論文の内容の要旨

論文題目　The regulatory mechanisms of hepatic stem/progenitor cell differentiation

(肝幹・前駆細胞の分化制御機構)

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<Background >

The liver is the largest internal organ in mammals and plays an important role in metabolism. It performs various functions including glycogen storage, decomposition of red blood cells, plasma protein synthesis, and detoxification. For intractable liver diseases, liver transplantation from living or brain-dead donors is the effective treatment. The immune rejection and donor shortages remain barriers to liver transplantation therapy. Artificial organ transplantation, for example artificial heart transplantation, is available for several tissue diseases. However, it is still difficult for an artificial organ to play many metabolic functions like a liver. Therefore, cell transplantation therapy might be useful for severe liver diseases.

Induced pluripotent stem (iPS) cells were generated from somatic cells by simultaneously introducing four genes (Oct3/4, Klf4, Sox2, and c-Myc) (Takahashi and Yamanaka., 2006). iPS cells have the ability of self-renew and pluripotency like ES cells. Because iPS cells are generated from somatic cells of individual persons, they would be good sources of cell transplantation therapy without immunosuppression and ethical issues of generation. For clinical application, establishment of the differentiation protocol of iPS cells into target cells or organs is necessary. For *in vitro* differentiation of iPS cells into target cells, several methods including gene transfer, cytokines additions, and cell aggregation were reported. However, the efficiency of differentiation from iPS cells into target cells is still low and the possibility of undifferentiated or other types of cell contamination remains. To achieve these, generation of more sophisticated differentiation methods and purification methods of target cells from heterogeneous population are necessary. Differentiation processes from iPS cells into differentiated specialized cells are similar to developmental processes. Understanding of developmental processes is useful to determine when and what kind of genes or stimuli are needed to induce iPS cells into specialized target cells.

Liver organogenesis begins at early embryonic stages from the foregut endoderm. Endodermal cells are known to receive inductive signals from the septum transversum mesenchyme and adjacent cardiac region, namely bone morphogenetic protein (BMP) and fibroblast growth factor (FGF). Subsequently, these cells commit to hepatoblasts that proliferate and migrate into the septum transversum (STM) to form the liver bud. Hepatoblasts are considered to be somatic stem/progenitor cells in fetal livers because

they have a high proliferative potential and the ability to differentiate into both hepatocytes and cholangiocytes during the middle to late fetal stages. Whilst in the fetal stage, the liver becomes the major fetal hematopoietic organ. Although the liver dramatically changes its function during development, the molecular mechanisms regulating its change are still unclear.

Fetal livers are consisted of not only hepatoblasts but also various kinds of cells including hematopoietic cells, mesothelial and submesothelial cells which cover liver lobes and are the origin of stellate cells and mesenchymal cells around vascular. Proliferation and differentiation of hepatoblasts are regulated by extrinsic factors from various cells in the liver and intrinsic factors by themselves. Some cyotokines were previously reported to regulate hepatoblasts maturatuon. Oncostatin M (OSM), one of the interleukin-6 family genes, is secreted from hematopoietic cells proliferated in the middle stage liver and induces the metabolic enzyme gene expression in hepatoblasts (Kamiya et al., 1999). Pleiotrophone (Ptn) and midkine (Mdk) secreted from mesenchymal cells are related to hepatoblasts proliferation (Onitsuka et al., 2010 and Asahina et al., 2002). Even if these maturation or proliferation stimulation factors are supplied *in vitro* culture, proliferation and differentiation level of hepatoblasts *in vitro* is less than hepatoblasts *in vivo*. There might be more unknown mechanisms of regulating hepatoblasts proliferation and differentiation. However, in humans, *in vivo* analysis of liver is difficult. It is necessary to establish the *in vitro* culture methods of human hepatic stem progenitor cells for the analysis of molecular mechanisms regulating these cells.

<Aim and Method>

To reveal the molecular mechanism regulating hepatic stem/progenitor cells proliferation and differentiation from the middle to the late fetal stages, two approaches were done in this study. (1) One is “an *in vitro* expansion system for generation of human iPS cell-derived hepatic progenitor-like cell exhibiting a bipotent differentiation potential”. (2) The other is “The function of $p57^{Kip2}$ in liver development”. In the former one, I examined human iPS cells differentiation into hepatic progenitor-like cells and defined the optimum *in vitro* culture condition of these hepatic progenitor-like cells for proliferation maintaining their characteristics. In the latest one, I focused on the difference of the proliferation ratio between fetal hepatic stem/progenitor cells and mature hepatocytes. I examined the cell cycle repression gene, $p57^{Kip2}$ functions in the liver during development using $p57^{kip2}$ null mice and $p57^{Kip2}$-/- and wild type (WT) chimera mice.

<Results>

(1) Human iPS cells were differentiated into hepatocytic lineage cells by serial cytokine stimulation

(activin A, basic FGF, BMP4, and hepatocyte growth factor). Differentiated cells were highly proliferative and expressed genes encoding α-fetoprotein (AFP) and hepatocyte nuclear factor 4α (HNF4α). To characterize human iPS cell-derived hepatic lineage cells, undifferentiated human iPS cells and cells cultured with or without cytokines were analyzed with antibodies against cell surface markers by flow cytometry. It was recently published that CD13 and CD133 are specific markers of hepatic stem/progenitor cells in fetal and adult mouse livers. Using fluorescence-activated cell sorting, purified $CD13^{high}CD133^{+}$ cells were co-cultured at a low density with mouse embryonic fibroblast cells (MEFs) in the presence of suitable growth factors and signaling inhibitors (ALK inhibitor A-83-01 and ROCK inhibitor Y-27632). Individual cells gave rise to relatively large colonies (more than 100 cells) expressing hepatocytic marker genes (AFP and HNF4α) and a cholangiocytic marker gene (cytokeratin 7). Next, colonies derived from $CD13^{high}CD133^{+}$ cells were trypsinized and re-plated onto fresh feeder cells. Cell proliferation continued for more than 1 month and colonies expressing HNF4α were found to express the proliferation marker Ki67. These hepatic progenitor-like colonies did not express the pluripotency marker Oct3/4, which is expressed in human iPS cells. We then considered the self-renewal potency of $CD13^{+}$ hepatic progenitor-like cells. Colonies derived from $CD13^{+}$ cells were composed mostly of $CD13^{+}$ cells. In contrast, hepatic progenitor-like cell colonies derived from $CD13^{-}$ cells barely contained any $CD13^{+}$ cells. Additionally, $CD13^{+}$ cells were more efficient than $CD13^{-}$ cells at colony formation. This suggests that $CD13^{+}$ cells could proliferate over time whilst maintain the ability to self-renewal. This method was validated through using human embryonic stem (ES) cells (KhES-3 line). When hepatic maturation was induced by the spheroid culture or cell-cell interaction, hepatic progenitor-like cells can further differentiate into cells comparable to mature hepatocytes capable of producing cytochrome P450 enzymes and albumin secretion. When hepatic progenitor-like cells were cultured in an extracellular matrix gel, they eventually formed cholangiocytic cyst-like structures with epithelial polarity. Mature cysts expressed cholangiocytic marker gene (cytokeratin 7) but did not express hepatocytic marker (AFP). Taken together, these results suggest that human iPS cell-derived hepatic progenitor-like cells have a bipotent differentiation ability.

(2) Expression analysis of cell cycle-related genes in purified hepatoblasts revealed that cyclin-dependent kinase inhibitor (CKI) $p57^{Kip2}$ was highly upregulated. $p57^{Kip2}$ was highly expressed in the mid to the late fetal liver, but its expression decreased in the adult liver. Immunofluorescence stainings and qPCR analysis showed that $p57^{Kip2}$ was expressed in both hepatoblasts and mesenchymal cells in the fetal livers. $p57^{Kip2}$-/- hepatoblasts had the similar levels of *in vitro* colony forming ability to $p57^{Kip2}$+/+ hepatoblasts for the short term culture. But in the case of long term culture, the number of colonies derived from $p57^{Kip2}$-/- hepatoblasts was higher than $p57^{Kip2}$+/+ hepatoblasts. During *in vivo*

liver development, the number of the proliferation marker Ki67 positive cells of $p57^{Kip2}$-/- hepatoblasts was higher than $p57^{Kip2}$+/+ hepatoblasts. In addition, expression levels of the functional genes in $p57^{Kip2}$ knockout ($p57^{Kip2}$-/-) livers were remarkably lower than $p57^{Kip2}$+/+ livers. Therefore, proliferation and differentiation of hepatoblasts might be regulated by intrinsic or extrinsic $p57^{Kip2}$ inactivation. Because $p57^{Kip2}$-/- mice are neonatal lethal, it is difficult to analyze $p57^{Kip2}$ functions in adult livers. Therefore, we generated $p57^{Kip2}$-/- and wild type (WT) chimera mice. $p57^{Kip2}$-/- chimera mice were able to grow into adult. The expression levels of liver functional genes were almost same between $p57^{Kip2}$-/- cells derived hepatocytes and host derived WT hepatocytes.

<Conclusion>

These data indicated that our *in vitro* differentiation and expansion system of human iPS cells-derived hepatic progenitor-like cells was useful for not only liver regeneration but also for the determination of molecular mechanisms regulating liver development in humans. These results also suggested that $p57^{Kip2}$ did not regulate hepatoblasts differentiation by intrinsic factors. Liver maturation defect of $p57^{Kip2}$-/- mice might be caused by extrinsic factors. $p57^{Kip2}$ was expressed not only in hepatoblasts but also in mesothelial and submesothelial cells in fetal liver. It was reported that mesothelial and submesothelial cells regulated the hepatoblasts proliferation and maturation. More detail analysis of $p57^{Kip2}$-/- mesothelial and submesothelial cells would be a potential finding to reveal unknown mechanisms of regulating hepatoblasts development.

<Reference>

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