論文内容の要旨

論文題目：**The role of adult liver stem/progenitor cells in liver regeneration and the mechanism of their emergence**
（成体肝幹／前駆細胞の誘導機構および肝再生における役割の解析）

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The liver is composed of diverse cell types that arise from various embryonic origins. Among those cell types, hepatocytes, the parenchymal cells performing the major functions of the organ, and cholangiocytes, lining the bile ducts, are the two epithelial cell lineages. Homeostasis of the adult liver and also regeneration after partial hepatectomy can be achieved by cell division of mature hepatocytes and cholangiocytes, whereas in damaged liver with defects in hepatocyte proliferation, adult liver progenitor cells (LPCs) emerge usually in the periportal area and have the potential to differentiate into both hepatocytes and cholangiocytes. However, whether LPCs indeed engage in liver regeneration has not been demonstrated.

The fibroblast growth factor (FGF) family members have diverse roles in physiological and pathological conditions both during development and in adulthood. Since little is known about the role of FGF signaling in LPC activation and/or liver regeneration, I examined by PCR analysis the expression of FGFs in the livers of mice fed a 3,5-Diethoxycarbonyl-1,4-Dihydrocollidine (DDC)-containing diet. I found the expression of *Fgf7* to increase significantly during the time course of DDC-induced liver damage, along with that of *Epcam* and *Krt19*, encoding the LPC/cholangiocyte markers epithelial cell adhesion molecule (EpCAM) and cytokeratin 19 (CK19), respectively. The results of immunostaining showed that FGF7 was detected only in the smooth

muscle of the arteries in the normal liver. On the other hand, there was a marked increase of FGF7 expression in the vicinity of LPCs under conditions of hepatic injury. To address the physiogical relevance of FGF7 expression, I used *Fgf7* knockout (KO) mice. *Fgf7* KO mice exhibit normal growth and mild phenotypes, and show no liver phenotype during development or in adulthood. In order to analyze the LPC response in *Fgf7* KO mice, adult littermates of wild type (WT) and KO mice were fed a normal or DDC-containing diet. I measured the degree of LPC activation by immunostaining CK19, a well-established LPC/cholangiocyte marker, and confirmed that $CK19^+$ LPC numbers were increased by DDC in the WT liver. However, the LPC response was almost completely suppressed in *Fgf7* KO mice at 2 weeks and later on (Figure 1). The KO mice were highly sensitive to DDC and had a low survival rate, whereas the WT mice were resistant to the hepatotoxin-induced liver injury. This study demonstrates, for the first time, that the extent of LPC activation via FGF7 signaling significantly affects the mortality and the regenerative capacity of the liver.

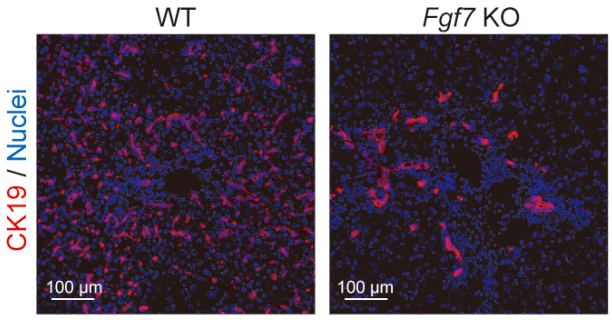

Figure 1. LPC response upon DDC administration is suppressed in *Fgf7* KO mice.

I then examined the relationship between FGF7 and LPCs in other models of liver injury. First, I checked the activation of LPCs and expression of FGF7 in liver-specific *Tak1*-deficient (*Tak1*-LKO) mice. Those mice have been reported as a liver injury model that eventually show cholestasis and carcinogenesis. LPC activation was observed in 8-week-old *Tak1*-LKO mice. The expression of *Fgf7* was augmented with the LPC activation. Second, the LPC response after ligation of the common bile duct was significantly inhibited in *Fgf7* KO mice. These findings suggest that FGF7 is necessary for LPC activation *in vivo,* and its expression and function may counter liver dysfunction.

I next performed gain-of-function experiments to further explore the function of FGF7 in regulating the LPC response. To begin with, I examined the effect of FGF7 on LPCs *in vitro*. I found that a recombinant FGF7 stimulated the proliferation of HSCE5, a cell line derived from $EpCAM^+$ LPCs of adult mice, in a dose-dependent manner. To reveal the effect of FGF7 *in vivo*, I examined *Alfp-Cre* ; *Rosa26-loxP-STOP-loxP-rtTA*; *tetO-CMV-FGF7* triple transgenic (Tg) mice in which liver-specific FGF7 overexpression could be achieved upon doxycycline (Dox) treatment. A significant increase in $CK19^+$ LPC-like cell numbers was observed in the liver of the triple Tg

mice, compared to control *Alfp-Cre*; *Rosa26-loxP-STOP-loxP-rtTA* double Tg mice. As an alternative system to achieve ectopic gene expressions in the adult mouse liver, I also exploited hydrodynamic tail vein injection (HTVi)-mediated gene transfer. It has been established that high levels of foreign gene expression in 10-40 % of mouse hepatocytes can be achieved by this method with little liver damage. Indeed, long-term expression of FGF7 was accomplished and continued for several weeks. Notably, the mice with FGF7 overexpression by HTVi showed similar phenotypes to epidermis-specific *FGF7* Tg mice, like a wet undercoat caused by excessive salivary secretions and retardation of weight gain, suggesting that the functional protein was adequately produced and secreted. I found that an $EpCAM^+$ LPC-like population was induced by overexpression of FGF7 via HTVi. While the LPC-specific marker Trop2 was not detected in the control, 22-29 % of the $EpCAM^+$ cells were Trop2-positive in the liver where FGF7 was overexpressed. The $EpCAM^+$ cells induced by overexpression of FGF7 also expressed other well-known LPC markers, CK19 and A6. Therefore, it seems reasonable to conclude that FGF7 alone is sufficient to generate an LPC-like population.

The severity of the liver damage induced by DDC was relieved by an excess of FGF7, according to blood tests. Though serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were markedly elevated after the administration of DDC in both control and FGF7-overexpressing mice, increases in alkaline phosphatase (ALP) and total bilirubin (TBIL) levels were greatly reduced by the HTVi of *Fgf7*. These results indicate that bile duct obstruction was alleviated by FGF7 overexpression under conditions where hepatocytes were persistently destroyed by DDC. Therefore, I considered that FGF7 could accelerate the functional recovery of hepatocytes and/or cholangiocytes through LPC activation.

It has been proposed that FGF7 is a mesenchymal cell-derived paracrine cytokine, which has specific mitogenic effects on epithelial cells in many organs. As LPCs are regarded as epithelial-type cells, I hypothesized that FGF7 was produced by some sort of mesenchymal cell. Immunostaining of the liver sections with several mesenchymal cell markers showed that $Thy1^+$ cells appeared in close proximity to LPCs after liver damage, as described in rats and humans. An established marker for fibroblastic cells, Elastin, and a stellate cell marker, Desmin, were partially expressed in $Thy1^+$ mesenchymal cells. Quantitative analysis of the Thy1 and CK19 immunostaining revealed that the expansion of $Thy1^+$ cells occurred prior to LPC activation. Intriguingly, the $Thy1^+$ cells were co-stained with FGF7 in the injured liver. I then analyzed the expression pattern of the FGF7 receptor, fibroblast growth factor receptor 2 isoform IIIb (FGFR2b). Among the non-parenchymal cell (NPC) populations, $EpCAM^+$ LPCs and $EpCAM^-$ cells isolated from

DDC-treated livers were subjected to immunostaining with an IIIb isoform-specific anti-FGFR2 antibody. I found that only EpCAM$^+$ cells expressed FGFR2b. To further confirm the FGF7-producing cells and receiving cells, I performed a quantitative PCR analysis using specific cell populations. Hepatocyte, NPC, EpCAM$^+$ LPC, Thy1$^+$ CD45$^-$ mesenchymal cell (Thy1$^+$ MC), Thy1$^+$ CD45$^+$ cell (T-cell) and Thy1$^-$ CD45$^+$ cell (blood cell) fractions were isolated from the livers of mice fed DDC. I checked for adequate cell separation by the specific expression of each marker. As expected, *Fgf7* and *Fgfr2* isoform IIIb were detected in Thy1$^+$ MC and LPC fractions, respectively. Since these FGF7-producing Thy1$^+$ CD45$^-$ cells strongly expressed *Elastin* (*Eln*), a fibroblastic cell marker, they are considered to be a mesenchymal cell population and distinct from T-cell populations. Fibroblast growth factor binding protein 1 (FGFBP1) is a soluble protein that can bind a subset of FGFs, including FGF7, and enhance their activities. Previous studies on skin and renal tube regeneration have shown FGFBP1 to be expressed in epithelial cells rather than mesenchymal cells and to be a target of FGF7 signaling. LPC-specific expression of *Fgfbp1* further strengthened the notion that LPCs are the primary target of FGF7 signaling from Thy1$^+$ cells. Interestingly, a quantitative analysis of the Thy1$^+$ area in *Fgf7* KO mice revealed little change when compared to the WT control in both normal and damaged liver. In other words, Thy1$^+$ cells were capable of increasing in number in response to liver damage irrespective of FGF7 function, while LPCs failed to proliferate due to a lack of FGF7 produced by Thy1$^+$ cells. In general, tissue stem cells are supported and regulated by their surrounding microenvironment, or the stem cell niche. While several molecules that participate in the LPC response have been reported, the possible involvement of niche signals has not. In this study, I have identified FGF7 as a key factor for the LPC response and that Thy1$^+$ periportal mesenchymal cells form the niche that produces FGF7 (Figure 2).

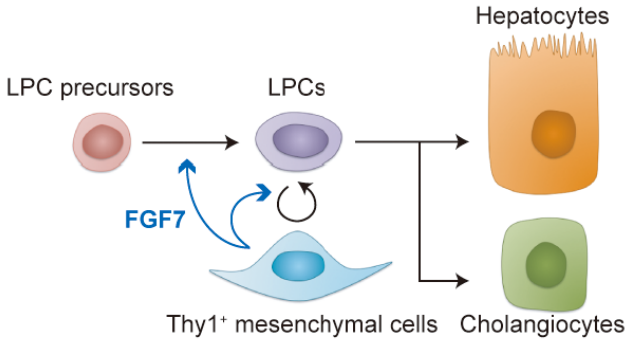

Figure 2. Schematic model.

While it has long been documented that LPCs appear and proliferate in injured and cancerous livers, whether LPCs indeed engage in regeneration has not been clear. My data, based on loss- and gain-of-function experiments with FGF7, demonstrate for the first time that the level of LPC activation correlates with resilience and survival in cases of severe liver injury, and strongly suggest that LPCs directly contribute to liver regeneration.