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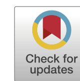
RAPID COMMUNICATION

Single-cell RNA sequencing reveals abnormal transcriptome signature of erythroid progenitors in pure red cell aplasia



Pure red cell aplasia (PRCA) is a bone marrow failure syndrome characterized by severe anemia, reticulocytopenia, selective reduction, and even the absence of bone marrow erythroid precursors, while leukocyte and platelet counts are normal.[1] Acquired PRCA can be divided into primary and secondary. Both are mediated by immune responses. Reduction in the quantity and dysfunction of committed erythroid progenitors (EPs), including burst forming unit-erythroid (BFU-E) and colony forming unit-erythroid (CFU-E), may be the source of reduced erythropoiesis and needs to be elucidated. High throughput sequencing is of great significance for the in-depth study of reduced or absent EPs. In this study, we used single-cell RNA sequencing (scRNA-seq) to analyze EPs from the bone marrow of PRCA patients, to explore the pathogenesis of PRCA and guide treatments.

Bone marrow aspirates were obtained from five PRCA patients, including two patients with newly diagnosed PRCA (ND-PRCA), two patients obtaining complete response (CR-PRCA) after treatment with sirolimus, and one refractory PRCA (R-PRCA) (Table S1). Lineage-negative (Lin^-) cells were sorted out by immunomagnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) from bone marrow aspirates for scRNA-seq. This study was approved by the Ethics Committee of the General Hospital, Tianjin Medical University, and all the patients signed informed consent. The scRNA-seq data of bone marrow mononuclear cells from five healthy controls were downloaded from the Gene Expression Omnibus database (GSE120221).[2]

A 10× Chromium Controller was used for barcoded and Illumina NovaSeq 6000 (Illumina, San Diego, CA, USA) was used for sequencing. After quality control and filtering, dimension reduction and clustering were obtained using principal component analysis, and t-distributed Stochastic Neighbor Embedding was used to visualize data structures. Then analyses of differentially expressed genes (DEGs), Gene Ontology (GO) functional enrichment, Gene Set Enrichment Analysis (GSEA), and trajectory inference were given. The details on data analysis are available in supplementary materials.

A total of 23,186 Lin^- cells were recruited for analysis, including 6652 erythroid/megakaryocytic (E/MK) cells. We divided E/MK precursors into megakaryocyte-erythroid progenitors (MEPs), BFU-E, CFU-E, erythroblasts, and megakaryocytic (MK) precursors (Fig. 1A). We found that the frequencies of MEPs and BFU-E were not significantly decreased in ND-PRCA patients compared with controls, while CFU-E and erythroblasts were significantly decreased and MK precursors were increased in ND-PRCA patients, which confirmed the block in erythroid development and the predominance of MK differentiation in PRCA (Fig. 1B). The ratio in CR-PRCA patients who were treated with sirolimus displayed significant improvement. In one R-PRCA patient who had no response to sirolimus treatment, the ratio of CFU-E and erythroblasts was still reduced, which was similar to the characteristics of ND-PRCA. The trajectories of E/MK development displayed the same result (Fig. 1C). We validated the proportion of EPs in the bone marrow of PRCA patients by multicolor flow cytometry and got similar results (Fig. 1D).

Subsequently, we focused on DEGs in EPs, including MEP, BFU-E, and CFU-E. Our results showed that ribosomal genes were widely down-regulated in the three stages of EPs in

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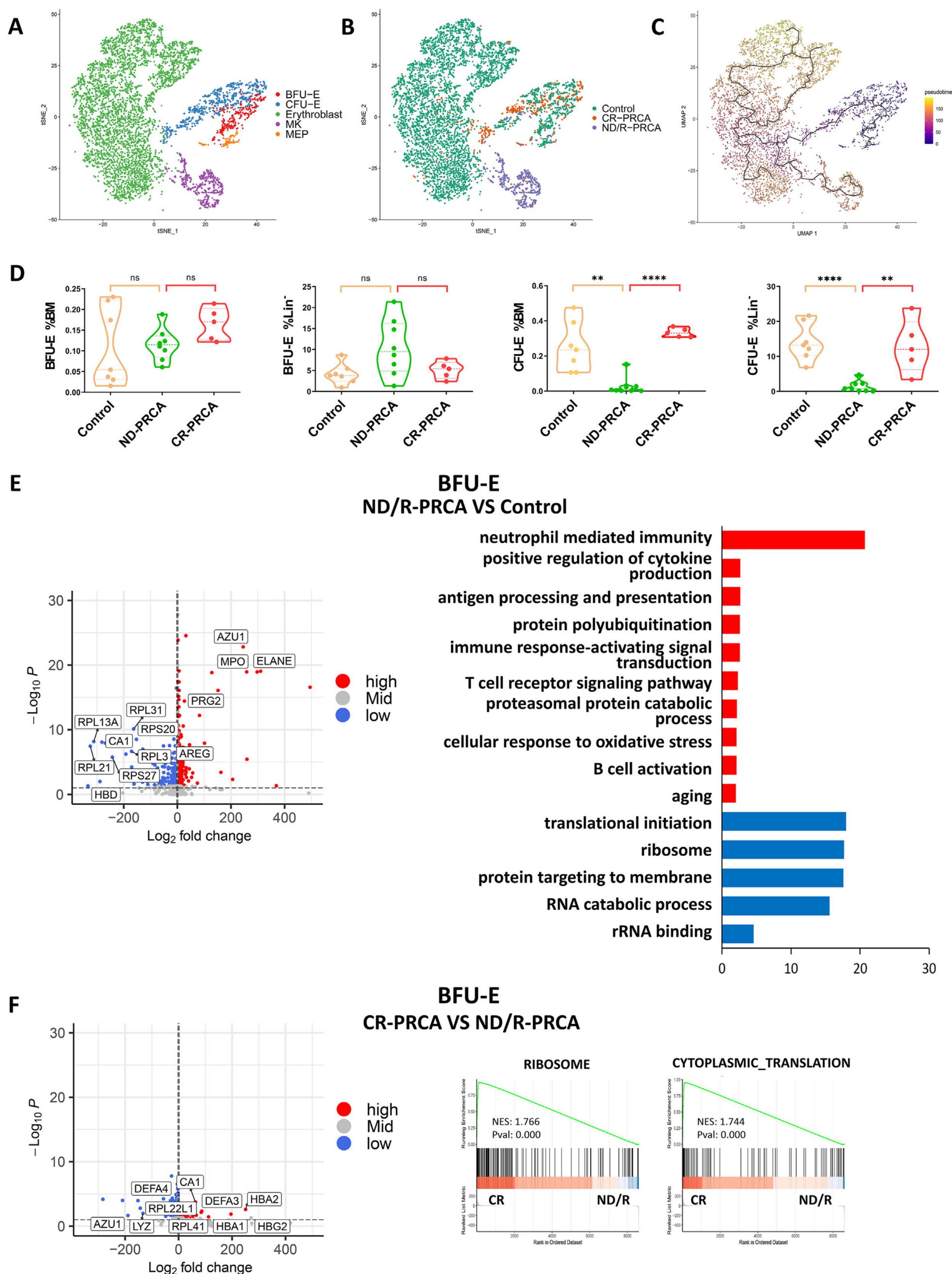

Figure 1 Transcriptome atlas of erythroid/megakaryocytic (E/MK) cells in controls and pure red cell aplasia (PRCA) patients, and analyses of differentially expressed genes (DEGs) in burst forming unit-erythroid (BFU-E) between groups. **(A)** t-distributed Stochastic Neighbor Embedding (tSNE) plot of E/MK cells. Cell clusters are colored to indicate cell types by expressed known markers. MEP, megakaryocyte-erythroid progenitor; BFU-E, burst forming unit-erythroid; CFU-E, colony forming unit-erythroid; MK,

ND/R-PRCA patients compared with those in controls (Fig. 1E; Fig. S1A, B), which suggested that defective ribosome biogenesis may be a key mechanism in the pathogenesis of decreased erythropoiesis. Our results revealed that acquired PRCA has a similar mechanism to congenital PRCA (Diamond-Blackfan anemia, DBA), a kind of ribosomopathy caused by gene mutations of ribosomal protein. Genes associated with neutrophil granules were up-regulated in all three stages of EPs (Fig. 1E; Fig. S1A, B), which suggested that inflammatory response may impair erythroid differentiation. In addition to the above DEGs, we identified that other up-regulated DEGs in BFU-E of ND/R-PRCA patients compared with controls were enriched in immune response, T/B cell activation, positive regulation of cytokine production, protein polyubiquitination, aging, *etc*. as indicated by GO enrichment analysis (Fig. 1E). Up-regulated DEGs in CFU-E of ND/R-PRCA patients were also enriched in immune response and inflammatory factor production, while genes encoding for hemoglobin showed decreased expression (Fig. S1B). These results supported that immune response can impair EPs and inhibit erythropoiesis; besides, aging and protein degradation by polyubiquitination may induce the apoptosis of EPs. After treatment with sirolimus, there was a significant improvement in the DEGs above in CFU-E of CR-PRCA patients compared with those of ND/R-PRCA patients (Fig. S2). However, only partial improvement was seen in BFU-E of CR-PRCA patients, including ribosomal genes, genes associated with neutrophil granules, and genes encoding for hemoglobin, which indicates the risk of recurrence if treatment is discontinued (Fig. 1F).

ScRNA-seq has been used to explore the pathogenesis of erythropoietic failure in congenital PRCA. Iskander et al[3] analyzed the hematopoietic cells of patients with RPS-DBA and RPL-DBA genotypes and found completely different developmental trajectories of these two genotypes. In RPS-DBA patients, erythroid cells are almost absent, while RPL-DBA patients have relatively preserved abnormal erythroid progenitors and precursors. Rio et al[4] found that there was a manifest imbalance between globin chain and heme synthesis in erythroid cells of DBA patients, especially in RPL5 or RPL11 depleted phenotypes, resulting in suppression of erythroid differentiation. Our results reveal an abnormal transcriptome signature of EPs in PRCA by scRNA-seq. Aberrant gene expression profiles of BFU-E may be the key component in blocked erythropoiesis, though the count of which did not decrease significantly. Wang et al[5] divided BFU-E into five clusters and found that erythroid development may be blocked at the stage of BFU-E in DBA patients. They found that the count of the terminal stage of BFU-E decreased significantly and the transcriptional profiles were aberrant.

Our results suggested that defective ribosome biogenesis may play an important role in impaired erythroid differentiation, which was involved in both congenital and acquired PRCA. Ribosomopathies are often characterized by hypoproliferative anemia, such as Schwachman-Diamond syndrome, dyskeratosis congenita, and cartilage hair hypoplasia. In addition, myelodysplastic syndrome with isolated del(5q) is characterized by erythroid hyperplasia, due to haploinsufficiency of the ribosomal protein RPS14. But the p53 signaling pathway is not activated in acquired PRCA, which is distinct from DBA. We need an in-depth investigation into the effects of defective ribosome biogenesis on erythropoiesis.

In addition to hyperinflammatory response and immune attack of EPs, aging and protein polyubiquitination were also involved in the process of arrested erythroid differentiation. It is worth noting that a complete hematologic response through treatment with sirolimus does not completely correct the abnormal transcriptional profiles of BFU-E. Our findings not only update new insights into the pathophysiology of PRCA but also guide the treatment strategies for PRCA.

Ethics declaration

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Tianjin Medical University General Hospital (No. IRB2022-KY-128). Informed consent was obtained from all individual participants included in the study.

Author contributions

YL, HN, JR, and ZW performed research and analyzed the data. HW designed the studies, ensured the correct analysis of the data, and drafted the manuscript. LY, LX, ZS, RF, and

megakaryocytic precursor. **(B)** tSNE plot of E/MK cells labeled by conditions. **(C)** Pseudo-time analysis from MEPs to erythroblasts and MKs estimated by Monocle 3. **(D)** Frequencies of BFU-E and CFU-E in total nucleated cells and lineage negative (Lin^-) hematopoietic cells by flow cytometry of controls, newly diagnosed/refractory PRCA patients (ND/R-PRCA) and PRCA patients achieved complete response (CR-PRCA). Bars show means $\pm$ standard error of the mean of biological replicates. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. ns, not significant. Comparisons between groups were performed using a one-way analysis of variance. **(E)** Volcano plot (left) of DEGs in BFU-E between ND/R-PRCA patients and controls. Red plot: up-regulated in ND/R-PRCA patients; blue plot: down-regulated in ND/R-PRCA patients. Gene Ontology (GO) functional enrichment bar chart (right) of DEGs in BFU-E between ND/R-PRCA patients and controls. Red bar: up-regulated in ND/R-PRCA patients; blue bar: down-regulated in ND/R-PRCA patients. **(F)** Volcano plot (left) of DEGs in BFU-E between CR-PRCA patients and ND/R-PRCA patients. Red plot: up-regulated in CR-PRCA patients; blue plot: down-regulated in CR-PRCA patients. Gene Set Enrichment Analysis (GSEA) maps (right) of ribosomal gene sets in BFU-E of CR-PRCA patients compared with those of ND/R-PRCA patients.

ZC assisted in design research, oversaw data collection, and contributed to the writing of the manuscript. All authors carefully revised the manuscript and finally approved the manuscript.

Conflict of interests

All authors declare no conflict of interests.

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Data availability

The raw data reported in this study are deposited in the NCBI Sequence Read Archive under bioproject No. PRJNA898870.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2023.03.002.

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