from irradiated, Rspo2-treated mice displayed 4-fold increased donor hematopoietic cell engraftment at 16 weeks compared to recipients of BM from irradiated, saline-treated mice (P=0.02, n=19/group). When we administered Rspo2 x 10 days to non-irradiated mice, we observed no change in blood counts, but decreased percentages of phenotypic HSCs compared to controls (P=0.0001) and mice transplanted competitively with 2 x 105 BM cells from Rspo2-treated mice demonstrated 3-fold decreased donor CD45.2+ cell engraftment at 16 weeks compared to mice transplanted with BM from saline-treated mice (P=0.005, n=5-7/group). Rspo2 promotes HSC regeneration following myelosuppression, but restrains HSC maintenance in homeostasis.

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Keywords: vascular, hematopoietic stem cell, regeneration

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DEVELOPMENTAL DYNAMICS OF ADULT B-CELL ACUTE LYMPHOBLASTIC LEUKEMIA (BCP-ALL) AT CLONAL LEVEL

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Adult B-cell precursor acute lymphoblastic leukemia (BCP-ALL) is a neoplastic disease characterized by phenotypical and functional heterogeneity. However, the cellular organization underlying this heterogeneity is poorly understood. Heterogeneity can arise from both clonal evolution and/or from cell differentiation (leukemia stem cell paradigm). Beyond BCP-ALL, the quantification of the temporal dynamics of leukemia development remains generally challenging. BCP-ALL patient-derived long-term cultures are our model of choice to tackle this problem. We have developed lentiviral expressible barcode vectors that can be detected together with the transcriptomes and cell surface markers. We perform single-cell CITE-seq at different stages of leukemic development in NSG mice to track the progeny of individually barcoded cells (see abstract by A. Gessner et al.). In this work, we combine barcode and multiome information to uncover an unknown differentiation architecture and also to quantify the temporal dynamics of leukemic cell differentiation at clonal level. We identified several large subpopulations of cells which developed after transplantation. Analysis of their transcriptomes revealed that these clusters reflected different stages of B-cell development. Our analysis of cell barcodes further showed that cells could differentiate between these clusters. The barcode information also revealed the likely cluster of origin, which showed the largest clonal diversity and comprised cells from each of the clones. This suggests the existence of a differentiation hierarchy in BCP-ALL. Using machine learning, we then identified expression patterns predicting with high accuracy the differentiation potential of each barcoded cell within the most immature cell compartment. To determine the dynamical properties of the clones, we have designed a mathematical model that simulates the development of these subpopulations from a clonally diverse stem cell compartment. The model, fitted using Bayesian inference, could notably validate the most likely cluster of origin and estimate the variability in proliferation between clones and between clusters. Our approach reveals the existence of a differentiation hierarchy and yields a comprehensive mechanistic view of the disease development at a clonal level.

Keywords: differentiation, leukemic stem cell, clonal development

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UNRAVELING THE EFFECTS OF PROLIFERATIVE STRESS AND GENOTOXICITY IN HEMATOPOIETIC STEM CELLS IN VIVO

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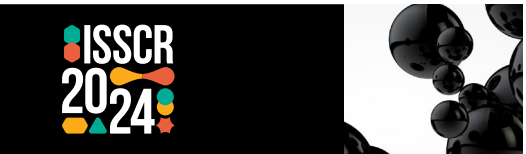
Hematopoietic Stem and Progenitor Cells (HSPCs) from patients affected by inherited disorders can be corrected with the use of Gene Therapy (GT), providing long term therapeutic benefit upon reconstitution of the entire hematopoietic system. However, how the replication stress, aging, vector driven oncogene activation and cancer predisposing mutations may impact the processes of hematopoietic reconstitution remains uncertain. Here we characterized the clonal dynamics of hematopoietic reconstitution and the acquisition of somatic mutations of lymphoid and myeloid cells in mice transplanted with wild (WT) type HSPCs transduced either with a lentiviral vector with active long terminal repeats which is highly genotoxic (group WT Genotox N=25) or with the safer self-inactivating long terminal repeats (group WT Non Genotox N=24). Additionally, the same HSPC-GT strategy was applied using mouse HSCPs lacking the tumor suppressor Cdkn2a gene (group Cdkn2a Genotox N=24, group Cdkn2a Non Genotox N=23 and Cdkn2a mock transduced N=20). Blood composition and vector integration sites (IS) of B, T, and myeloid cells were monitored overtime (up to 2.5 years). Somatic mutations were identified analyzing the genomic portion of the mouse genome flanking each IS, and a new Mutation Index (MI) was developed to assess mutation accumulation rates. As expected, the group Cdkn2a Genotox showed an accelerated tumor onset when compared to control groups ($p < 0.0001$), caused by activation of Braf oncogene. Moreover, mice from all groups showed a marked myeloid skewing at the expense of lymphoid lineages at the latest time points, specifically in the group WT Genotox. More than 250,000 IS were



identified, corresponding to 9 Gb of sequence genomic information. We found that the MI in both Genotox groups was significantly higher when compared to Non Genotox groups ($p < 0.001$). Notably, myeloid clones exhibited a higher mutation frequency compared to B and T cell lineages. Moreover, the MI of the WT Genotox group in the myeloid compartment was significantly higher than Cdkn2a Genotox ($p < 0.01$). Overall, our data unveils a previously unappreciated effect of genotoxicity by vector insertions which have a profound negative impact on hematopoiesis and accumulation of somatic mutations even in absence of oncogenesis.

Keywords: hematopoietic reconstitution, somatic mutations, gene therapy

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IDENTIFICATION OF IMPORTANT FACTORS INVOLVED IN TP53-ASSOCIATED PATHWAYS IN EARLY STAGES OF HUMAN HEMATOPOIETIC DIFFERENTIATION

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The TP53-related pathway has been reported to be involved in cell proliferation, maturation and function in B cells and other tissues as well as the inhibition of cell death. Published data indicated that this pathway is also upregulated during early hematopoietic differentiation, but its detailed role in cells during this period remains unclear. Therefore, we used human cord blood-derived lineage(Lin)-CD34+ very immature hematopoietic progenitor cells to investigate their role in differentiation, particularly how they contribute to cell fate decision during early hematopoiesis. First, we amplified the most immature Lin-CD34+ cells from human cord blood-derived cells in vitro to knock down (KD) a gene known to be involved in the TP53 pathway at the early hematopoietic cell stage. After confirming that Lin-CD34+ cells amplified in this system maintained their ability as multipotent progenitor cells, including colony-forming capacity, the effect of KD was evaluated. The results showed that KD reduced the total number of colonies, particularly Mix (24.7±21.5 colonies to 5.78±3.27 colonies / 200 cells) and BFU-E (18.9±10.3 colonies to 11.1±10.9 colonies / 200 cells). These results indicate that the TP53 pathway is required for early progenitor cells to maintain their ability to differentiate into non-myelomonocytic lineages. We next evaluated whether this pathway is involved in commitment to each lineage cell and cell proliferation; KD reduced erythrocyte counts (from 27428±20889 to 3803±5242 / 100 cells) but not monocyte counts (from 192±91 to 286±270 / 100 cells). On the other hand, KD reduced the number of NK cells, as well as the percentage and number of NK cells expressing endogenous CD16 (from 25.2±8.4 to 6.64±10.7 % gated on CD56+ cells) (from 69313±20535 to 18958±34271 / 100 cells) in vitro. These results indicate that TP53-related factors affect lineage-specific cell differentiation and maturation, in addition to suppressing cell death, during the early stages of hematopoietic differentiation.

Keywords: hematopoietic progenitor cell, TP53, differentiation

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EVALUATION OF FEEDER-FREE METHODS FOR T CELL MANUFACTURING FROM HUMAN INDUCED PLURIPOTENT STEM CELLS (HIPSC)

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The emergence of human-induced pluripotent stem cell (hiPSC)-derived T (iT) cells marks a pioneering advancement in adoptive cell therapies, providing solutions to crucial limitations inherent in conventional autologous manufacturing methods. hiPSCs serve as a readily available source of therapeutic T cells, offering advantages of limitless expansion and genetic manipulation. However, current protocols for iT cell development are burdened by lengthy and labor-intensive processes. Additionally, a decrease in efficiency results from the efforts to develop clinically compatible protocols in xeno- and feeder-free conditions compared to the OP9-DL1/4 standard research protocol. To address this challenge, we have developed scalable and clinically compatible methods for generating hematopoietic stem/progenitor cells (HSPCs) using both monolayer (2D) and embryoid body (EB)-based (3D) protocols. We evaluated the efficiency of these protocols to generate CD34+CD43+ HSPCs capable of further feeder-free T cell differentiation, progressing towards the double-positive CD4+CD8+ population and eventually yielding functionally single-positive cytotoxic CD8αβ+ and helper CD4+ iT cells. We found that the monolayer method demonstrates the capability to yield CD34+CD43+ HSPCs within 8 days with higher efficiency than the EB-based protocol (1 iPSC-to-4 HSPC versus 1 iPSC-to-0.2 HSPC) and higher purity (60-80% CD34+ versus 10-40%). HSPCs generated by both methods displayed similar potential in producing CD4+CD8+ DP, CD8αβ+, and CD4+ iT cells in a feeder-free system (1 HSPC produced up to 150 DP, 750 CD8αβ+, and 150 CD4+ iT cells). Future studies aim to compare the functional potential of iT cells derived by the two different methodologies. Our results support further efforts to scale up these approaches to enable the high cell yield necessary for clinical-grade production.

Keywords: T cells, hematopoietic stem/progenitor cells, iPSC