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Thesis/dissertation Title

Investigation of CRISPR-Cas9 as a novel method to generate organ-deficient mouse model for blastocyst complementation

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The field of regenerative medicine aims to overcome organ shortage through methods like blastocyst complementation. This technique requires organ-deficient embryos to generate transplantable organs. By injecting pluripotent stem cells (PSCs) into organ-deficient embryos, organs can be generated solely of stem cell-origin, depending on the type of organ agenesis of the embryo.

The gene knockout model for blastocyst complementation provided methods to generate organ-deficient embryos. However, many genes are involved in the developmental processes of multiple organs and tissues, thus reducing the specificity to target a single organ of interest. Conditional cell ablation technique combining *Cre/loxP* technology and diphtheria toxin A (DTA) has also been frequently used to generate organ-deficient embryos. However, there are challenges such as toxicity from DTA affecting some other tissues and inducing unspecific cell death. Thus, this study investigates a novel CRISPR-Cas9-based strategy to generate organ-deficient mouse models by inducing cell death with single-guide RNA targeting at multiple sites (sgRNA^{ms}) in the genome.

First, doxycycline-induced *Cas9-EGFP* HEK293T cell lines were established for *in vitro* experiments to investigate the downstream effects of the Cas9-sgRNA^{ms} system. Upon Cas9 expression, an increase in γH2AX intensity was observed in the cell lines expressing sgRNA^{ms}. Furthermore, sgRNA^{ms}-expressing cell lines also showed an increase in the early and late apoptosis cell population, suggesting cell death was due to extensive DNA damage. These cell lines also showed a decrease in cell proliferation rate using the MTT assay and BrdU immunostaining. Additionally, the Cas9-sgRNA^{ms}

system contributed to cell ablation in mouse ESCs. The knockout of *p53* in mESCs showed a decrease in apoptosis population upon expression of *Cas9-sgRNA*^{ms}. The evidence supports the hypothesis that the designed *Cas9-sgRNA*^{ms} system managed to induce cell death via the *p53* pathway *in vitro*.

Through the expression of Cas9 under an organ-specific promoter, coupled with constitutive expression of *sgRNA*^{ms}, cell death was hypothesized to occur in cells of the targeted organ. This study aimed to produce a thymus-deficient mouse model as a proof of concept. A mouse model was established with *Cas9* knocked in under the *Foxn1* promoter, and *hU6-sgRNA*^{ms} knocked in at the *Rosa26* locus, the feasibility of thymic epithelial cells ablation to produce a thymus-deficient mouse model was investigated in this study. *Foxn1*^{Cas9}*;Rosa26_ms1* and *Foxn1*^{Cas9}*;Rosa26_ms2* mice were evaluated for the ablation of the thymus. These progenies showed an athymic phenotype, similar to the nude mouse, and $CD3^+/CD45^+$ T cells were not detected in the peripheral circulatory system, confirming the lack of a functional thymus in these mice. Blastocyst complementation of *Foxn1*^{Cas9}*;Rosa26_ms* embryos with rat ESCs showed the successful generation of rat cells-derived thymus in mouse embryos.

This study provides a novel technique to generate organ-deficient embryos by CRISPR-Cas9-based method, using multiple-site targeting sgRNA.