論 文 の 内 容 の 要 旨

論文題目　Regulation of Mouse Liver Development by Transmembrane Proteins
（和訳　細胞膜タンパク質によるマウス胎生肝細胞の分化・成熟の制御）

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The liver is a central metabolic organ in adult, and consists of parenchymal hepatocytes and various nonparenchymal cells that include sinusoidal endothelial cells, Kupffer cells, hepatic stellate cells and blood cells. Hepatocytes carry out most of the metabolic functions. However, the liver functions as a major hematopoietic tissue in fetus and lacks most of the metabolic functions of adult liver. Thus, the characteristics of the liver and hepatocytes drastically change along with development from mid-gestation to perinatal stage. The liver is a unique organ and understanding the mechanism of liver development is an interesting and important subject not only for developmental biology, but also for development of therapeutics for liver diseases.

The liver primordium emerges from the foregut endoderm at embryonic day (E) 8 in mice. Immature hepatocytes are characterized by the expression of serum proteins, alpha-fetoprotein (AFP) and albumin (Alb). Several factors involved in liver development have been identified, such as cytokines and cell surface proteins. Fibroblast growth factor (FGF) and bone morphogenetic protein (BMP) are involved in the liver specification from the foregut endoderm. Hepatocyte growth factor (HGF) stimulates proliferation of fetal liver cells in mid-gestation and oncostatin M (OSM), an IL-6 family cytokine, induces the maturation of fetal hepatocytes in vitro. In the primary culture of mouse liver at mid-gestation, OSM promotes morphological and functional maturation of fetal hepatocytes as evidenced by the expression of various liver metabolic enzymes, accumulation of glycogen and lipids, clearance of ammonia and formation of junction structures, indicating that this culture system mimics the liver development from the mid- to late-stage. Although OSM is a potent factor promoting the

differentiation of hepatocyte, the liver develops normally in mice deficient for OSM or the OSM receptor, indicating that additional factors are involved in hepatic development. In fact, hepatocytes were shown to differentiate in high-density cultures of fetal liver cells without OSM, suggesting that some membrane-bound proteins play a role in the differentiation of hepatocytes. While several factors involved in liver development have been identified, the characteristics of immature hepatocytes during liver development have not been elucidated. Cell sorting by using antibodies against membrane proteins will be a powerful technology to characterize immature fetal hepatocytes. To use cell sorting for studies on liver cells, our laboratory has been identifying cell surface molecules expressed on liver cells and previously identified a type I transmembrane protein, Delta like (Dlk)/Pref-1, that was expressed in fetal hepatocytes. Further identification of extracellular molecules in fetal hepatocytes would help us to study the mechanisms of liver differentiation. To this end, we have taken two strategies; 1) generation of a panel of monoclonal antibodies (mAbs) against mouse fetal liver cells by immunizing rats, and identification of the antigen by expression cloning, 2) molecular cloning of cDNAs encoding a signal sequence by the Signal Sequence Trap (SST) method using a retroviral expression system. Here, I describe two transmembrane proteins, Lutheran blood group glycoprotein (Lu) and T-cell immunoglobulin and mucin domain 2 (Tim2), identified by these two screenings.

First, we generated over 600 hybridomas by immunizing rats with mouse fetal liver cells deprived of hematopoietic cells and selected those that recognize fetal liver cells by flow cytometric analysis. Among 273 candidates, we focused on Abs that recognized fetal hepatoblasts but not adult hepatocytes, because such antigens could be implicated in liver development. We then identified the antigens by expression cloning using the retrovirus expression system and revealed that one Ab recognized Lu, a type I transmembrane protein belonging to the immunoglobulin superfamily. Lu was first identified as an antigen expressed in human erythrocytes and later it was also shown to be expressed in human fetal liver and placenta and so on. In mice, Lu was reported to be expressed in some adult tissues such as heart, lung, skeletal muscle and kidney. Using anti-Lu mAb for flow cytometric analysis, we revealed that Lu is expressed in mouse fetal liver Dlk^+ cells, i.e. hepatoblasts, but not in adult hepatocytes, suggesting that Lu is a marker for immature hepatocytes and might be involved in mouse liver development. Lu is known to bind Laminin $\alpha5$ (Lama5) chain that is a component of Laminin10/11; extracellular matrix (ECM) proteins. We found that fetal hapatocytes produce Lama5. Laminin10/11 bind several different receptors, including integrin $\alpha3\beta1$, $\alpha6\beta1$ and $\alpha6\beta4$. Because $\beta1$ integrin signaling is implicated in the maturation of fetal hepatocytes, expression of Lu in fetal hepatocytes may contribute to liver differentiation by retaining laminins on the cell surface.

In the second strategy, we screened cDNA library derived from mouse fetal liver cells deprived of hematopoietic cells by the SST method. Among 640 SST clones we identified 108 different cDNA clones. A half of the cDNA clones encoded a secreted protein or hypothetical secreted protein including known abundant

secreted proteins such as AFP and Alb. One fourth of the clones were transmembrane proteins or hypothetical transmembrane proteins and the rest of them were intracellular membrane proteins. Among transmembrane proteins, I focused on the ones whose function in fetal liver had never been studied. I examined the expression of these clones and revealed that Tim2 was highly expressed in liver. Tim2 is a type I transmembrane protein with an immunoglobulin domain and a mucin domain in the extracellular region, and has a putative tyrosine-phosphorylation site in the intracellular region. Tim2 belongs to the Tim gene family, which consists of 8 members including putative family members, Tim5 to Tim8, in the mouse. It was reported that Tim2 was expressed on activated T-cells and bound to its ligand, semaphorin 4A (Sema4A), which was expressed on activated macrophages, B-cells and dendritic cells (DC). Another report showed that Tim2 was expressed in B-cells, and in adult liver and kidney, and bound ferritin heavy chain (H-ferritin). Recently, it was reported that proliferation of activated T-cells and production of Th2 cytokines were increased in Tim2 deficient mice. Though Tim2 was well investigated in immune response and it was recently reported to be expressed in the adult liver, Tim2 function in the fetal liver development had not been addressed. I found that Tim2 was highly expressed in fetal and adult liver and weakly expressed in kidney, but was undetectable in the other fetal tissues. Furthermore, flow cytometric analysis using anti-Tim2 mAb revealed that almost all fetal liver Dlk^+ fetal hepatocytes and also adult hepatocytes expressed Tim2. In addition, by immunohistochemistry strong Tim2 signal was observed between fetal hepatocytes, suggesting that Tim2 might be involved in the interaction between hepatocytes. To investigate the function of Tim2 in liver development, I prepared a recombinant soluble Tim2 fusion protein consisting of its extracellular domain and the Fc domain of human IgG (Tim2-hFc). Binding analysis revealed that Tim2-hFc bound to fetal and adult hepatocytes, indicating that both Tim2 and its ligand/receptor are expressed on hepatocytes. The addition of Tim2-hFc in the fetal hepatocyte culture inhibited the expression of mature hepatic enzymes; tyrosine aminotransferase (TAT), carbamoyl phosphate synthetase (CPS), and accumulation of glycogen. Simultaneously, Tim2-hFc reduced the number of hepatocytes that were positive for Ki67, a proliferation marker, revealed that Tim2 binding to its ligand/receptor results in cell cycle arrest. Next, I attempted to knockdown the expression of Tim2 by small interference RNA (siRNA) in the primary culture. Interestingly, downregulation of Tim2 expression by siRNA enhanced the expression of liver differentiation marker genes in the culture, suggesting that Tim2 may negatively modulate differentiation of hepatocytes. Consistent with this idea, a recent report showed that transfection of Tim2 cDNA in a T-cell line resulted in inhibition of transcriptional activity of NFAT and AP-1. Taken together these results, I have proposed the following model. Tim2 delivers a negative signal for maturation of fetal hepatocytes at an early stage of liver development when the liver is a major hematopoietic organ. At this stage, the interaction between Tim2 and its ligand/receptor is expected to be loose, because fetal hepatocytes are sparsely distributed in the liver and numerous blood cells are surrounding fetal hepatocytes. However, as liver development proceeds, fetal

hepatocytes gradually come into contact with each other to make the hepatic cord and the increase of Tim2 binding to its receptor/ligand on the neighboring cells attenuates the negative signal of Tim2 for the hepatic differentiation. At the same time, Tim2 suppresses the proliferation of neighboring hepatocytes. It was previously reported that hepatic membrane molecule(s) modulated cell growth and liver function via cell-cell contacts. As Tim2 is expressed on hepatocytes and Tim2-hFc binds to hepatocytes in fetal and adult liver, Tim2 appears to mediate cell-cell contacts between hepatocytes, which may affect the differentiation of hepatocytes. Thus, the binding between Tim2 and its receptor/ligand could contribute to liver differentiation.

In conclusion two transmembrane proteins, Lu and Tim2, were found to be expressed in hepatocytes and were shown to be involved in liver development. Lu was suggested to be involved in liver development by interacting with ECM. Tim2 was shown to contribute to cell-cell interactions between hepatocytes and to regulate differentiation of hepatocytes. These results reveal previously unrecognized important function of these proteins in liver development.