells of decidua; the difference was significant. (f) COLEC12 expression in decidual endothelial cells (five clusters). **D,** t-Distributed stochastic neighbor embedding diagram showing the distribution of decidual endothelial cell subgroups. Decidual endothelial cells were divided into five clusters, which are labeled with different colors. The numbers represent the cluster numbers. **E,** Pie chart showing the composition ratio of the cell number in each cluster. **F,** Heat map of differences in gene expression among the five clusters.

involved the greatest number of terms, such as the interleukin 17 pathway, AP1 pathway, and response to peptide. In addition to the functional heterogeneity of ECs, these results suggest that they may have multiple roles in the onset of labor (Figure 2E, 2F).

Table I. Main functions of each cluster.

Cluster gene ontology	Count	%	Log10 (P)
Cluster 1			
Peptide chain elongation	4	17	-5.84
Positive regulation of apoptotic process	6	26	-4.49
AP1 pathway	3	13	-4.4
Response to inorganic substance	5	22	-3.79
Wound healing	5	22	-3.75
Cluster 2			
Transport of small molecules	4	80	-5.4
Response to reactive oxygen species	3	60	-5.04
Cluster 3			
Actin filament-based process	40	12.42	-12.4
Regulation of cell adhesion	36	11.18	-11.02
Ameboidal-type cell migration	28	8.7	-10.29
Response to growth factor	35	10.87	-10.06
Platelet degranulation	15	4.66	-9.74
Cell junction organization	21	6.52	-9.14
Muscle system process	25	7.76	-8.42
Endothelium development	14	4.35	-8.38
Cluster 4			
AP1 pathway	4	21.05	-6.62
Body fluid secretion	4	21.05	-6.04
Fluid shear stress and atherosclerosis	4	21.05	-5.38
Positive regulation of cell adhesion	5	26.32	-4.85
Negative regulation of cell proliferation	6	31.58	-4.7
Cluster 5			
Blood vessel development	67	15.16	-25.68
Extracellular matrix organization	39	8.82	-21.14
Response to wounding	54	12.22	-18.68
Response to growth factor	53	11.99	-16.79
Cytokine-mediated signaling pathway	55	12.44	-16.63
Response to oxygen levels	38	8.6	-16.17

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

The numbers of downregulated genes differed among the clusters (**Supplementary Table III**). Cluster 3 was more inactive; i.e., no specific downregulated gene was found. Most terms were in cluster 5, such as the scavenger receptor. In general, the downregulated genes had functions in response to mechanical stimulus, response to oxygen levels, and cell-substrate adhesion, suggesting that this process was influenced by numerous factors (Figure 2G, 2H).

Discussion

Labor has been studied from the perspective of tissue (decidua) and cell type (ECs) [1,9,13]. The internal heterogeneity of decidual ECs needs to be clarified and discussed. However, the complexity of decidua tissue and ECs hampers investigation of their role in labor[9,14]. Single-cell sequencing enables insight into cell heterogeneity[15]. We investigated the heterogeneity of ECs at single-cell resolution and explored decidual EC subtypes.

Good support of endothelial tissues (ECs) is important for health[16]. ECs (endothelial tissues) cover the inner surface of blood vessels (lymphatics) and are the junctions of vessels (lymphatics) and blood (lymph). ECs in the blood-vessel wall regulate blood flow and release nitric oxide (related to preterm labor) to dilate blood vessels[17,18]. However, preterm birth is a syndrome with multiple causes, including breakdown of maternal-fetal tolerance, decidual senescence, and vascular disorders[14]. Onset of labor may involve many factors (such as inflammation and immune factors) and various cell types (such as ECs and T cells)[9,14,19]. Therefore, nitric oxide and ECs may be associated with onset of labor, partly. Integration of endothelial

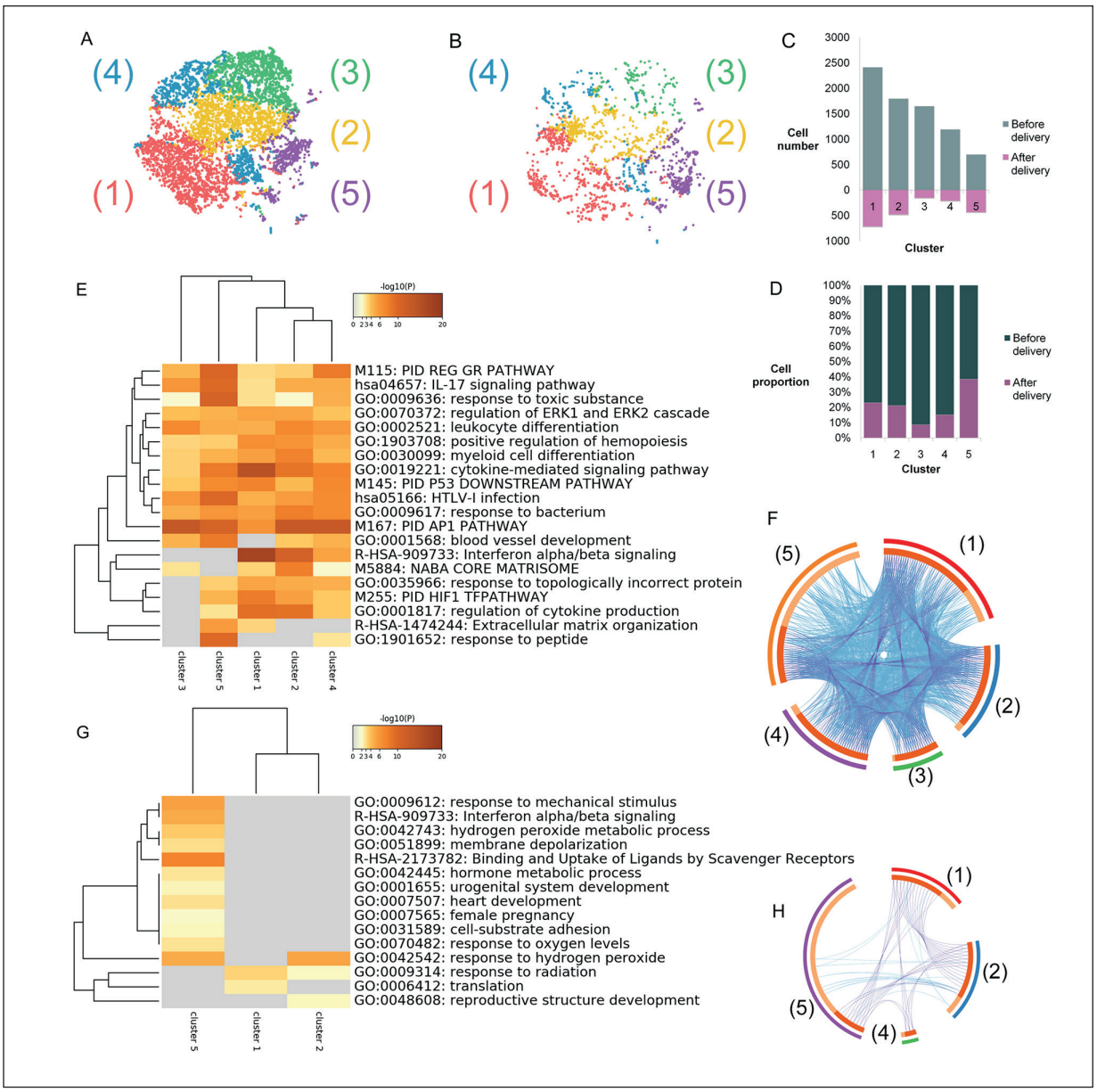

Figure 2. Changes in decidual endothelial cell subgroups after onset of labor. **A,** t-Distributed stochastic neighbor embedding diagram showing the distribution of decidual endothelial cell subgroups before labor. **B,** t-Distributed stochastic neighbor embedding diagram showing the distribution of decidual endothelial cell subgroups after labor. **C,** Cell number in the clusters before and after delivery. **D,** Cell proportion in the clusters before and after delivery. **E,** Heat map of enriched terms among upregulated genes in the decidual endothelial cell subgroups after delivery. Log10 (P) is the p-value in log base 10. **F,** Overlap between upregulated genes of decidual endothelial cell subgroups (five clusters), including at the shared-term level. Blue curves link genes that belong to the same enriched ontology term. The inner circle represents gene lists; hits are arranged along the arc. Genes that hit multiple lists are in dark orange, and genes unique to a list are in light orange. **G,** Heat map of enriched terms among downregulated genes in the decidual endothelial cell subgroups after delivery. Log10 (P) is the p-value in log base 10. **H,** Overlap between downregulated genes of decidual endothelial cell subgroups (five clusters), including at shared-term level. Blue curves link genes that belong to the same enriched ontology term. The inner circle represents gene lists; hits are arranged along the arc. Genes that hit multiple lists are in dark orange, and genes unique to a list are in light orange.

and perivascular stromal signaling can determine pregnancy outcomes[9]. Therefore, decidual ECs may be partly involved in labor.

EC cluster 5 expressed ACKR1 and SPARCL1 at a high level. In early pregnancy, ACKR1 plays a role in recruiting leukocytes to pro-

mote implantation, and SPARCL1 might affect embryo implantation and placenta development[20,21], revealing functions of this cluster in early pregnancy. IFITM1 was highly expressed in cluster 5, and is reportedly related to angiogenesis[22]. It is possible that cluster 5 interacts with trophoblasts[23]. GO analysis showed that cluster 5 contributed to vascular development and response to growth factor. These results reflect the role of cluster 5 in pregnancy maintenance.

To study the role of ECs in labor, we compared EC clusters before and after labor onset. There were more upregulated genes than downregulated genes, indicating that ECs may be more involved in labor onset via activation rather than inhibition. Cluster 5 showed enhanced activity after labor. The interleukin 17 pathway was activated after delivery, especially in cluster 5. In this cluster, CCL2 was also upregulated after labor. Interleukin 17 is related to angiogenesis by ECs and acts via the CXCL8 and CCL2 pathway[24,25]. This suggests that cluster 5 participates in labor via the above-mentioned pathways. The ERK1 and ERK2 cascade clustered almost equally among the five clusters. This pathway triggers endothelial homeostasis and migration[26-28], suggesting involvement of all EC clusters. After labor, most downregulated genes were in cluster 5. One GO term involved the scavenger receptor, a negative regulator of endothelial activation[29]. This indicated that onset of labor is accompanied by endothelial activation by ECs and that ECs play heterogeneous roles in labor. More precisely, cluster 5 may be more actively involved in labor onset than the other clusters.

This study was limited by the small number of samples and potential for sampling error, because it is difficult to obtain all ECs. In addition, the data are merely preliminary. Therefore, more-detailed biological assessments are required in future studies. We provide gene lists and hope that our work provides insight into decidual ECs at the single-cell level and will act as a reference for further research.

In summary, we evaluated ECs and their heterogeneity at the single-cell level. Different subgroups had different regulatory functions during labor, reflecting the complexity of the labor process. Our results reflect the process before uterine smooth muscle activation and provide information on specific changes in decidual tissue during pregnancy.

Conclusions

Peripartum decidual ECs are heterogeneous and participate in labor to varying degrees. One of the five subtypes of ECs may be more actively involved in labor onset. Our findings may enable assessment of decidual ECs and labor onset.

Conflict of Interest

The authors declare that they have no conflict of interests.

Acknowledgements

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