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# Liver maturation deficiency in $p57^{Kip2}-/-$ mice occurs in a hepatocytic $p57^{Kip2}$ expression-independent manner

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ABSTRACT

Fetal hepatic stem/progenitor cells, hepatoblasts, are highly proliferative cells and the source of both hepatocytes and cholangiocytes. In contrast, mature hepatocytes have a low proliferative potency and high metabolic functions. Cell proliferation is regulated by cell cycle-related molecules. However, the correlation between cell cycle regulation and hepatic maturation are still unknown. To address this issue, we revealed that the cell cycle inhibitor $p57^{Kip2}$ was expressed in the hepatoblasts and mesenchymal cells of fetal liver in a spatiotemporal manner. In addition, we found that hepatoblasts in $p57^{Kip2}-/-$ mice were highly proliferative and had deficient maturation compared with those in wild-type (WT) mice. However, there were no remarkable differences in the expression levels of cell cycle- and bipotency-related genes except for *Ccnd2*. Furthermore, $p57^{Kip2}-/-$ hepatoblasts could differentiate into mature hepatocytes in $p57^{Kip2}-/-$ and WT chimeric mice, suggesting that the intrinsic activity of $p57^{Kip2}$ does not simply regulate hepatoblast maturation.

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## 1. Introduction

The adult liver plays an important role in the maintenance of metabolic homeostasis. In contrast, the fetal liver has few metabolic functions and supports hematopoiesis during embryogenesis. Thus, the liver undergoes dramatic functional changes during development. However, the underlying molecular mechanisms are still unclear.

Hepatoblasts are fetal hepatic stem/progenitor cells that possess a high proliferative potential and have the ability to differentiate into both hepatocytes and cholangiocytes. Fetal livers not only consist of hepatoblasts but also various kinds of cells including hematopoietic, endothelial, mesothelial, and submesothelial cells. Mesothelial and submesothelial cells cover liver lobes and are the origin of stellate cells and mesenchymal cells around vasculature (Asahina et al., 2011). Proliferation and

*Abbreviations:* Afp, α-fetoprotein; Bmp, bone morphogenic proteins; C/EBPα, CCAAT/enhancer-binding protein α; CK, cytokeratin; CKI, cyclin-dependent kinase inhibitor; Cyp, cytochrome P450; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; Dlk1, delta-like 1 homolog; DMEM, Dulbecco's modified Eagle's medium; Dox, doxycycline; E, embryonic day; ES cell, embryonic stem cell; FBS, fetal bovine serum; Fgf, fibroblast growth factor; Foxa, forkhead box protein a; Gapdh, glyceraldehyde 3-phosphate dehydrogenase; GFP, green fluorescent protein; GMEM, Glasgow's modified Eagle's medium; Hlx, H2.0-like homeobox; Hnf, hepatocyte nuclear factor; Hprt1, hypoxanthine phosphoribosyltransferase 1; HSC, hematopoietic stem cell; IMDM, Iscove's modified Dulbecco's medium; iPS cell, induced pluripotent stem cell; KuO, Kusabira Orange; KO, knockout; Lhx2, LIM homeobox protein 2; Lif, leukemia inhibitory factor; MEF, mouse embryonic fibroblast; OSM, oncostatin M; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; Pdgfr, platelet-derived growth factor receptor; PFA, paraformaldehyde; RT-PCR, reverse transcription-polymerase chain reaction; SD, standard deviation; Tat, tyrosine aminotransferase; TLDA, TaqMan Low Density Array; Wnt, wingless-type MMTV integration site family; WT, wild-type

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differentiation of hepatoblasts are regulated by both intrinsic factors in hepatoblasts themselves and extrinsic factors derived from non-parenchymal cells during liver development. Hepatic transcription factors such as forkhead box protein a1 (Foxa1), Foxa2, and CCAAT/enhancer-binding protein α (C/EBPα) are known to regulate the proliferation and differentiation of hepatoblasts (Lee et al., 2005; Tomizawa et al., 1998; Yamasaki et al., 2006). H2.0-like homeobox (Hlx) and LIM homeobox protein 2 (Lhx2), which are mainly expressed in mesothelial and submesothelial cells of embryonic livers, are important for progression of liver development (Hentsch et al., 1996; Porter et al., 1997; Wandzioch et al., 2004). Midkine and pleiotrophin secreted from mesothelial and submesothelial cells, and oncostatin M (OSM) secreted from hematopoietic cells promote hepatoblast differentiation and proliferation (Asahina et al., 2010; Kamiya et al., 1999; Onitsuka et al., 2010). In addition, several soluble factors and cytokines are important for *in vitro* hepatic differentiation of pluripotent stem cells and somatic stem/progenitor cells (Gerbal-Chaloin et al., 2014). However, their proliferation and differentiation levels are lower than those *in vivo*. This discrepancy implies that there may be unknown mechanisms regulating hepatoblast proliferation and differentiation.

The cell cycle is a series of events that lead to cell division. Differentiated cells are considered to have a low proliferative ability. The cell cycle is controlled by cyclins and cyclin-dependent kinase inhibitors (CKIs). CKIs include two families, the Ink4 family ($p16^{Ink4a}$, $p19^{ARF}$, $p15^{Ink4b}$, $p18^{Ink4c}$, and $p19^{Ink4d}$) and the Cip/Kip family ($p21^{Cip1}$, $p27^{Kip1}$, $p57^{Kip2}$). Each Cip/Kip family member is expressed in a dynamic and restricted pattern in embryos and is involved in the regulation of proliferation and differentiation during the development of several tissues. Although *p21*$^{Cip1}$ −/− and *p27*$^{Kip1}$ −/− mice do not show developmental defects, *p57*$^{Kip2}$ −/− mice have severe developmental abnormalities and die soon after birth (Lee et al., 1995; Matsuoka et al., 1995; Nakayama et al., 1996; Parker et al., 1995; Zhang et al., 1998, 1999). *p57*$^{Kip2}$ mRNA and protein are exclusively expressed in rat livers at middle to late fetal stages and their expression levels decrease remarkably after birth (Awad et al., 2000; Ilyin et al., 2003). Therefore, $p57^{Kip2}$ may be important for the differentiation of proliferative progenitor cells into mature cells through cell cycle retardation in late liver development. However, whether $p57^{Kip2}$ regulates hepatoblast differentiation remains unknown because *p57*$^{Kip2}$ −/− mice do not survive postnatally.

To reveal the mechanisms regulating hepatoblast development, we focused on the relationship of cell cycle regulation and differentiation. Among cell cycle-related genes, we found that $p57^{Kip2}$ was the most highly expressed in hepatoblasts but not in adult hepatocytes. To examine $p57^{Kip2}$ function in liver development, we compared functional hepatic gene expression and hepatoblast proliferation ratios in the late stage fetal liver of $p57^{Kip2}$-knockout (KO) and $p57^{Kip2}$-wild-type (WT) mice. The expression levels of functional hepatic genes in *p57*$^{Kip2}$ −/− livers at the late fetal stage were lower than in WT livers. In addition, compared with WT hepatoblasts, *p57*$^{Kip2}$ −/− hepatoblasts were highly proliferative in late stage *p57*$^{Kip2}$ −/− fetal livers. To investigate *p57*$^{Kip2}$ −/−-derived hepatoblast differentiation potential in the adult liver, we analyzed *p57*$^{Kip2}$ −/− and WT chimeric mice. *p57*$^{Kip2}$ −/− and WT chimeric mice survived after birth and *p57*$^{Kip2}$ −/−-derived hepatocytes expressed functional hepatic genes at levels similar to those in WT cell-derived hepatocytes. We also found that $p57^{Kip2}$ was expressed in hepatoblasts, mesothelial cells, and submesothelial cells in the fetal liver. These results suggest that the intrinsic expression of $p57^{Kip2}$ in hepatoblasts was not important for the defective liver maturation in $p57^{Kip2}$ knockout mice.

## 2. Material and methods

### 2.1. Mouse strains

*p57*$^{Kip2}$−/− mice were obtained by breeding *p57*$^{Kip2}$+/− females and *p57*$^{Kip2}$+/− males (Takahashi et al., 2000). Fetuses were genotyped by polymerase chain reaction (PCR) using a RED Extract-N-Amp™ Tissue PCR kit (Sigma, St. Louis, MO). Genotyping primers are listed in Table 1. Mice were kept in standard laboratory cages and maintained in a 12 h light/dark cycle with free access to food and water. BDF1, enhanced green fluorescent

**Table 1**
PCR primers for detection of mouse gene expression.

| Primer name | Sequence (5′–3′) | | |
|---|---|---|---|
| $p57^{Kip2}$ genotyping1 | TGCACTGAGAGCGAGTAGAGATT | | |
| $p57^{Kip2}$ genotyping2 | AGCGGACCGATGGAAGAACTCTG | | |
| $p57^{Kip2}$ genotyping3 | GCGAAGGAACAAAGCTGCTATTG | | |
| **Genes** | **Forward primer (5′–3′)** | **Reverse primer (5′–3′)** | **Probe number** |
| Albumin | AGTGTTGTGCAGAGGCTGAC | TTCTCCTTCACACCATCAAGC | 27 |
| Afp | CATGCTGCAAAGCTGACAA | CTTTGCAATGGATGCTCTCTT | 63 |
| $p15^{Ink4b}$ | AATAACTTCCTACGCATTTTCTGC | CCCTTGGCTTCAAGGTGAG | 93 |
| $p16^{Ink4a}$ | AATCTCCGCGAGGAAAGC | GTCTGCAGCGGACTCCAT | 91 |
| $p18^{Ink4c}$ | AAATGGATTTGGGAGAACTGC | AAATTGGGATTAGCACCTCTGA | 79 |
| $p19^{Ink4d}$ | ACACCTGTCCATTGAAGAAGG | CCCCAAACACACACACTCAA | 52 |
| $p19^{ARF}$ | GGGTTTTCTTGGTGAAGTTCG | TTGCCCATCATCATCACCT | 106 |
| $p21^{Cip1}$ | AACATCTCAGGGCCGAAA | TGCGCTTGGAGTGATAGAAA | 16 |
| $p27^{Kip1}$ | GTTAGCGGAGCAGTGTCCA | TCTGTTCTGTTGGCCCTTTT | 62 |
| $p57^{Kip2}$ | CAGGACGAGAATCAAGAGCA | GCTTGGCGAAGAAGTCGT | 17 |
| Tat | GGAGGAGGTCGCTTCCTATT | GCCACTCGTCAGAATGACATC | 82 |
| Hprt1 | CCTGGTTCATCATCGCTAATC | TCCTCCTCAGACCGCTTTT | 95 |
| Cyp3a11 | CCATGTCGAATTTCCATAAACC | GGGACTCGTAAACATGAACTTTT | 53 |
| Cyp7a1 | GGCTGCTTTCATTGCTTCA | TCTCAAGCAAACACCATTCCT | 50 |
| Cyp1a2 | CCTGGACTGACTCCCACACC | CGCAATCTGTACCACTGAAG | 19 |
| Ccnd2 | CTGTGCATTTACACCGACAAC | CACTACCAGTTCCCACTCCAG | 45 |

Afp, α-fetoprotein; Cyp, cytochrome P450; Hprt1, hypoxanthine phosphoribosyltransferase 1; Tat, tyrosine aminotransferase.

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