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学 位 論 文 名	CRISPR/Cas9 を用いた造血幹細胞遺伝子修復技術の開発
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## 論文内容の要旨

### 1 研究目的

Gene therapy in hematopoietic stem cells (HSCs) has been successfully conducted to treat genetic disorders such as the X-linked form of severe combined immunodeficiency (X-SCID). Retroviral vectors used in gene therapy randomly integrate transgene into the genome and may cause dysregulated gene expression. Thereby, targeted repair of mutations causing disease is highly desired. Here we designate the gene correction strategy for SCID mice which with the clustered regularly interspaced short palindromic repeat (CRISPR)-associated protein 9 (Cas9) system. To correct SCID regardless of mutations, the one possible strategy is to insert exon 2-8 of complementary DNA (cDNA) to interleukin-2 receptor gamma chain (*Il2rg*) intron 1, which we call universal gene correction. First, we investigated the delivery methods of Cas9 and single guide RNA (sgRNA) to hematopoietic stem and progenitor cells (HSPCs.). We found that electroporation of Cas9 ribonucleoprotein (RNP) can induced a DSB in mouse HSPCs with up to 20% of insertion/deletion (InDel). Next, we then tested homology-independent targeted integration (HITI) to insert exon 2-8 cDNA into the *Il2rg* intron1 site. Only with HITI, we detected the insertion of cDNA that is approximately 1kb. These results suggest that, with combined application of Cas9-RNP and HITI, adequate levels of insertion of cDNA in site-specific manner can be achieved.

### 2 研究方法

#### 1. SCID mice generation by CRISPR/Cas9.

Genotyping with Surveyor assay and sequencing. Phenotypical characterization of mice were detected by peripheral blood T and B lymphocyte evaluation with flow cytometry.

#### 2. Cas9 *in vitro* delivery

Transfection of Cas9-expressing lentiviral vector, plasmid and Cas9 mRNA or Cas9 ribonucleoprotein (RNP) complex into NIH3T3 cell, X-SCID immortalized stromal cells

and mouse Lin- HSPCs. Validation of Cas9 transduction efficiency by flow cytometry and DSB was detected by Surveyor assay and deep sequencing.

3. *Il2rg* exon 2-8 cDNA knock-in into CRISPR/Cas9 induced DSBs in genome

We designed codon optimized *Il2rg* exon 2-8 cDNA with reverse directed sgRNA sequences for *Il2rg* intron 1 at the both end of the cDNA as HITI construct. cDNA insertion was confirmed by genomic PCR with designed primers for 5' and 3' ends.

4. Cas9-RNP delivery and cDNA insertion into mouse Lin- HSPCs *Il2rg* intron 1.

Cas9-RNP and cDNA plasmid were inserted into mouse Lin- HSPCs via electroporation and Cas9-RNP mediated DSB was detected by Surveyor assay.

### 3 研究成果

#### 1. Generation of X-SCID mice by CRISPR/Cas9.

We generated X-SCID mice with point mutations in the *Il2rg* by CRISPR/Cas9, which are closer to the mutations of human X-SCID. *Streptococcus pyogenes* Cas9 (SpCas9) mRNA and sgRNA targeting the exon 2, 3 or 4 of *Il2rg* were injected into fertilized mouse eggs. In the F0 generation, we obtained total 35 pups and 25 of them displayed positive Surveyor assay. We confirmed the lack of T and B lymphocytes in the peripheral blood. In germ line transmission, we chose one male founders from each three strains and mated them with the wild-type female mice. In the F1 generation, we performed DNA sequencing to confirm gene mutations in all female offspring. We detected the 7-bp deletion in the all mutant females in the strain targeting exon 2 (SCID Ex2) and 1-bp nucleotide insertion in the strain targeting exon 2, 3 and 4. In summary, we generated novel strains of X-SCID mice with the mutations in *Il2rg* by CRISPR/Cas9.

#### 2. Targeted DSB in NIH3T3 cells and X-SCID immortalized stromal cells

We used NIH3T3 and X-SCID immortalized stromal cells to setup the appropriate protocols for Cas9 delivery in mammalian cells. First, we used *Staphylococcus aureus* (SaCas9) expression lentiviral vector. SaCas9-expressing lentiviral vector showed 60-90% transduction efficiencies. We designed 2 types of sgRNAs and determined the best sgRNA by surveyor assay in NIH3T3 cells. Second, we compared delivery methods of SaCas9/sgRNA-expressing plasmid, SaCas9 mRNA, and SpCas9 RNP complex. SaCas9 expressing plasmid and mRNA resulted in less transduction efficiency than lentivirus, while DSBs were successfully detected in both methods. Compared to the other methods, electroporation of SpCas9-RNP showed better efficiency to generate DSBs in NIH3T3 cells. We performed targeted deep sequencing, showing 18.96 to 43.12% InDel rate. Finally, we tested Cas9-RNP in immortalized stromal cells derived from the three X-SCID strains carrying the mutations in *Il2rg* exon 2, 3 or 4. With Cas9-RNP electroporation, targeted DSBs at the *Il2rg* intron 1 in the X-SCID

immortalized stromal cells was detected by Surveyor assay and indicating that Cas9-RNP efficiently generated DSBs at the *Il2rg* intron 1 in X-SCID genome.

### **3. *Il2rg* exon 2-8 cDNA knock-in into CRISPR/Cas9 induced DSBs in genome**

We next tried to insert *Il2rg* exon 2-8 cDNA into intron 1 so that complete *Il2rg* mRNA can be transcribed from exon 1 and the inserted exon 2-8. HITI construct was electroporated with the Cas9-RNP targeting the *Il2rg* intron 1. The successful insertion of the *Il2rg* exon 2-8 cDNA was detected by genomic PCR in both NIH3T3 cells and immortalized X-SCID stromal cells. Further, we sequenced the 5' ends of the insertion, and found that the *Il2rg* exon 2-8 cDNA was successfully inserted into intron 1. Although NHEJ-based insertion resulted the short InDel mutations around sgRNA target site, the InDel mutations did not alter inserted cDNA sequence. In summary, we successfully integrated *Il2rg* exon 2-8 cDNA into the intron 1.

### **4. Gene editing of mouse HSPCs**

To achieve an efficient gene correction in mouse HSPCs, we used mouse Lin<sup>-</sup> cells as a HSPCs. We tested different delivery methods such as lentiviral vector, plasmids, Cas9-mRNA. However these methods were too inefficient to cause detectable DSBs in mouse Lin<sup>-</sup> HSPCs. As electroporation of Cas9-RNP was more efficient than the other delivery methods for mouse Lin<sup>-</sup> HPSCs. Electroporation of Cas9-RNP showed efficient DSB by Surveyor assay and InDel frequency was approximately 20%.

Next, we performed knock-in of the *Il2rg* exon 2-8 cDNA into *Il2rg* intron 1 through electroporation of Cas9-RNP with the *Il2rg* exon 2-8 cDNA in the HITI plasmid. We confirmed the insertion of cDNA by genomic PCR and DNA sequencing. Sequencing result showed partial deletion in both 5' and 3' ends, near the insertion site of *Il2rg* exon 2-8 cDNA, however cDNA sequence was remained intact. Taken together, we successfully performed the universal gene correction by targeted knock-in of the *Il2rg* exon 2-8 cDNA into *Il2rg* intron 1 of X-SCID HSPCs.

## **4 考察**

In this study, we successfully demonstrated the universal gene correction of X-SCID mutations in mouse HSPCs. The universal gene correction, consisting of Cas9-RNP and HITI-based partial cDNA construct, effectively integrated the cDNA into the target intron by NHEJ-based repair following Cas9-mediated DSBs.

Our results indicate that electroporation of Cas9-RNP is most effective in HSPCs and it is meritorious because genome-integrating viruses such as lentivirus and retrovirus could cause leukemia, and rapid degradation of Cas9 causes less immune responses in hosts and, more importantly, less off-target DSBs. We estimated possible off-target sites using off-target detection software such as Cas-OFFinder and CCTop. All of possible off-targets sites were

located in introns or no gene region sites.

There are some issues to be addressed in the future study. First, it is important to assess gene-editing efficiency in *bona fide* HSCs, which is only assessed after transplantation, and perhaps improve gene-editing efficiency. Second, we need to evaluate if the expression can be fully restored, and the HSPCs are cured and produce T and NK cells *in vivo*. Finally, we have not compared HITI-based construct with HDR-based or non-HITI NHEJ methods.

In conclusion, we demonstrated that the combination of electroporation with Cas9-RNP and the HITI-based partial cDNA insertion through NHEJ.

## 5 結論

1. We generated three strains SCID mice with mutation in *Il2rg* gene exon 2, 3, and 4 by CRISPR/Cas9 system successfully which mimic to SCID patients.
2. Cas9 was successfully delivered to NIH3T3 cell by SaCas9-expressing lentiviral vector, plasmid, Cas9 mRNA and SpCas9-RNP via electroporation. However, in mouse HSPCs, we could detect DSB, only with SpCas9-RNP electroporation. SpCas9-RNP showed up to 42% of gene editing efficiency in NIH3T3 cell and 21% in mouse HSPCs.
3. *Il2rg* exon 2-8 cDNA were inserted efficiently via NHEJ-mediated knock-in with HITI construct in NIH3T3 cell, SCID immortalized stromal cell and mouse HSPCs respectively.

## 論文審査の結果の要旨

重症複合免疫不全症 severe combined immunodeficiency (SCID) に対する治療として骨髄移植が推奨されているが、適切なドナーが見つからないなどの問題がある。そこで、自己の造血幹細胞を体外で培養し、ウイルスベクターを使用して治療用の遺伝子を add on する *ex vivo* 遺伝子治療が開発されてきた。2017 年には、SCID の一型である adenosine deaminase (ADA) 欠損症に対する遺伝子治療製剤として、レンチウイルスベクターにより ADA 遺伝子を形質導入した自己由来の CD34 陽性細胞が欧州委員会に販売承認され、最近、発展の著しい遺伝子治療のなかでも期待される分野となっている。

一方、SCID の中で最も頻度が高い、IL-2 受容体  $\gamma$  遺伝子 (*Il2rg*) の変異に起因する X 連鎖型 SCID (X-SCID) の遺伝子治療では、染色体にランダムに組み込まれたレトロウイルスベクターによる LMO2 遺伝子の活性化に伴い白血病が発生した有害事象が問題となった。

申請者は、X-SCID に対して最新のゲノム編集技術を応用した遺伝子治療の開発研究を行った。*Il2rg* の exon 2-8 の cDNA を hematopoietic stem and progenitor cells (HSPCs) の intron 1 に挿入し多様な変異に対応する (universal gene correction) ことを目標としている。

実験の概要は下記の通り、

1. *Il2rg* の exon 2, 3, 4 をそれぞれ標的とした sgRNA と、SpCAS9 をマウス受精卵に注入し、X-SCID のモデルマウスを作製した。
2. マウスの HSPCs で、double-strand break を誘導するため、Lentivirus、Plasmid、mRNA、ribonucleoprotein (RNP)を試みた。電気穿孔法により CAS9-RNP を導入する手法により、20%に

insertion/deletion (InDel)を誘導可能であった。

3. exon 2-8 の cDNA を *Il2rg* intron1 に挿入する手法として、homology-independent targeted integration (HITI) と homology directed repair (HDR) を比較したところ、HITI のみが 1kb の cDNA を挿入可能であった。

実験は、標準的な手法を踏襲し HITI などの新規技術も取り入れて適切に実施されている。効率の良い電気穿孔法で CAS9-RNP を蛋白質として供給することにより、HSPCs で 20% という高いゲノム編集を実現しており、今後の研究の発展が期待される。

なお、本論文の骨子は *Scientific Reports* 誌に投稿され、現在、審査中である。

以上より、博士（医学）の学位に十分な資質を有していると考えられ、審査委員全員一致で合格とした。

## 最終試験の結果の要旨

申請者は、1. モデルマウスの作製、2. マウス HSPCs における電気穿孔法による CAS9-RNP の導入、3. HITI 法による *Il2rg* の修復実験について順次、発表を行った。

内容の要点は「論文審査の結果」にまとめたとおりである。

審査員からは以下のような質問が出された。

- 1) exon 2-8 の挿入に際し、逆向きの遺伝子発現やフレームシフトなどが生じる可能性はないか？
- 2) off target はどの程度、解析しているか？
- 3) 従来の電気穿孔法に比べて高効率であるが、どのような工夫をしたのか？
- 4) 今後、臨床応用を行う際の課題は何か？

申請者はいずれの質問に対しても的確に返答し有意義な議論が行われた。発表および質疑応答から、申請者が研究者として十分な資質・能力を有すると考えられ、医学博士号を受けるに値すると審査員全員が判断し最終試験に合格とした。