博士論文（要約）

Gene Editing in Murine Hematopoietic Stem Cells Using a Novel Ex Vivo Expansion System

(マウス造血幹細胞増幅法を用いたゲノム編集法の確立)

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博士論文の要旨

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Background

Hematopoietic stem cells (HSC) are a rare population of tissue stem cells that are responsible for the life-long maintenance of all blood cell lineages. HSCs combine two defining characteristics, self-renewal and multipotency, which together are conceptualized as "stemness". Due to their potency, HSCs are a particularly attractive target for gene therapy. Previous and ongoing studies have targeted this cell population to treat hereditary diseases such as immune deficiencies and hemoglobinopathies. With the advent of versatile and genetic precision tools, such as the CRISPR/Cas9 system, HSC-focused gene therapies are set to become an established treatment modality for a broad array of genetic conditions. However, the low frequencies of HSCs in the bone marrow currently are a limiting factor to broad application. To illustrate this, estimations posit that the fraction of functional HSCs in the murine bone marrow is 0.01% to <0.004%. Unfortunately, attempts to induce proliferation of HSCs outside of their physiologic niche, as currently required when performing gene editing, often result in the loss of stemness. Thus, there is a great need for culture systems that promote the *ex vivo* expansion of *bona fide*, gene edited HSCs.

In recent work, a defined, serum-free, recombinant protein- and polyvinyl alcohol (PVA)-based culture system has been established that selectively facilitates *ex vivo* proliferation of murine HSCs. In this thesis, it is shown that HSCs from immunodeficient mice can be gene edited, expanded, and transplanted, resulting in correction of the immunodeficient phenotype. Furthermore, application of a novel polymer enabled the expansion of single, gene edited HSC clones. This single cell expansion protocol allowed for genotype-based selection of individual clones before transplantation.

Aims and addressed questions

This thesis explored the feasibility of HSC gene editing in combination with *ex vivo* expansion strategies using defined culture conditions. A disease model as well as healthy cells were studied. The aim was to determine if such strategies complement the gene

editing process by enhancing efficiency and safety.

Results

To demonstrate the compatibility of *ex vivo* HSC expansion with gene editing, the causative mutation of the *Prkdc*^{SCID} immunodeficiency phenotype was targeted for correction using CRISPR/Cas9. CD150^{+}CD201^{+}c-Kit^{+}Lin^{-} (CD150^{+}CD201^{+}KL) HSCs from C.B17/IcrJcl-Prkdc^{scid} (CB17/SCID) mice were isolated, edited and expanded before autologous transplantation into conditioned immunodeficient recipients. Cas9 protein and optimized guide RNAs (gRNAs) were delivered as ribonucleoprotein complexes (RNP) via electroporation, yielding high targeting efficiencies (>80%). Correction of the SCID mutation was performed with a corrective template, supplied as a single-strand oligonucleotide (ssODN). Homology-directed repair (HDR) rates could be improved to 25% of total cells 7 days after editing by optimizing donor template design. Interestingly, the frequency of the corrected allele increased in culture over time, suggesting a survival advantage for corrected over uncorrected cells. Importantly, absolute numbers of primitive CD150^{+}CD201^{+}KL cells increased by 9-fold in the week post editing, suggesting that immature HSCs are supported by the culture conditions.

Peripheral blood (PB) samples from transplanted mice revealed robust reconstitution of B and T lymphocytes up to 24 weeks post transplant. Thymi and spleens were shown to be repopulated with physiological levels of B and T lymphocytes. Sequencing of the *Prkdc* locus in PB lymphocytes showed >50% HDR alleles, suggesting that the rescued lymphoid compartment had been repopulated by corrected HSCs rather than leaky progenitor cells. T cell receptor (TCR) diversity was assayed by spectratyping, a method to quantify the length distributions of the third complementary determining regions (CDR3s) coding sequences in CD4^{+} T cell-derived Tcrb transcripts. Results showed a broad CDR3 length diversity, suggesting active VDJ recombination in these cells, which is a process highly dependent on *Prkdc* activity. Next, the humoral immune response was tested by immunizing transplanted mice with a T-dependent antigen. Positive IgG and IgM titers confirmed that B and T cell interaction was restored, suggesting comprehensive immune reconstitution in transplanted mice. Importantly, lymphocytes could also be detected in the PB of secondary recipients transplanted with bone marrow from primary recipients, indicating that long-term HSCs (LT-HSCs), the most primitive known HSC population, had been corrected. Taken together, these experiments demonstrate that *ex vivo* expansion supports the generation of gene edited HSCs to levels relevant for disease phenotype correction.

Next, it was asked if the expansion system could be extended to allow for expansion of single HSC clones. To this end, the ability of Soluplus, a novel synthetic polymer, to support HSC growth in culture was evaluated. Soluplus outperformed PVA with regards to single cell culture survival and stemness support in native, unedited HSCs. To explore the feasibility of an expansion system for single cell HSCs, a different gene editing model was required, since *Prkdc*^{SCID} was considered too inefficient to reliably evaluate the single cell expansion approach. To this end, the fact that two major alleles of the panleukocyte marker CD45, CD45.1 (Ly5.1, Ptprc^{a}) and CD45.2 (Ly5.2, Ptprc^{b}), diverge only minimally on a genetic level was exploited. Indeed, only a single nucleotide substitution in CD45.2^{+} cells is necessary to enable the binding of the CD45.1-specific antibody clone A20 and prevent engagement of the CD45.2-specific clone 104. By introducing this mutation (K302E) into CD45.2^{+} HSCs, editing events could be easily quantified by flow cytometry and successfully targeted HSCs could be simply cloned by FACS. Editing efficiency was 6% and 20.4% for CD45.1^{+}CD45.2^{+} and CD45.1^{+}CD45.2^{-} phenotypes, respectively. CD45.2^{+} HSCs converted to the CD45.1^{+} phenotype by CRISPR/Cas9 editing were cloned and expanded for 2 weeks. For >40% of sorted clones, expansion resulted in growth of HSC colonies, many of which contained cells with the CD201^{+}CD150^{+}KSL HSC phenotype. Single HSC colonies were transplanted into CD45.2^{+} recipients, resulting in long-term (16 week) engraftment by ~30% of the transplanted clones. Interestingly, the degree of donor chimerism 16 weeks after transplantation correlated with the fraction of CD201^{+}CD150^{+}KSL cells in the transplanted graft. Importantly, preservation of self-renewal in the edited HSCs was evident after transplanting the graft into secondary recipients. Finally, the single cell expansion approach was applied to the SCID editing model. As expected, expansion rates of edited SCID HSC clones were not as high as in the healthy counterpart model, but 10% of clones did produce viable, single cell-derived colonies 2 weeks post sort. Immune phenotyping and genotyping of the *Prkdc* locus resulted in 1.4% candidate clones that showed a high CD201^{+}CD150^{+}KL cell frequency as well as the desired SCID-correcting knockin. These candidate clones were combined and transplanted into immunodeficient recipients. As in the bulk SCID editing experiments, lymphocytes emerged in PB samples rapidly, albeit with less robust B cell constitution.

Conslusion

This is the first demonstration that the immunodeficient phenotype of the SCID model can be corrected by transplanting expanded, Cas9-edited HSCs. *Ex vivo* expansion

complements gene editing and facilitates the generation of edited, long-term HSCs in numbers sufficient for transplantation and meaningful engraftment. Furthermore, single cell expansion allows for marker-free, sequence-based selection of individual HSC clones. This strategy permits quality control of edited cells, e.g. by screening for on-target edits and potential off-target lesions produced by gene editing. This level of direct selection has so far only been possible in cell lines such as induced pluripotent stem cells (iPSC). Taken together, this system holds great potential to significantly advance the field of hematopoietic gene therapy and other HSC-centric therapeutic strategies.