Rapid Response of Neutrophils Determined by Shortest Differentiation Trajectories and Absence of Self-Renewal in committed Progenitors

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Abstract

Despite substantial advances in our understanding of hematopoietic stem cell self-renewal and differentiation, the underlying mechanisms have remained elusive. Neutrophils are the primary and rapid responders during infections of innate immunity and can quickly generate an enormous number of mature neutrophils for immune defense. The underlying mechanisms have been investigated but still present conflicts. Our previous study clearly redefined progenitors and reformed the hierarchy of hematopoiesis. Here, we aim to reveal the underlying mechanisms and provide a new interpretation of immune phenomena. We describe the conversion of the cell cycle from differentiation to proliferation in peripheral hematopoiesis. The differentiation hematopoietic progenitor cells (HPCs) were maintained in cell cycle G1 phase, controlled by the DNA replication of cell cycle. DNA replication switched and controlled the progenitor status from differentiation to proliferation. Our study showed that the initial progenitors in adult peripheral blood exhibit self-renewal capabilities but absent after lineage commitment is completed during hematopoiesis. Furthermore, GATA2 and CSF3R were identified as the key transcription factors which determine the co-segregation among neutrophils and other lineages, deciphering the underlying molecular mechanisms. Neutrophil present the shortest

differentiation trajectories in the hematopoietic hierarchy, which decide their role as rapid responders. Our results revealed the straightforward characterization of HPCs' differentiation and proliferation activities, facilitating advancements in inflammation and disease treatment.

Keywords: cell cycle, hematopoiesis, self-renewal, neutrophils, shortest differentiation trajectories

Introduction

Hematopoietic stem cells (HSCs) possess the potential of self-renewal, generating daughter HSCs by cell division, and can differentiation into various blood cell lineages. Despite substantial advances in our understanding of HSC self-renewal and differentiation, the underlying mechanism has remained elusive(Laurenti and Gottgens, 2018). The inefficiently capture and lineage heterogeneity impede standardized definitions and the revelation of molecular mechanisms underlying fate decision and lineage commitment (Eaves, 2015; Paul et al., 2015). Moreover, the single-cell RNA (scRNA) sequencing transcription atlas attempts to redefine progenitors and reform the classical hematopoietic hierarchy, but contradictions persist (Drissen et al., 2019; Zheng et al., 2018). These contradictions are influenced by progenitor original, capture methods, inefficient definitions, and analysis algorithms (Pellin et al., 2019). It widely accepted that progenitors continuously differentiate into all lineages during hematopoiesis (Buenrostro et al., 2018; Velten et al., 2017). This continuous differentiation makes it difficult to define transitioned progenitors by surface marker due to the lack of reliable boundaries among them (Zheng *et al.*, 2018). Therefore, owing to inconsistent definitions and conflicting hematopoietic hierarchical models (Jacobsen and Nerlov, 2019), the regulatory mechanisms underlying self-renewal and differentiation remain unclear. Investigating the state transition from quiescent to activation during hematopoiesis is challenging.

Our previous study redefined the entire spectrum of original hematopoietic progenitor cells (HPCs) and reformed the hematopoietic hierarchy roadmap using a

comprehensive transcriptional atlas (Chen et al., 2024). Hematopoietic system can be reconstituted by HSC transplantation, suggests that the self-renewal potential in HPCs must exist in peripheral blood following G-CSF mobilization. The states of differentiation, proliferation, and self-renewal of progenitors can be captured simultaneously, as progenitors continuously differentiate into all lineages during hematopoiesis. Here, based on the clearly redefinition and reformed hierarchy of hematopoiesis, we aim to reveal the underlying mechanism of self-renewal and differentiation during peripheral hematopoiesis. Furthermore, we purpose to provide a new interpretation of immune phenomena, such as the causation of neutrophils as the primary and rapid responders during infections. Our study showed the initial progenitors in adult peripheral blood exhibit the self-renewal capabilities but gradually absent after lineage commitment is completed during the hematopoiesis, a process controlled by the DNA replication of cell cycle. This study makes significant breakthroughs in the fundamental discipline of immunity development. Collectively, our results highlight numerous possible avenues for achieving precise control of progenitor cell differentiation, facilitating advancements in drug development and leukemia treatment.

Results

The co-segregation of neutrophils progenitor cells (NPCs) was decide by GATA2 and CSF3R

Based on the efficient capture and unsupervised dimensionality-reduction analysis, all stages of HPC were redefinition by the scRNA transcriptional atlas in our previous study (Figure 1A)(Chen *et al.*, 2024). To delineate the two stages of neutrophilic progenitors, a re-analysis was conducted to identify marker genes for distinguishing them. We identify new efficient marker genes of neutrophilic progenitors, such as *MPO*, *MGST1*, *IGLL1*, *C1QTNF4*, and *NPW* (Figure 1B). The specific genes of *ELANE* and *AZU1* were rarely expressed in these stages, indicating that some markers may only be active during the mature differentiation stages, but not in progenitor

stages. The initial differentiation stages of hematopoiesis simultaneously primed into megakaryocytic–erythroid, lymphoid, and neutrophilic linegaes. The characterization of *CSF3R* and *GATA2* activation clearly demonstrated the co-segregation of neutrophils progenitor cells (NPCs) with other lineages (Figure 1C). *CSF3R* expression was continuously high during the differentiation of neutrophils, but gradually restrained in the megakaryocytic–erythroid and lymphoid lineages differentiation process. *GATA2* activation was gradually up-regulated in the megakaryocytic–erythroid lineages but was suppressed in the other two lineages. The modulation of these two genes displayed a synergistic function in fate decision during the initial stage of hematopoiesis.

The shortest differentiation trajectories of NMPs

The expression of the MCM complex genes, component of the pre-replication complexes (pre-RC), was highly expressed in the late stages of the megakaryocytic–erythroid (Pro-ME), mast cell-basophil progenitors (MBPs), and neutrophilic lineages (NMPs) (Figure 2B). This indicates that the three lineage progenitors may switch from differentiation (lineages commitment) to proliferation (reproduction). The lineage commitment of these three progenitor types was continuous processes without distinct boundaries. Cell cycle analysis was conducted to confirm the transformation during hematopoiesis. Our results showed that the cell cycle stages of most progenitors (NMPs, MBP, and Pro-ME) were already in the S phase, while lymphoid was not (Figure 2A). This means that the differentiation of neutrophil progenitor was almost complete and shifted to proliferation after the initial priming from MPCs. However, the differentiation of megakaryocytic–erythroid progenitor went through two transitional stages (GAP and MEP) before moving to the proliferation stage (Pro-ME). Compared with the process of MEP lineages, differentiation process of neutrophils is the shortest in hematopoiesis (Figure 1A). Furtheremore, the proportion and numbers of neutrophil progenitors are second only to the megakaryocytic–erythroid lineages. This phenomenon straightforwardly

provides an explanation why neutrophils are the second most abundant type of cell in whole blood, only behind to erythrocytes. The expression characterization of CSF3R, highly expressed throughout the differentiation trajectories of NMPs (Figure 1C), indicates that CSF3R-activated signaling may be highly consistent with the differentiation signaling of neutrophils. This suggests that the mobilization transformation of initial progentiros (MPCs) can differentiate quickly into neutrophil with few signaling changes.

The switch from G1 to S phase of progenitors cell cycle

Quiescent state hematopoietic progenitors were maintained at the G0 stages of cell cycle. G-SCF mobilization caused the switch from a quiescent state to an activated state, which showed lineages commitment activity of differentiation. Since the switch of G1 to S phase in progenitors was identified, we aim to identify the genes that control cell cycle and differentiation. The genes of *CDT1*, *TYMS*, *UNG*, *DHFR*, *PCNA*, *GINS2*, and *DTL* (CDT2) were highly expressed in the S phase progenitors (Figure 2B)(Li et al., 2022). Interestingly, the genes of *CDC6* and *CDC45*, which encoding proteins essential for the initiation of DNA replication, were rarely expressed in the S phase progenitors (Figure 2C). Furthermore, the up-regulation of these cell cycle genes confirmed that most NMPs and Pro-ME were already in S stages, and the status of progenitors switched from differentiation to proliferation (Figure 2A, B). By switching the sequential expression of DNA replication initiation elements, the transitioned progenitor cells were primed into final differentiation stages of progenitors and completed lineage commitment (Figure 2A). CDK4 and CDK6 were highly expressed in all lineages of progenitor, and CDK4 being up-regulated in S phase of progenitors (Figure 2B). However, CDK1 was absent in all progenitors, and CDK2 was uniquely expressed in Pro-ME. The status of Pro-ME had already initiated CDT1 destruction process of S phase, which highly expressed the factors of PCNA, GMNN, and DTL (CDT2, CRL4). The CRL4 (CDT2) complex mediates the polyubiquitination and subsequent degradation of CDT1 and other cell cycle regulated

genes. The transformation of uracil to thymine was very important during DNA replication. Our results showed that three enzymes involved in thymidylate synthase were up-regulated, including *DUT*, *TYMS*, and *DHFR* (Figure 2B). DUT catalyzes the cleavage of dUTP to dUMP, and TYMS catalyzes the reductive methylation of dUMP to dTMP. Uracil DNA Glycosylase (UNG) plays an important role in preventing mutagenesis by eliminating uracil from DNA molecules. In summary, the expression of DNA replication-controlled genes of the cell cycle indicated that cell proliferation was obviously occurred at NMPs, MBP, and Pro-ME, but rarely present in other progenitor clusters.

Absence of progenitor self-renewal following lineages commitment in adult peripheral blood

HSCs were maintained at the G0 phage and kept in a quiescent state in the bone marrow. The mechanism that drives progenitor cells into cell cycle remains unknown. Our results showed that the majority of the progenitors in the three clusters--NMPs, Pro-ME, and MBPs— had already entered the S phase of cell cycle, which was markedly different from other initial and transitional state clusters. Most of the progenitors in the initial and transitional state clusters were maintained in the G1 phase of activated state, indicating that these progenitors tend to undergo lineages commitment without cell division. It is impossible for progenitors lacking DNA replication activity to have self-renewal ability. Conversely, the progenitors in the S and G2/M phases in the initial and transitional state clusters may engage in asymmetric or symmetric self-renewal division, maintaining their self-renewal potential. Approximately 30% of the rarely cells in these clusters that entered into S and G2/M phases exhibited self-renewal division activity. The expression of cell cycle genes was highly activated in most NMPs, Pro-ME, and MBPs (Figure 2B). This reminds us that during the lineage commitment of progenitor cells, each lineage progenitors should complete the differentiation (lineages commitment) process before transitioning to proliferation (cell division). Further Gene Ontology (GO) and Kyoto

Encyclopedia of Genes and Genomes (KEGG) signaling results showed that DNA replication initiation and cell cycle DNA replication were obviously enriched in NMPs, MBPs, and Pro-ME(Figure 3A, B). Additionally,, we identified only low signaling of DNA replication activation in the original progenitor group of MPCs. Progenitors even did not exhibit signaling of DNA replication activity indicates that the progenitors do not possess self-renewal ability. The results revealed that along the trajectory of hematopoiesis, progenitors were gradually differentiation into committed stages and transitioning into DNA replication of cell cycle before proliferation (Figure 3C). In conclusion, HPCs were maintained at the G1 phase by controlling DNA replication initiation and the DNA replication of cell cycle. Undifferentiated progenitors maintained in the G1 phage tend to activate lineages commitment procedures, whereas those that undergo DNA replication of cell cycle exhibit self-renewal activity. In contrast, differentiated progenitors do not have self-renewal ability and transition into proliferation.

Discussion

Overall, we revealed the underlying mechanisms of progenitor cell cycle state maintenance and provide new interpretations of immune phenomena through cell cycle analysis based on the clearly redefinition and reformed hierarchy of hematopoiesis (Chen *et al.*, 2024). Our study showed that NMPs, MBPs, and Pro-ME were derived into DNA replication of cell cycle, indicating that they had completed differentiation of lineages commitment and switched to proliferation. Critical DNA replication initiation factors were identified. We further verified and confirmed the shortest differentiation trajectories of neutrophils in hematopoiesis. Our findings showed that the progenitors completed lineages commitment in adult peripheral blood did not exhibit self-renewal capabilities during hematopoiesis. HPCs were maintained at G1 phage (differentiation), controlled by the initiation of DNA replication and DNA replication of cell cycle. The state switching from differentiation to proliferation among lineage-committed cells during hematopoiesis was revealed.

Bone marrow hematopoietic progenitors remained at G0 phase of the cell cycle. After the initial mobilization by G-SCF, progenitors differentiated into multipotent progenitor cells, and self-renewal was lost (Wilkinson et al., 2020). The mechanisms of the transformation and the switch from a quiescent state to an activated state remain unclear. Our study showed that the CSF3R, expressed in the initial subsets of progenitor, was the key signaling receptor for activation. Furthermore, our previous study revealed that transcription factor (TFs) of *SOX4*, *CDK4*, *XBP1*, *FOXP1*, and *CDK6* were highly expressed across all progenitor clusters during hematopoiesis (Chen *et al.*, 2024; Laurenti et al., 2015). The expression of these TFs, which generally showed a synergistic relationship with key receptor CSF3R signal activation, indicated that quiescent state progenitor cells were mobilized and assigned to the next stages of differentiation. We also clearly identified the lineages-specific fate decision transcription factors during lineages commitment based on the reliable redefinition (Chen *et al.*, 2024). Since these factors were uniquely expressed and activated in differentiation stages of progenitor but occurred later than the mobilization, this confirmed that lineages-specific fate decision transcription factors were unrelated to quiescent state switching.

Due to the limitation of unclear progenitor definitions, the transitioned stages during hematopoiesis were unknown. Here, we detailed the differentiation to proliferation transformation of progenitors during hematopoiesis. The mini-chromosome maintenance (MCM) complex, including *MCM2*, *MCM6*, *MCM5*, *MCM4*, *MCM3*, and *MCM7* genes, was the component of pre-replication complexes (pre-RC) (Cook et al., 2004). CDT1 cooperates with CDC6 and the origin recognition complex (ORC) during the G1 phase of the cell cycle to promote the loading of the MCM complex onto DNA to generate pre-RC (Cook *et al.*, 2004). The pre-RC plays an essential role in the eukaryotic cell mitosis. The genes of pre-RC were up-regulated in the proliferation state progenitors (NMP, MBP, Pro-ME). Additionally, GINS complex, a core component of CDC45-MCM-GINS (CMG) helicase, plays an essential role in the initiation of DNA replication and the progression of DNA

replication forks (Kamada et al., 2007). Our study showed the cell cycle S phase-related genes were activated in the NMP, MBP, and Pro-ME. However, other cluster only conducted low DNA replication activity. The state of the three types of progenitors has switched from differentiation to proliferation.

Self-renewal of HSCs supports blood system homeostasis. Generally, progenitor self-renewal must involve DNA replication during cell division, regardless of the type of division occurring. However, our study demonstrated that most HPCs were maintained at the G1 phage (differentiation), which was controlled by the DNA replication of cell cycle. Progenitors should complete the differentiation procedure (without cell division) before entering the proliferation (with cell division) stage. This process is akin to a tree that must grow to maturity before it can bloom and bear fruit. Since we have clearly redefined all stages of differentiation trajectories, that manifested that self-renewal did not occur after the lineages commitment is completed in adult peripheral blood but proliferation did. This provides solid interpretation of why progenitors cannot proliferate extensively *in vitro*. It is clear that a healthy blood system can be reconstituted by HSC transplantation, which is explained by the self-renewal potential of HSC. Our previous study constructed the expression atlas by utilizing both mobilized and quiescent state progenitors, yet we did not observe self-renewal after lineages commitment was completed during hematopoiesis. One possible explanation is that self-renewal occurs in uncommitted transplanted progenitors, but robust proliferation abilities are retained in committed progenitor cells. Another possibility is that differentiated cells (NMP, Pro-ME) may also possess self-renewal capabilities, conflicting with the notion that only early progenitors have self-renewal potential. It can be concluded that multipotent self-renewal progenitor do not exist after lineages commitment is completed in peripheral blood. Whether Pro-ME and NMPs possess self-renewal capabilities still requires further investigation. Furthermore, lineages commitment factors, which play a role in unique cell type fate decisions, may not be related to self-renewal, such as SCF, GM-CSF, and TPO. Since the cell cycle occurs in differentiated lineages, this indicates that lineages specific TFs

are not key factors in cell cycle regulation.

Neutrophils are the primary and rapid responders during infections and can quickly generate an enormous number of mature neutrophils for immune defense (Burn et al., 2021; Ley et al., 2018). Our study showed that the continuous differentiation process of neutrophils is the shortest in the hematopoietic hierarchy. The progenitors quickly completed differentiation and derived into proliferation at the second stages. CSF3R , the receptor of G-CSF, mobilizes progenitors from the bone marrow to peripheral blood. Progenitors switch from a quiescent state to an activated as G-CSF activates the receptor signaling, initiating the process of lineages commitment. Our study revealed that the expression characterization of CSF3R indicated that the initial mobilization transformation of progentiros (MPCs) can quickly differentiate to neutrophils with few signaling changes. The two characteristics of neutrophil differentiation, the shortest differentiation process and minimal signaling changes, facilitate their role as primary and rapid responders against infections and enable them to quickly generate an enormous number of mature neutrophils. A two step pipeline would be faster and more efficient that a lengthy procedure for generation.

Overall, we revealed the cell cycle phases of progenitor lineages during hematopoiesis. Our study further confirmed the shortest differentiation trajectories of neutrophils, facilitate their role as rapid responders during infections. Our study provides detailed insights into the cell cycle of HPCs, revealing the molecular mechanism underlying the transformation from differentiation to proliferation. Although HPCs self-renewal did not occur after the lineages commitment is completed in adult peripheral blood, proliferation did. Collectively, the cell cycle activity of hematopoiesis perfectly explains the physiological phenomenon of immunity. This study makes significant breakthroughs in the fundamental discipline of immunity development. Often, the truth is simple, yet we have been confused for a long time.

Methods

HPC enrichment and ScRNA sequencing

Original peripheral blood HPCs from quiescent donors who had not received any treatment (11 individuals) and mobilized (G-CSF) donors (three individuals) were enriched using a surface marker panel (Lineage−) via negative deletion (Chen *et al.*, 2024).10× Genomics RNA sequencing was performed on human peripheral blood enriched HPCs. In total, 27,566 CD34+ progenitor cells were extracted and re-analyzed to evaluate diversity, resulting in 17 clusters, including 21,244 mobilized and 6,322 quiescent progenitors.

ScRNA sequencing data processing

Single-cell unsupervised dimensionality reduction and clustering functions were performed using Seurat (Stuart et al., 2019). Progenitor definition and annotation were followed previous results (Chen *et al.*, 2024). Gene Ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of genes were conducted using the clusterProfiler package in R software(Wu et al., 2021). Score of cell cycle phases were analyzed by Seurat.

Figure Legends

Figure 1. The co-segregation of neutrophils and megakaryocytic–erythroid progenitor cells commitment.

A, UMAP illustrating 17 redefined clusters of adult peripheral blood CD34+ progenitor cells(Chen *et al.*, 2024). B, Violin plots showing the expression of neutrophil marker genes, revealling that *MPO*, *IGLL1*, and *MGST1* were more efficient for definition. C, Expression-feature plots of the CSF3R and GATA2 clearly demonstrated co-segregation among neutrophils and megakaryocytic–erythroid progenitor cells. UMAP, uniform manifold approximation and projection; MPC, multi-potent progenitor cells; GAPs, gene-controlled progenitors; CLPs, common lymphoid progenitors; MEPs, megakaryocytic–erythroid progenitors; MBPs, mast cell,

basophil, or eosinophil progenitors; Pro-MEs, megakaryocytic and erythroid progenitors; NMPs, neutrophilic and monocytic progenitors.

Figure 2. Cell cycle analysis and characterization of cell cycle markers genes. A, UMAP illustrating the cell cycle phases of progenitor cells, displaying the NMPs, MBPs, and Pro-ME were already in S phases in adult peripheral blood. B, Dot plots showing the expression of representative cell cycle markers genes. C, Violin plots showing the expression of cell cycle markers and unique marker genes, indicating that *PCNA*, *TYMS*, and *CDT1* were up-regulated in S phase progenitors.

Figure 3. GO and KEGG analysis of cell differentiation. A, Bar plot displaying enriched categories in the GO analysis, showed that DNA replication initiation and DNA replication of cell cycle were significantly enriched. B, Enrichment results of three signaling pathways in progenitor cells. C, Schematic showing the progenitor cell switch from a quiescent state to differentiation and proliferation of the cell cycle, illustrating that progenitor differentiation occurs prior to proliferation.

Authors' contributions

Y.Y. conceived the original idea, designed the study, and conducted the bioinformatics and statistical analyses of the sequencing data. Y.Y. wrote and revised the manuscript. All authors approved the final draft of the manuscript.

Declaration of interests

The authors declare no competing interests.

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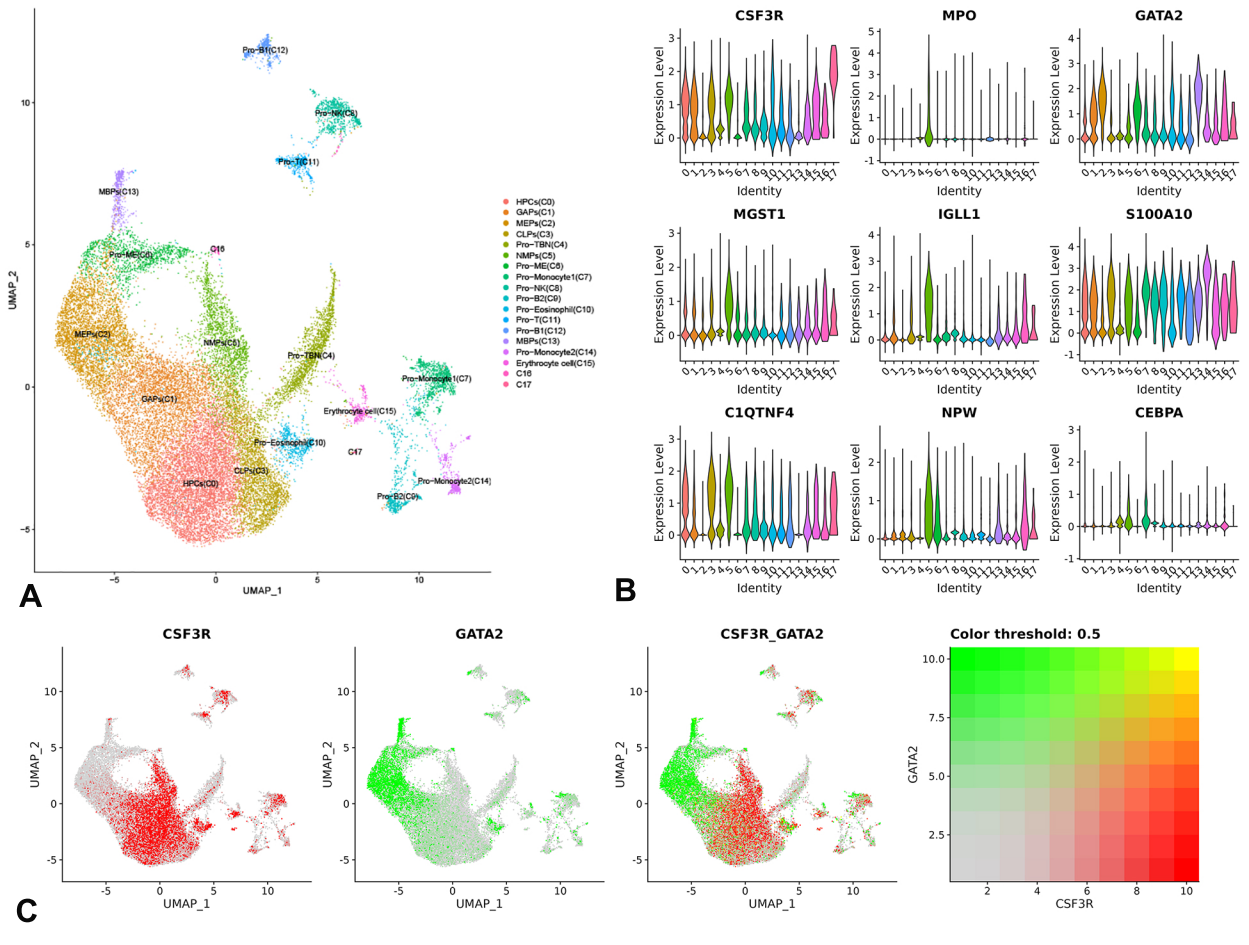
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MEPs(C2)
NMPs(C5)
Pro-TBN(C4)
Pro-Monocyte1(C7)
GAPs(C1)
Erythrocyte cell(C15)
Pro-Eosinophil(C10)
C17
CLPs(C3)
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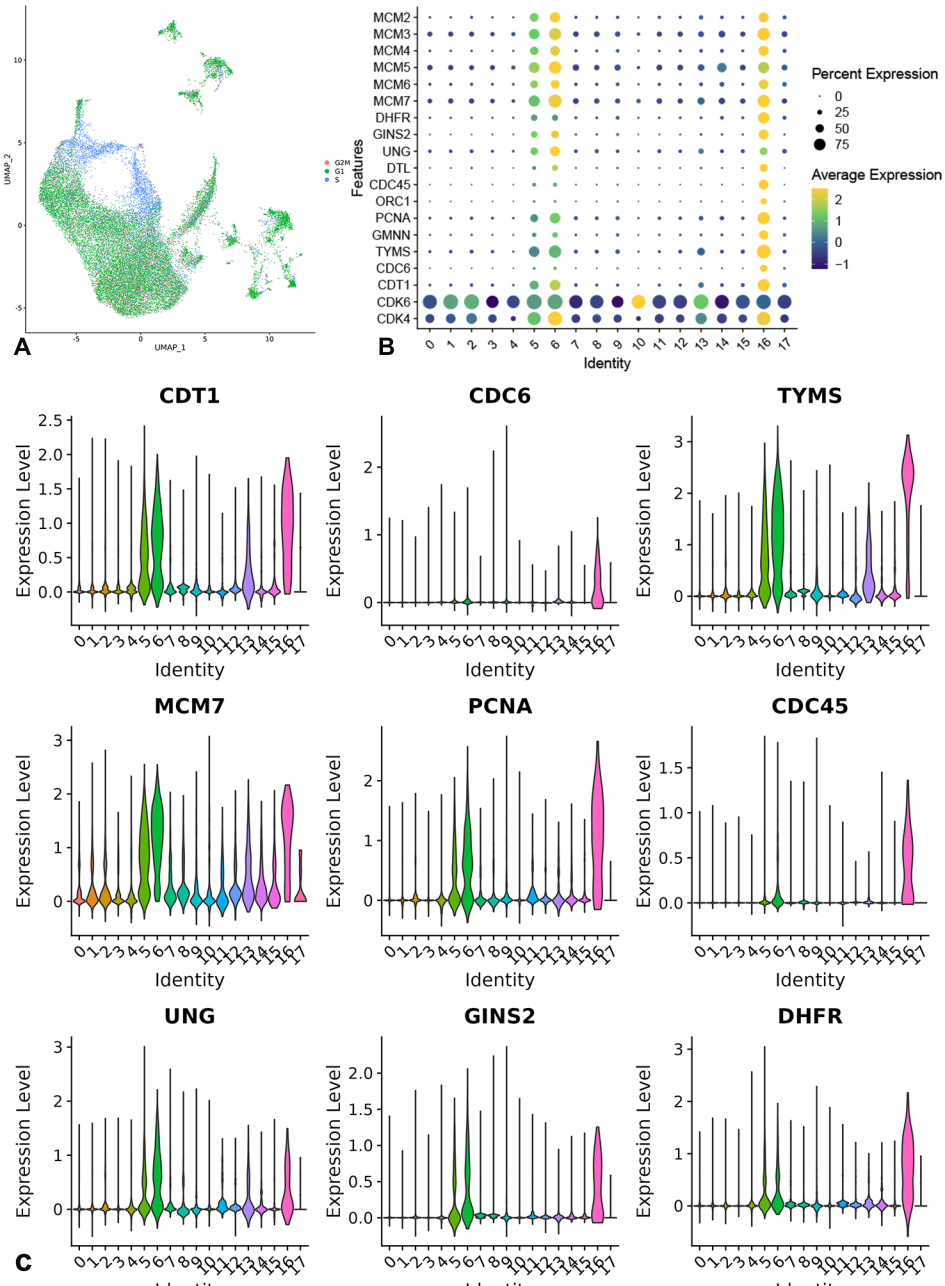
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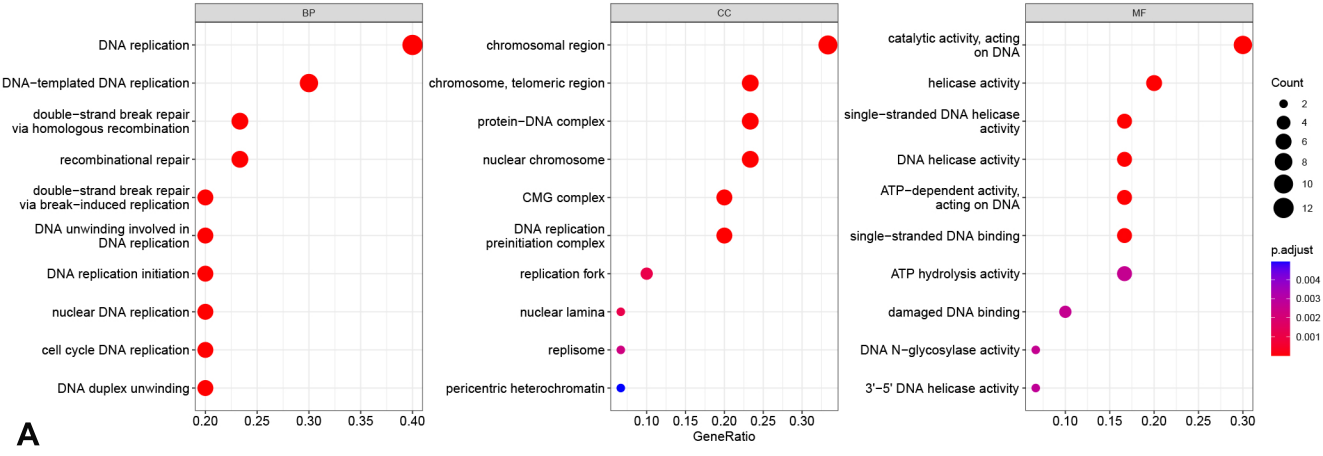
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DNA−templated DNA replication
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recombinational repair
double−strand break repair via break−induced replication
DNA unwinding involved in DNA replication
DNA replication initiation
nuclear DNA replication
cell cycle DNA replication
DNA duplex unwinding
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CC
chromosomal region
chromosome, telomeric region
protein−DNA complex
nuclear chromosome
CMG complex
DNA replication preinitiation complex
replication fork
nuclear lamina
replisome
pericentric heterochromatin
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GeneRatio
MF
catalytic activity, acting on DNA
helicase activity
single−stranded DNA helicase activity
DNA helicase activity
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single−stranded DNA binding
ATP hydrolysis activity
damaged DNA binding
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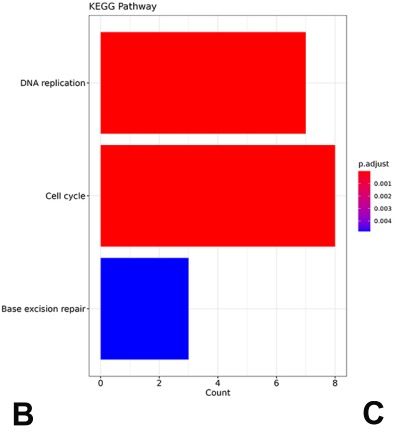
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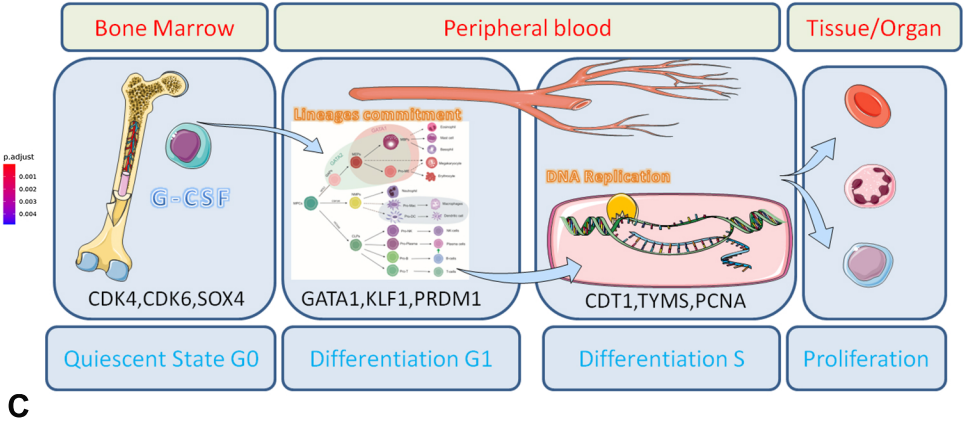
0.004
B


Bone Marrow
Peripheral blood
Tissue/Organ
G-CSF
Lineages commitment
DNA Replication
CDK4,CDK6,SOX4
GATA1,KLF1,PRDM1
CDT1,TYMS,PCNA
Quiescent State G0
Differentiation G1
Differentiation S
Proliferation
C