Molecular subtypes of vascular endothelial cell differentiation in keloid by single-cell sequencing

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To the Editor: Keloid is a fibroproliferative disorder characterized by invasive growth beyond wound margins, causing significant physical and psychosocial morbidity. Persistent inflammation and aberrant extracellular matrix deposition by activated fibroblasts and myofibroblasts are central to keloid pathogenesis. Increasing evidence highlights the pivotal role of vascular endothelial cells (ECs) in disease progression.[1] Features of pathological angiogenesis in keloids include disorganized microvasculature, luminal flattening, and functional impairments that paradoxically induce local hypoxia despite high blood flow. This dysfunctional vasculature increases vascular permeability, promoting perivascular accumulation of inflammatory cells and mediators, thereby amplifying inflammation and fibrotic signaling. Notably, clinical associations between endothelial dysfunction—such as that observed in hypertension—and increased keloid susceptibility underscore the central role of ECs in disease predisposition. These findings position vascular ECs not only as contributors to the profibrotic microenvironment but also as potential diagnostic and prognostic indicators. Thus, assessing endothelial function holds significant clinical promise for risk prediction, molecular classification, and the development of precision interventions in keloid management.

This study integrated microarray, single-cell RNA sequencing, and bulk RNA sequencing data to reconstruct EC subpopulations and their putative *de novo* differentiation trajectories in keloids. By combining multidimensional bioinformatic analysis with experimental validation, we established a novel molecular classification system with potential implications for prognostic assessment and precision therapeutic development. To further validate the clinical relevance of this classification framework, we performed comprehensive histopathological and immunohistochemical analyses on discarded keloid tissue specimens obtained from 19 patients undergoing day surgery, along with their matched clinical data, following approval by the Ethics Committee of Changhai Hospital, Shanghai (No. CHEC2023-088) and after obtaining written informed consent from all participating patients.

The study design is summarized in Supplementary Figure 1, http://links.lww.com/CM9/C755. A total of 13,162 ECs were classified into six subpopulations (EC 1–6) based on the similarity of the gene expression profiles across individual cells. Significant differences in subpopulation distribution were observed between normal scars and keloids. EC 5 (highly expressing von willebrand factor, [VWF]) and EC 2 were predominantly present in normal scars, whereas EC 1 and EC 6 (highly expressing collagen type I alpha 2 [COL1A2]) each constituted more than 50% of the endothelial population in keloids. Notably, EC 3 and EC 4 exhibited similar proportional distributions in both normal scars and keloids, suggesting that they may fulfill relatively stable functional roles across these two tissue states. Robust intercellular communication was observed among the six EC clusters. EC 3 occupied a central position in the communication network and primarily mediated signaling to other cell types, with chemokine ligand 12 (CXCL12) identified as a key ligand [Supplementary Figure 2, http://links.lww.com/CM9/C755].

Pseudotime trajectory analysis reconstructed the differentiation order of the six EC subpopulations. EC 1 resides at the origin of the differentiation trajectory and progressively transitions toward EC 2; EC 2 bifurcates into two distinct differentiation branches, both of which initially share EC 3 as a common progenitor. As illustrated in Supplementary Figure 3, http://links.lww.com/CM9/C755, the upper branch ultimately differentiates into EC 6, whereas the lower branch terminally differentiates into EC 4. Key differentially expressed genes (gap junction protein alpha 4 [*GJA4*], hes family bHLH transcription factor 4 [*HES4*], lysosomal associated protein

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transmembrane 5 [*LAPTM5*], podoplanin [*PDPN*], retinol binding protein 4 [*RBP4*], and stathmin 1 [*STMN1*]) were found to play decisive roles in determining cell fate [Supplementary Figures 3 and 4, http://links.lww.com/CM9/C755].

Integrated analysis of single-cell and microarray transcriptomic data identified differentially expressed endothelial fate-related genes (DEEFRGs) that show significantly higher expression levels in normal scar tissues compared to keloids, suggesting potential diagnostic utility [Supplementary Figure 5, http://links.lww.com/CM9/C755]. Based on non-parametric tests and Pearson correlation analysis, we constructed a co-expression regulatory network. In this network, nodes were color-coded to highlight key genes governing cellular differentiation into five distinct trajectories: More than one-third of the genes (shown in green) were identified as critical determinants driving endothelial cell transition toward cell state 1; additionally, 7 genes (purple), 1 gene (brown), 3 genes (yellow), and 1 gene (gray) were found to specifically promote differentiation toward cell states 2, 3, 4, and 5, respectively. Furthermore, the genes extracted from this regulatory network were subjected to subsequent clustering analysis. According to the consensus clustering plot, these genes were robustly partitioned into three distinct clusters. A heatmap illustrated the expression levels of these fate-related genes across the three clusters, as well as across different time points, sex groups, and age groups. This visualization revealed significant associations between the three consensus clusters and the aforementioned clinicopathological features, further underscoring a strong correlation between endothelial cell fate determination and consensus-based clustering: Fate-determining genes were highly expressed in consensus cluster 3, whereas their expression levels in consensus clusters 1 and 2 were comparable but significantly lower than in cluster 3. Most of these genes—such as phospholipid phosphatase 3 (*PLPP3*), bone marrow stromal cell antigen 2 (*BST2*), caveolae-associated protein 2 (*Cavin-2*), and DIX domain-containing 1 (*DIXDC1*)—are linked to angiogenesis and endothelial cell (EC) function: PLPP3 enhances cell–cell adhesion and endothelial barrier integrity;[2] BST2 promotes endothelial progenitor cell adhesion;[3] Cavin-2 supports EC function via eNOS regulation;[4] and DIXDC1 mediates VEGF–Wnt/β-catenin crosstalk to coordinate vascular development.[5] The high expression of these genes is associated with enhanced angiogenesis and greater vascular stability. This supports our hypothesis that higher endothelial maturity—stronger angiogenic capacity and more stable EC function—is associated with better prognosis in patients with keloid [Supplementary Figure 6, http://links.lww.com/CM9/C755].

Chi-squared tests further confirmed significant differences among the three consensus clusters with respect to both time of diagnosis (P <0.001) and diagnostic category (P <0.001). The data also indicated that cluster 3 was associated with the most favorable prognosis and was predominantly observed in healthy individuals; cluster 2 conferred an intermediate prognosis; whereas cluster 1, which was most common in keloid patients, was significantly associated with the worst outcomes. On these findings, we classified ECs into three distinct clinical subtypes: low-risk group (consensus cluster 3), middle-risk group (consensus cluster 2), and high-risk group (consensus cluster 1). Moreover, a principal component analysis model constructed using 11 DEEFRGs selected by least absolute shrinkage and selection operator regression demonstrated robust prognostic performance in both training and test sets (area under the curve [AUC] >0.86) [Supplementary Figure 6, http://links.lww.com/CM9/C755].

Given the significant association between the expression levels of DEEFRGs and clinical subtypes, marker genes for each subgroup were identified based on biological function and expression patterns: Low-risk group (bone marrow stromal cell antigen 2 [BST2]$^+$), middle-risk group (phosphatidylinositol-3,4,5-trisphosphate Rac exchange factor 1 [PREX1]$^+$), and high-risk group (BST2$^-$) [Supplementary Figure 7, http://links.lww.com/CM9/C755].

By performing co-expression analysis of key biomarkers, including differentlly expressed transcription factors, DEEFRGs, signaling pathways, and immune cells, we constructed subtype-specific regulatory networks for distinct keloid clinical subtypes and identified transcription factors and downstream pathways significantly associated with key DEEFRGs. The core genes and their upstream–downstream regulatory relationships within the molecular network varied significantly across risk groups. In the low-risk group, BST2 and transmembrane protein 70 (TMEM70) act as central regulators—BST2, positively correlated with the transcription factor transcription factor 7 like 1 (TCF7L1), suppresses the androgen response pathway, while TMEM70, co-expressed with Ring1 and YY1 binding protein (RYBP), B-cell-specific Moloney murine leukemia virus insertion site 1 (BMI-1), and TCF7L1, activates Wnt/β-catenin signaling. In the middle-risk group, von Willebrand factor A 1 (VWA1) (regulated by BMI-1) and matrix metalloproteinase 10 (MMP10) (controlled by tumor protein P63 (TP63)) are pivotal: VWA1 strongly promotes bile acid metabolism and protein secretion pathways, whereas MMP10 markedly inhibits mechanistic target of rapamycin complex 1 (mTORC1) signaling. In the high-risk group, synaptotagmin 1 (SYT1) and transmembrane protein 178A (TMEM178A) dominate—SYT1, co-regulated by homeobox C9 (HOXC9), Runt-related transcription factor 1 (RUNX1), and TP63, activates transforming growth factor-β (TGF-β) signaling, while TMEM178A, governed by GATA binding protein 3(GATA3) and Runt-related transcription factor 1 (RUNX1), robustly stimulates both Notch and Wnt/β-catenin pathways [Supplementary Figures 8, http://links.lww.com/CM9/C755]. Integrated chemical compound analysis indicated that levomepromazine may reverse aberrant gene expression in the high-risk group, demonstrating potential therapeutic value [Supplementary Figures 9, http://links.lww.com/CM9/C755].

The reliability of our findings was further supported by immunohistochemical analyses of 19 clinical samples. In terms of vascular morphology, we observed that patients in the high-risk group had a greater number of blood vessels but significantly narrower lumens, whereas the vascular

morphology in the low-risk group more closely resembled that of normal tissue. The distribution of various marker genes in these immunohistochemical results also corroborated our earlier findings, providing additional evidence for the accuracy of our analysis, which revealed that the low-risk group exhibited significantly higher expression levels of BST2, MMP10, and TMEM70 compared to the other subgroups, whereas PREX1 was most abundantly expressed in the middle-risk group. In contrast, both BST2 and PREX1 expression were markedly reduced in the high-risk group. These correspondences between morphological and molecular features further validate the clinical interpretability of the "endothelial maturity" concept proposed in this study [Supplementary Figures 10, http://links.lww.com/CM9/C755].

Correlation with clinical phenotypes indicated that patients in the high-risk group were more prone to sensory abnormalities, including pain and pruritus, and displayed a significantly higher prevalence of keloids with abnormal pigmentation. Histopathological evaluation of keloid tissues further demonstrated that the low-risk group contained a greater abundance of morphologically normal blood vessels, while vascular occlusion was predominant in the high-risk group. The middle-risk group exhibited an approximately equal proportion of normal and aberrant vasculature. In terms of prognosis, the high-risk group showed the highest recurrence rate, followed by the middle-risk group, whereas no recurrence events were observed in the low-risk group (despite partial loss to follow-up). Consistently, vancouver scar scale (VSS) scores were significantly elevated in the high-risk group compared to both the low- and middle-risk groups, reflecting greater scar severity—a finding that further substantiates the robustness and clinical validity of the proposed molecular classification system.

Using single-cell transcriptomic analysis, we identified six distinct endothelial cell subpopulations in both keloid and normal scar tissues, and pseudotime trajectory analysis further revealed a continuous developmental trajectory from an immature to a mature endothelial state. Furthermore, based on DEEFRGs, we propose—for the first time—the concept of "endothelial maturity" and establish three molecular subtypes of keloids that are significantly associated with clinical prognosis. This classification system not only elucidates the intrinsic relationship between endothelial developmental states and disease severity but also demonstrates strong clinical feasibility: key marker genes (e.g., *BST2* and *PREX1*) can be readily detected in routine pathological specimens via immunohistochemistry or RT-qPCR, making the approach applicable across healthcare settings for postoperative risk stratification and early intervention against scar deterioration. Notably, the molecular subtypes show a strong correlation with VSS scores, underscoring their potential utility in guiding personalized therapeutic decisions—high-risk patients may benefit from intensified combination therapies, whereas low-risk individuals might be effectively managed with conservaive or localized interventions.

However, this study has some limitations. Due to the reliance on purely bioinformatic analyses, it is challenging to fully capture the complexity of EC developmental trajectories and differentiation states. Future work should integrate cellular and animal experiments to validate the causal relationships between the identified DEEFRGs and phenotypic outcomes and to evaluate the clinical value of the novel keloid molecular classification from multiple dimensions. In addition, the current clinical sample size is limited, and further inclusion of more samples is necessary for experimental validation. The feasibility and therapeutic potential of drug development targeting this classification will also be a focus of subsequent research. We also aim to detect DEEFRGs at early stages of scar formation to enable molecular classification and provide early warning for keloid risk.

In summary, the molecular classification of keloids based on endothelial fate-related genes proposed in this study not only deepens the understanding of vascular endothelial heterogeneity in keloids but also provides a theoretical foundation for more precise prevention and treatment of scars, holding significant clinical translational potential.

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Conflicts of interest

None.

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