論文の内容の要旨

論文題目　Establishment of induced Pluripotent Stem Cells Mediated Organ Replacement Therapy

和訳　　iPS 細胞を用いた臓器構築とその移植モデルの確立

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Primary immunodeficiency diseases are caused by inherited genetic defects leading to intrinsic defects in the immune system. Some of the immunodeficiency diseases (e.g. X-SCID, ADA, Jak3, and RAG) are prime candidates for gene therapy approaches in which desired genes are introduced into bone marrow cells to rescue the mutated genes and the treated cells are returned to the patients. DiGeorge syndrome (DGS) is also one of the primary immunodeficiency diseases results from gene deletions in the DiGeorge chromosomal region at 22q11.2 and mutations in genes at chromosome 10p13. The features of this syndrome vary wildly including craniofacial and cardiovascular anomalies, hypoplasia or aplasia of the thymus with associated deficiency of matured T cells, hypocalcemia with hypoplasia or aplasia of the parathyroids, and a variety of central nervous system abnormalities. Thus, since the immunodeficiency of DGS is marked by organ defects, gene therapy approach described above is not suitable for the treatment of disease. Therefore, transplantation of thymus is considered to be most

promising cure of immunodeficiency in DGS patients. The early development of the thymus and parathyroids is dependent on inductive interactions between pharyngeal endodermal cells and cranial neural crest cells originating in the hindbrain. These early interactions are likely to be critical for the proper development of a subset of cells in the thymus and the thymic epithelial cells (TEC), which are important for T-cell education in the mature thymus. Although previous studies have demonstrated that in vitro differentiation of embryonic stem cells into thymic epithelial cell progenitors or pharyngeal endoderm, it has been impossible to generate completely differentiated thymus in vitro because of the complicated cell-cell interactions during development and organogenesis. In mice, the thymus and parathyroids develop from the third pharyngeal pouch endoderm at E11-11.5 and a transcription factor *foxn1* begins to express in the common primordium. *foxn1* belongs to the forkhead box (fox) gene family and plays a critical role in the productive differentiation of thymic epithelium since the loss of function mutations result in severe immunodeficiency. However, it is still unclear which signals determine the location and the size of the thymic primordium and how these signals initiate *foxn1* gene expression. On the other hand, some studies showed generated thymus in nude mouse by way of blastomere aggregation and blastocyst injection. In this study, to establish a novel organ replacement therapy model for DGS, we generated the thymus by way of "*nude mouse blastocyst complementation*" with the iPS cells derived from nude mice. Moreover, we developed xenogenic blastocyst complementation method in which rat-induced pluripotent stem cells (R-iPSCs) cells were injected into the blastocyst of nude mice. Transplantation of the thymus derived from R-iPSCs to nude rat could generate repopulation of rat T lymphocyte subsets in the peripheral blood This experimental system not only highlight a tool to investigate the mechanism of thymus formation and a new therapy model for DGS, but may also provide potential use of iPS cell for novel organ replacement therapy.

Establishment of Foxn1 over expressed iPSCs from the fibroblast of the KSN nude mouse.

The concept of organ replacement therapy is a generation of the organs from autolgous tissue and replacement damaged or defective organs with the regenerated autologous organs. To establish a small animal model for the organ replacement therapy, we used

athymic nude mice and tried to regenerate thymus by way of blastocyst complementation technique.

In order to generate thymus in nude mice, we initially established the iPS cell lines from tail-tip fibroblast of adult KSN nude mouse by way of Moloney murine leukemia virus (MMLV) based retroviral vector mediated transduction of four reprogramming factors (Oct4, Klf4, Sox2, c-Myc) as described previously. To address whether the generated nude mouse iPSC (N-iPSC) colonies expressed pluripotency markers, the colonies were stained with antibodies to Nanog. The N-iPSC colonies expressed Nanog and were morphologically indistinguishable from mouse embryonic stem cells (mESCs). Furthermore, RT-PCR analysis revealed that N-iPSCs expressed mESC marker genes, including Nanog, ECAT1, ZFP296, Eras, Rex1, and Gdf3, in quantities comparable to those in mESCs. These results indicate that the N-iPSCs were reprogrammed into a pluripotent mESCs-like state.

In this study, to rescue nude phenotype, we transduced N-iPS with lentiviral vector which carries Ubc promoter driven wild type transduced N-iPS with lentiviral vector which carries Ubc promoter driven wild type *foxn1*gene and IRES connected Kusabira orange as a marker. Transduced iPS (F-OE iPS) efficiently expressed Kusabira orange which means that *foxn1* was also expressed in N-iPS.

Generation of thymus in nude mice.

Although a recent study showed that in vitro differentiation of mESCs into thymic epithelia cell progenitors, it has not been possible to generate completely differentiated functional thymus from mESCs *in vitro*. In contrast to "*in vitro differentiation method*", a previously described "*blastocyst complementation method*" allows ESCs or iPSCs could develop into fully differentiated thymus through the cellular interactions that take place between cells, tissues and organs. We therefore explored the generation of thymic organ, by the strategy based on blastocyst complementation method for organ regeneration. The GFP marked wild-type ESCs and N-iPSCs as well as Kusabira orange marked F-OE iPSCs were injected into *foxn1nu/nu* blastocyst, and then chimeric mice were generated in the frequency of 37.7% for ES, 38.4% for N-iPSC and 23.3% for F-OE iPSC. Among ESC injected chimeric mice, we detected thymus in the frequency of 30% as previously reported. On the other hand, although high efficiency of chimera formation was observed, no thymus

was detected in the N-iPSC injected mice. In the F-OE iPSC injected chimeric mice, we also detected thymus in the frequency of 36.1%. Although the regenerated thymus in the F-OE iPS injected mice was relatively smaller than the case in wild-type mice, it was morphologically and histologically normal. Furthermore, we could detect Kusabira orange positive cells in the cortex and medulla in regenerated thymus. On the other hand, we also detected kusabira orange negative cells in the regenerated thymus. Fluorescence-activated cell sorter (FACS) analysis of PBMC from chimeric mice revealed that CD4 single positive cells (3.82±3.0%) and CD8 single positive cells (2.59±2.75%) are efficiently produced from the regenerated thymus. These ratios are comparable to PBMC from wild type mice (CD4: 5.32±2.5%, CD8: 5.61±2.54%) and ESC injected mice (CD4: 5.81±2.59%, CD8: 2.90±1.71%) (Fig. 2-3c, 2-5). However, some of chimeric mice fail to generated thymus even though high formation of chimerasim.

It has been recently reported that there is a second thymus (cervical thymus) in mice, which contains a cortex-medulla structure and could correct T cell development in nude mice upon transplantation Interestingly, we also found the Kusabira orange positive cervical thymus in F-OE iPSC chimeric mice, indicating that *foxn1* overexpression under the control of the ubiquitous expression promoter could efficiently regenerate thymic organs and produce thymic T cells in nude mice.

Generation of chimeric mice carrying xenogenic thymus.

To apply blastocyst complementation technique to the treatment of human diseases, it is inevitable to generate xenogenic chimeric animals (e.g., pig-human chimera, monkey-human chimera) carrying human organs. The first xenochimeric livestock animal was generated between goat and sheep then before, many groups have been trying to generating xenochimeras between rat and mouse, but failed to generation of live chimeric animal. Finally, our group successfully generated rat-mouse xeno chimeras. We therefore explored to generate xenogenic animal model by injecting GFP labeled wild type Rat-iPSCs (R-iPSC) into blastocysts of nude mice. In order to achieve this xenogenic blastocyst complementation successfully, we initially generated R-iPSCs by infecting rat embryonic fibroblast with lentiviral vector containing mouse Oct4, Klf4, Sox2, and GFP under the condition. Pluripotency of R-iPSC was analyzed by alkaline phosphatase staining. Of the 558 blastocyst injections, the mouse-rat-chimeras

expressing GFP were born in the frequency of 17.3%, and 33.3% of GFP positive thymic organs were detected in these chimeras. Moreover, these chimeric mice had amount of hair on the body. FACS analysis of PBMC from the mouse-rat chimera having GFP positive thymus revealed that mouse CD4,CD8 single positive cells were efficiently produced (8.2%, 3.2%, respectively) from the regenerated thymus. On the other hand, we could not detect rat CD4, CD8 single positive cells in PBMC. Because GFP positive cells were not detected in bone marrow, R-iPSCs do not appear to contribute to regenerating of rat hematopoietic stem cells. These results indicate that R-iPSCs can generate xenogenic thymic organs in nude mouse by blastocyst injection and the reconstituted thymus can produce mouse- derived mature T lymphocytes in PBMC.

The regenerated thymus develops lymphocyte subset.

To examine the functionality of regenerated thymus derived from R-iPSCs, we transplanted small pieces of the regenerated thymus into kidney capsule of nude rat and analyzed the existence of mature T lymphocyte in Peripheral Blood. FACS analysis of PBMC from the nude rat at 8 weeks after transplantation revealed that the T lymphocytes did not express GFP indicating that the produced T lymphocytes derived from the recipient bone marrow. These results clearly indicate that regenerated thymic organ derived from R-iPSCs in nude mice is functional thymus capable of educating rat immature thymocytes to differentiate into mature T lymphocytes.

Discussion

Here we have described a small animal model of organ replacement therapy for DGS by way of blastocyst complementation. Recently, it has been reported that the thymic epithelial progenitor cells are generated from mouse ES cells *in vitro*. Thymic epithelial cells have a pivotal function in determining the fate of thymocytes and direct contact with hematopoictic precursor cells are known to be necessary for the development of a normal thymic architecture. It is still impossible to generate functional thymus from pluripotent stem cells *in vitro*. Moreover, although ES cells may provide treatment for DGS, their unlimited self-renewal and high differentiation potential poses the risk of tumor induction after engraftment. Thus, more care must be taken before using ES cell transplantation as a therapeutic option for patients with degenerative diseases.

In contrast the blastocyst complementation technique enabled us to generate completely differentiated functional thymus and is relatively safe technique compared to *in vitro* generated progenitor cells, because pluripotent stem cells injected into blastocyst cells can differentiate through normal developmental stage. Previously blastocyst complementation technique has been used for understanding the development of thymus epithelium, compensation of cardiac defects and understanding the determinants of pancreas and liver organ size. However, there have been no previous reports on the use of this technique to regenerate donor-derived functional organs and for curing fatal genetic diseases.

Less than 1% of infants with DGS is born with no detectable. This form of DGS is called “complete DGS and is usually fatal due to infection by 2years of age. Although, thymus transplantation is a promising investigational therapy for treating the disease, a severe shortage of organ donors for transplant is the major problem. There are two phenotypes associated with complete DGS. Nearly two-thirds of the patients, have no T cells and no symptoms of rash or lymphadenopathy. This is called ‘‘typical’’ complete DGS. The remaining one-thirds of the patients develops a rash and lymphadenopathy associated with oliogoclonal ‘‘host’’ T cells. This is called ‘‘atypical’’ complete DGS. Because these oligoclonal T cells can destroy the thymus allograft before immune reconstitution occurs, atypical DGS patients usually require adequate immunosuppression by pulse steroid therapy. However, negative effects on thymopoiesis unpredicted transplant rejection is inevitable due to even under the use of immunosuppression. The use of autologous iPSC-derived thymic tissues as presented in this study can eliminate the necessity of immunosuppression and overcome the problems mentioned above. More importantly, we unlike the allogeneic setting could ignore the risk of graft versus host disease (GVHD) in the case of autologous transplantations. In consistent with previous report, however, the thymus generated by our system are chimeric and may contain host derived tissues (e.g. blood vessel and nerve tissue), therefore, further improvement for eliminating host tissue in thymus would be necessary to overcome these problems.

To generate human thymus for the treatment of DGS by way of blastocyst complementation, it is necessary to challenge the generation of xenochimeric animal containing human thymus. However there have been no reports of the successfull generation of xenochimera. In this paper, we introduced a new paradigm for organ

regeneration by the establishment of xenochimeric animal, and this is the first report that demonstrated a generation of rat thymus in nude mice. Although regenerated thymuses were chimera, we found that those were able to support thymocyte differentiation in both mouse and rat.

There are a large number of similarities between porcine and human, implying a potential use of porcine for generation of human organ by way of blastocyst complementation.

Our results presented here demonstrate a comprehensive proof of principle approach that combines organ replacement with cell therapy to repair a genetic disorder and may offer a new avenue for a novel strategy for the treatment of DGS.