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Disruption of polyubiquitin gene *Ubc* leads to defective proliferation of hepatocytes and bipotent fetal liver epithelial progenitor cells

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ABSTRACT

We have previously demonstrated that disruption of polyubiquitin gene *Ubc* leads to mid-gestation embryonic lethality most likely due to a defect in fetal liver development, which can be partially rescued by ectopic expression of Ub. In a previous study, we assessed the cause of embryonic lethality with respect to the fetal liver hematopoietic system. We confirmed that $Ubc^{-/-}$ embryonic lethality could not be attributed to impaired function of hematopoietic stem cells, which raises the question of whether or not FLECs such as hepatocytes and bile duct cells, the most abundant cell types in the liver, are affected by disruption of *Ubc* and contribute to embryonic lethality. To answer this, we isolated FLCs from E13.5 embryos and cultured them *in vitro*. We found that proliferation capacity of $Ubc^{-/-}$ cells was significantly reduced compared to that of control cells, especially during the early culture period, however we did not observe the increased number of apoptotic cells. Furthermore, levels of Ub conjugate, but not free Ub, decreased upon disruption of *Ubc* expression in FLCs, and this could not be compensated for by upregulation of other poly- or mono-ubiquitin genes. Intriguingly, the highest *Ubc* expression levels throughout the entire culture period were observed in bipotent FLEPCs. Hepatocytes and bipotent FLEPCs were most affected by disruption of *Ubc*, resulting in defective proliferation as well as reduced cell numbers *in vitro*. These results suggest that defective proliferation of these cell types may contribute to severe reduction of fetal liver size and potentially mid-gestation lethality of $Ubc^{-/-}$ embryos.

1. Introduction

Ubc, one of two stress-regulated polyubiquitin genes in mammals, plays an essential role in maintenance of cellular ubiquitin (Ub) levels, especially under stress conditions [1,2]. Maintenance of cellular Ub levels, which is achieved by regulation of poly- (*Ubb* and *Ubc*) or mono-ubiquitin gene (*Uba52* and *Uba80*) expression and the dynamic equilibrium between Ub conjugation/deconjugation, is important for cellular function and survival [3–7]. In mammals, *Ubc* is relatively highly expressed in the liver, and disruption of *Ubc* has been shown to result in mid-gestation embryonic lethality along with defective fetal liver development [2]. Although providing extra Ub has been shown to delay the onset of $Ubc^{-/-}$ embryonic lethality, the exact cause and the nature of defective cell types responsible for this embryonic lethality remain to be identified, despite the fact that the $Ubc^{-/-}$ phenotype is known to be directly caused by reduction of cellular Ub levels.

To identify the exact cause of $Ubc^{-/-}$ embryonic lethality, we first determined whether or not the hematopoietic system is impaired in the $Ubc^{-/-}$ fetal liver, which is the major site of hematopoiesis during mid-gestation [8,9]. However, using non-competitive and competitive reconstitution methods, we previously showed that cell autonomous hematopoietic function in the $Ubc^{-/-}$ fetal liver is almost completely intact. Therefore, $Ubc^{-/-}$ embryonic lethality is not due to intrinsic failure of the hematopoietic system but rather to decreased numbers of hematopoietic stem cells or other cell types that are essential for fetal liver development. There are several different cell types in the fetal liver, including epithelial, endothelial, and hematopoietic cells [8]. Among these, hepatocytes are hepatic parenchymal cells and the most abundant epithelial cell type, and bile duct cells are also of epithelial origin [10]. In addition, the presence of hepatic progenitor cells has been suggested. These cells include uncommitted bipotent fetal liver epithelial progenitor cells (FLEPCs), which can proliferate and differentiate into committed fetal liver epithelial cells (FLECs) such as hepatocytes and bile duct cells [11–13]. Bipotent FLEPCs play an important role during liver regeneration after injury for the repopulation (proliferation and differentiation) of epithelial cells.

Abbreviations: Ub, ubiquitin; AFP, α-fetoprotein; CK-19, cytokeratin-19; FLCs, fetal liver cells; FLECs, fetal liver epithelial cells; FLEPCs, fetal liver epithelial progenitor cells.

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In this study, we focused on FLECs, which are the most important cells for liver architecture and function as well as the most abundant cell type in the fetal liver [10]. Especially, we investigated the characteristics of bipotent FLEPCs, specifically whether or not their proliferation/differentiation capacity is affected by disruption of *Ubc* expression. We hypothesize that an insufficient number of FLEPCs could result in impairment of fetal liver development along with reduced fetal liver size (liver hypoplasia), potentially contributing to embryonic lethality. In fact, a previous report showed that fetal liver hypoplasia leads to embryonic lethality, which further supports our hypothesis that reduced liver size is closely related to $Ubc^{-/-}$ embryonic lethality [14].

Here, we observed that proliferation capacity was significantly reduced in bipotent FLEPCs as well as hepatocytes during culture *in vitro*. This reduction of proliferation capacity resulted in lower cell numbers. These findings identify the defective cell types that are responsible for reduction of fetal liver size in $Ubc^{-/-}$ embryos and provide insights into the roles of Ub in fetal liver development and liver regeneration after injury.

2. Materials and methods

2.1. Approval of animal procedures

All mouse procedures were approved by the University of Seoul Institutional Animal Care and Use Committee (UOS IACUC; UOS-091201-1 and UOS-121025-2).

2.2. Isolation and culture of fetal liver cells (FLCs)

FLCs were isolated as previously described [15]. For *in vitro* culture, cells were plated on gelatin-coated dishes at 2×10^5 cells/cm^2, incubated in DMEM containing 10% FBS for 24 h, and then changed to hepatocyte media containing amino acids, vitamins, hormones, and growth factors (ScienCell). As previously reported [16], about 5% of all FLCs were adherent when cultured in DMEM for 24 h, after which they were grown for 5 days.

2.3. Immunofluorescence microscopy

Cells on cytospin slides were prepared and stained as previously described [9]. Antibodies used were anti-E-cadherin (E-cad) (1:20, BD Pharmingen), anti-α-fetoprotein (AFP) (1:400, Thermo Scientific), anti-CD45 (1:20, BD Pharmingen), and anti-cytokeratin 19 (CK-19) (1:100, Novocastra). To detect apoptotic FLCs, fluorescence TUNEL assay was performed using an Apoptag® red *in situ* apoptosis detection kit according to the manufacturer's protocol (Millipore).

2.4. Flow cytometric analysis of FLCs

FLCs were fixed in 2% paraformaldehyde, permeabilized with 0.5% Tween 20, and stained with anti-AFP (1:1000) and anti-CK-19 (1:20) antibodies, followed by Alexa Fluor® 647-conjugated anti-mouse IgG (1:1000, Invitrogen) and phycoerythrin with cyanin-7 (PE-Cy7)-conjugated anti-rabbit IgG (1:100, Santa Cruz). To determine the range of background fluorescence, secondary antibodies alone were used as a control. Using FACSCanto II (BD Biosciences), FLCs were analyzed for AFP^+, $CK\text{-}19^+$, $AFP^+/CK\text{-}19^+$, and $AFP^-/CK\text{-}19^-$ populations to obtain the frequencies of specific cell types. To determine the frequency of $E\text{-}cad^+$ cells, anti-E-cad (1:20) antibody and Alexa Fluor® 647-conjugated anti-mouse IgG (1:1000) were used.

2.5. EdU (5-ethynyl-2′-deoxyuridine) incorporation assay

EdU incorporation assay was carried out using a Click-iT® EdU flow cytometry assay kit according to the manufacturer's protocols (Invitrogen). After EdU incorporation, cells were stained with anti-AFP (1:200) and anti-CK-19 (1:50) antibodies, followed by Alexa Fluor® 488 azide (to detect EdU^+ cells), Alexa Fluor® 647-conjugated anti-mouse IgG (1:1000, Invitrogen), and PE-Cy7-conjugated anti-rabbit IgG (1:100, Santa Cruz). Using FACSCanto II, FLCs were first analyzed for AFP^+, $CK\text{-}19^+$, and $AFP^+/CK\text{-}19^+$ populations, after which the frequency of EdU^+ cells in each population was determined.

2.6. Quantitative real-time RT-PCR and immunoblot analysis

Quantitative real-time RT-PCR (qRT-PCR) was carried out as previously described [2]. For immunoblot analysis, cell lysates were prepared in hypotonic buffer (10 mM sodium phosphate, pH 7.4, 1 mM PMSF, 1 μg/μl aprotinin, and 1 μg/μl leupeptin) with 0.1% digitonin and processed as previously described [2]. Briefly, cell lysates (10 μg) were subjected to SDS–PAGE, followed by immunoblot analysis with anti-Ub antibody (1:1000, Enzo Life Sciences). Blots were then stripped and reprobed with anti-α-tubulin antibody (1:10,000, Sigma–Aldrich).

3. Results and discussion

3.1. Reduced proliferation capacity of $Ubc^{-/-}$ FLCs during culture in vitro

We isolated cells from E13.5 fetal livers and plated them onto gelatin-coated dishes. One day after plating, we observed that about 5% of total FLCs were adherent, which was consistent with a previous report [16]. These adherent cells were then grown in hepatocyte media for 5 days. We next stained cells with E-cadherin (E-cad), a surface marker for epithelial cells, to confirm the epithelial origin of cultured cells (Fig. S1A). Culture conditions were optimized for epithelial cells since the percentage of epithelial cells increased dramatically after culture (Fig. S1B). We found that many epithelial cells stained positive for AFP, a hepatocyte marker, whereas the presence of non-epithelial cells such as hematopoietic cells was also confirmed by positive staining for CD45, a pan-hematopoietic marker (Fig. S1C). We cultured these unfractionated FLCs instead of fractionating cells to select epithelial cells before plating, due to the reduced availability of total liver cells from $Ubc^{-/-}$ embryos. Nonetheless, unfractionated FLCs formed clusters composed of epithelial cells during the *in vitro* culture period (see Fig. S1A and S1C).

During culture *in vitro*, we measured the fold-increases in numbers of control ($Ubc^{+/-}$) and $Ubc^{-/-}$ FLCs. Although the proliferation rate was indistinguishable between wild-type ($Ubc^{+/+}$) and $Ubc^{+/-}$ FLCs (data not shown), it was dramatically reduced in $Ubc^{-/-}$ FLCs (Fig. 1A). Proliferation capacity was defined as the number of cells increased during the last 24 h culture period. We found that proliferation capacity of control cells was much higher during the early culture phase (day 2) than late phase (day 5) (Fig. 1B). Reduced proliferation capacity in late phase was expected as the culture media contained growth factors and hormones that induce not only proliferation but also differentiation of FLCs. Intriguingly, the proliferation capacity of $Ubc^{-/-}$ FLCs in early phase had already diminished and was not significantly different from cells in late phase (Fig. 1B).

To determine whether or not the reduced proliferation capacity of $Ubc^{-/-}$ FLCs could be due to increased apoptosis, we performed TUNEL assay using cytospin slides with an equal number of

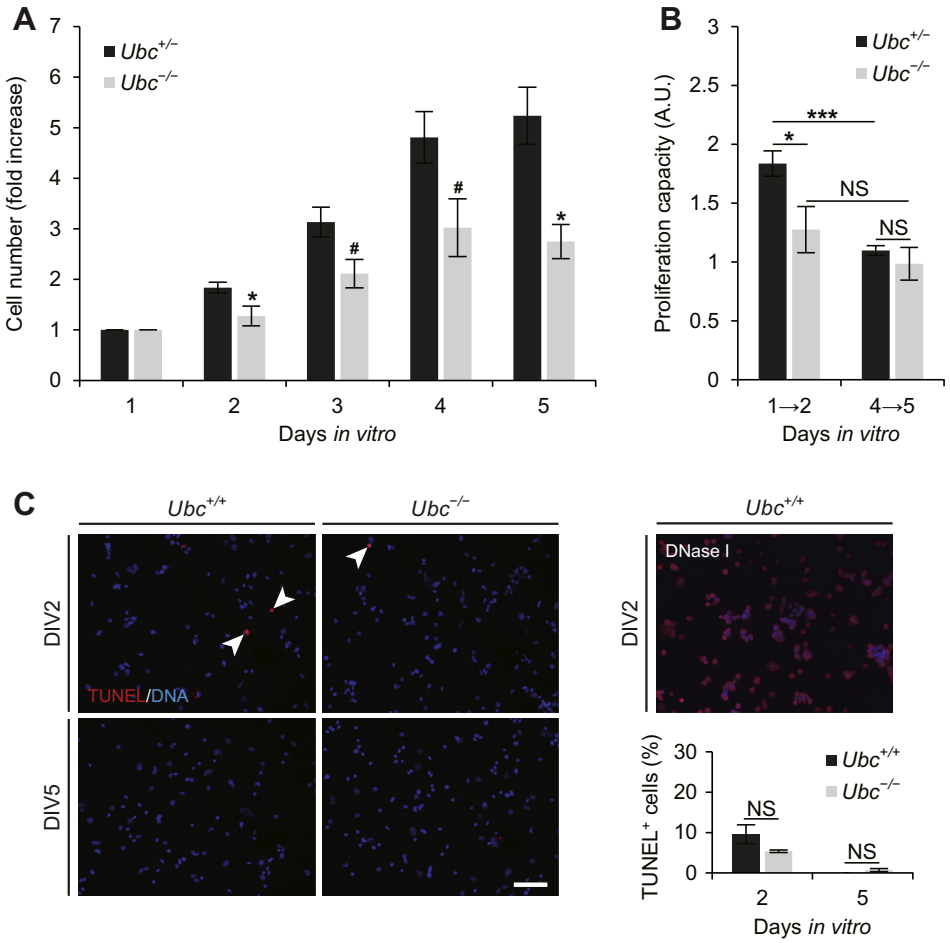

Fig. 1. Reduced proliferation capacity of $Ubc^{-/-}$ FLCs cultured *in vitro*. (A) FLCs were isolated from $Ubc^{+/-}$ ($n = 12$) and $Ubc^{-/-}$ embryos ($n = 4$) on E13.5 and DIV5. The number of cells was counted using a hematocytometer, and fold-increase in cell number was expressed relative to cell number on day 1 for each genotype. (B) Proliferation capacities of $Ubc^{+/-}$ ($n = 12$) and $Ubc^{-/-}$ FLCs ($n = 4$) on day 2 (1 → 2) and day 5 (4 → 5) are shown. (C) Cytospin slides were prepared from $Ubc^{+/+}$ and $Ubc^{-/-}$ FLCs ($n = 3$ each) on DIV2 and DIV5, and subjected to TUNEL assay. Arrowheads indicate $TUNEL^+$ apoptotic cells. DNase I treatment served as a positive control in the TUNEL assay. To determine the percentage of $TUNEL^+$ cells, the number of $TUNEL^+$ cells was divided by the number of $DAPI^+$ cells in three randomly selected fields. More than 100 $DAPI^+$ cells were counted per field. All data are expressed as the means ± SEM from the indicated number of samples. $^{\#}P < 0.1$; $^{*}P < 0.05$; $^{***}P < 0.001$ *vs.* control ($Ubc^{+/-}$) on each day (A) or as indicated (B). NS, not significant. Scale bar, 100 μm.

attached FLCs. $TUNEL^+$ cells were very small in number, and we did not observe any difference in $TUNEL^+$ cell fractions between control ($Ubc^{+/+}$) and $Ubc^{-/-}$ FLCs on either 2 days or 5 days *in vitro* (DIV2 or DIV5) (Fig. 1C).

3.2. Disruption of Ubc in FLCs results in reduction of Ub levels due to insufficient compensation by other ubiquitin genes

As previously reported [9], the proliferation capacity of $Ubc^{+/-}$ FLCs (see Fig. 1B) was highly correlated with the mean GFP fluorescence of a GFP-$puro^r$ fusion protein knocked in to the *Ubc* locus (Fig. 2A), which was used as a direct indicator of *Ubc* transcriptional activity. Therefore, highly proliferating cells generally exhibited high GFP fluorescence, and $Ubc^{+/-}$ FLCs showed a general decrease in GFP fluorescence that was correlated with reduced proliferation capacity in late phase of culture.

In accordance with the reduced GFP fluorescence in $Ubc^{+/-}$ FLCs in late phase, the coding potential of *Ubc*, which was determined by multiplying the number of Ub moieties per coding unit (nine or four Ubs per *Ubc* or *Ubb* transcript, respectively) by the mRNA levels measured by qRT-PCR, was reduced in $Ubc^{+/+}$ FLCs on DIV5 compared to cells on DIV2 (Fig. 2B top). The coding potential of *Ubb* as well as the mRNA levels of *Uba52* and *Uba80* were also reduced in $Ubc^{+/+}$ FLCs as culture progressed (Fig. 2B). These results suggest that overall Ub levels were reduced in late-phase cells, which was expected as highly proliferating cells in early phase may require additional Ub for acceleration of cell cycle progression with timely degradation of cell cycle regulators [17]. Although the coding potential of *Ubb* increased in $Ubc^{-/-}$ FLCs, its contribution to increase total Ub levels was quite minimal due to its low level compared to that of *Ubc*, as observed in $Ubc^{+/+}$ FLCs (Fig. 2B top). In addition, we could not confirm the upregulation of *Uba52* and *Uba80* in $Ubc^{-/-}$ FLCs (Fig. 2B bottom).

This result was also confirmed by immunoblot analysis in which Ub conjugate levels were much lower in late phase of culture as well as by disruption of *Ubc* in FLCs (Fig. 2C). Interestingly, free Ub levels were not reduced but rather slightly increased in $Ubc^{-/-}$ FLCs. Since we previously reported that cell death occurs when free Ub levels are not maintained [18], maintenance of free Ub levels in cultured $Ubc^{-/-}$ FLCs could explain the apparent lack of apoptosis. This result seems to contradict our previous result in which $Ubc^{-/-}$ FLCs isolated from E13.5 embryos were shown to display increased apoptosis [9]. Since only 5% of total cells isolated from E13.5 embryos were adherent and cultured *in vitro*, it is highly likely that apoptotic cells did not attach to the plate.

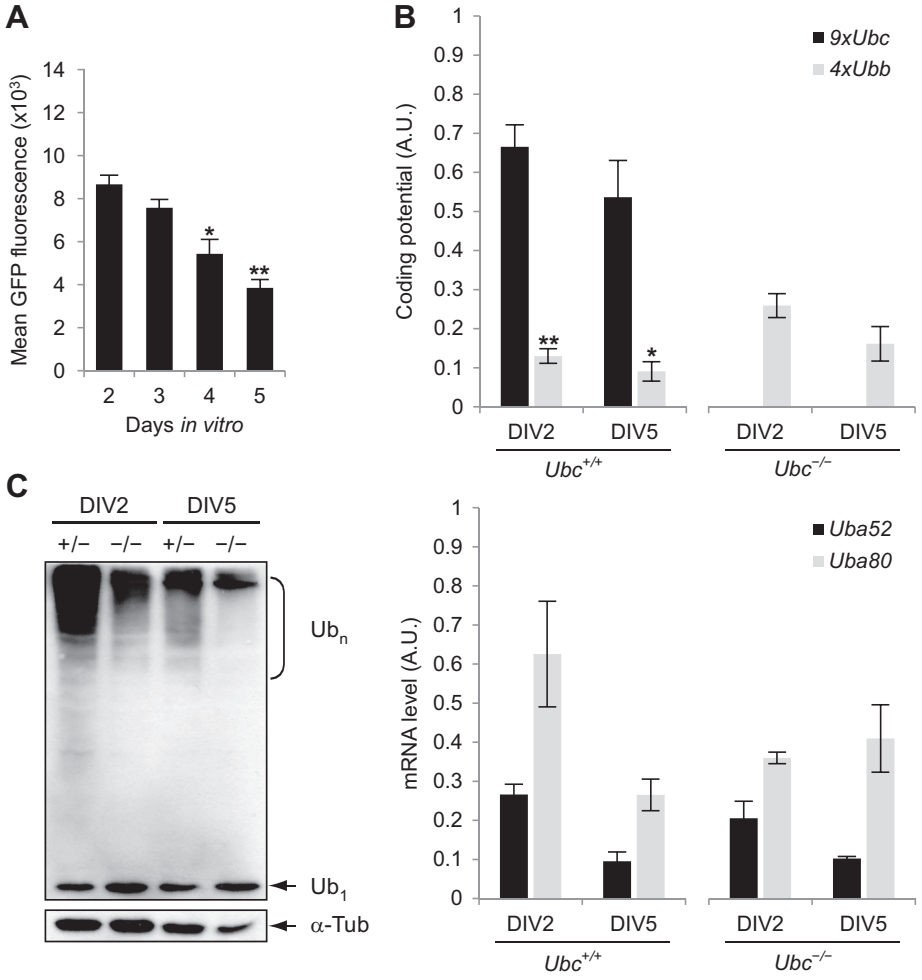

Fig. 2. Decreased overall coding potentials and Ub conjugate levels in $Ubc^{-/-}$ FLCs cultured *in vitro*. (A) Mean GFP fluorescence in $Ubc^{+/-}$ FLCs ($n = 3$) cultured *in vitro* for 2–5 days, as determined by flow cytometry. (B) (Top) Coding potentials of *Ubc* and *Ubb* in $Ubc^{+/+}$ and $Ubc^{-/-}$ FLCs ($n = 3$ each) on DIV2 and DIV5. (Bottom) *Uba52* and *Uba80* mRNA levels in $Ubc^{+/+}$ and $Ubc^{-/-}$ FLCs ($n = 3$ each) on DIV2 and DIV5 were determined by qRT-PCR and normalized to β-actin mRNA levels. (C) Ub conjugate (Ub_n) and free Ub (Ub_1) levels in $Ubc^{+/-}$ and $Ubc^{-/-}$ FLCs on DIV2 and DIV5 were determined by immunoblot analysis using anti-Ub antibody. α-Tubulin (α-Tub) was used as a loading control. Representative results from three different fetal livers for each genotype are shown. All data are expressed as the means ± SEM from the indicated number of samples. $^{*}P < 0.05$; $^{**}P < 0.01$ *vs.* day 2 (A) or *9xUbc* on each day (B).

3.3. Hepatocytes and bipotent FLEPCs are affected by disruption of Ubc during culture in vitro

Next, to identify the exact cell types present during culture *in vitro*, we prepared cytospin slides with cells on DIV2 and stained them with AFP, a hepatocyte marker, and CK-19, a bile duct cell marker (Fig. 3A). Interestingly, in addition to AFP^+, $CK\text{-}19^+$, and $AFP^-/CK\text{-}19^-$ cells, we observed a fourth cell type that was positive for both AFP and CK-19 ($AFP^+/CK\text{-}19^+$ cells) (Fig. 3A and data not shown for $AFP^-/CK\text{-}19^-$ cells). We also confirmed the presence of $AFP^+/CK\text{-}19^+$ cells by flow cytometry using markers specific for AFP and CK-19 (Fig. 3B top). $AFP^+/CK\text{-}19^+$ cells are known to be uncommitted bipotent FLEPCs, as they can differentiate into committed unipotent AFP^+ or $CK\text{-}19^+$ FLECs [19]. During culture, FLCs underwent differentiation (especially in late phase), therefore we observed reduced frequency of bipotent FLEPCs on DIV5 compared to DIV2, as determined by flow cytometry (Fig. 3B bottom). Interestingly, cell frequency was not affected by disruption of *Ubc*, as both control ($Ubc^{+/-}$) and$Ubc^{-/-}$ bipotent FLEPCs showed similar frequencies on specific days *in vitro* (28–30% on DIV2 *vs.* 16–17% on DIV5). These results suggest that differentiation capacity of bipotent FLEPCs might not be affected by disruption of *Ubc*.

To measure *Ubc* expression levels in four different cell types during culture, we monitored GFP fluorescence of specific cell populations on days 2 and 5 by flow cytometry (Fig. 3C). We observed the highest GFP fluorescence, i.e. highest *Ubc* transcriptional activity, in bipotent FLEPCs on DIV2. Surprisingly, this was maintained until DIV5, at which point GFP fluorescence was significantly diminished in total cell population (Fig. 3C).

By multiplying the frequencies of specific cell populations, as determined by flow cytometry using appropriate markers, by the total numbers of cells in specific growth stages, we found that control cell numbers increased dramatically from DIV2 to DIV5 regardless of cell type (Fig. 3D). This dramatic increase in cell number was completely abolished in $Ubc^{-/-}$ hepatocytes and $Ubc^{-/-}$ bipotent FLEPCs, but not in $Ubc^{-/-}$ bile duct cells or non-epithelial cells. It is also interesting that the number of $Ubc^{-/-}$ bipotent cells was comparable with that of control cells on DIV2. This cell number did not increase at all until DIV5, whereas the number of control

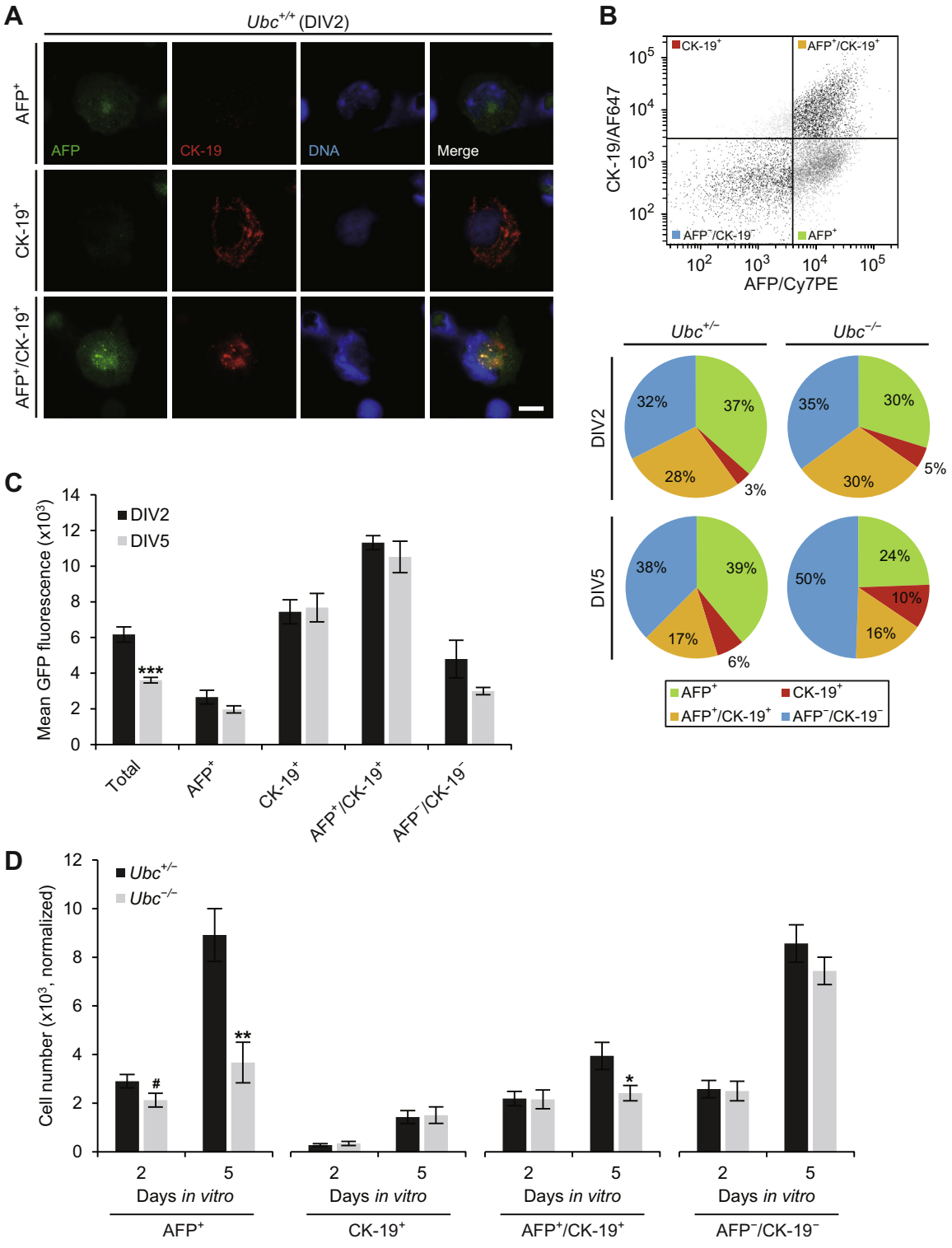

Fig. 3. Reduced numbers of hepatocytes and bipotent FLEPCs due to disruption of *Ubc* during culture *in vitro*. (A) Cytospin slides were prepared from $Ubc^{+/+}$ FLCs on DIV2 and stained with AFP (hepatocyte marker) and CK-19 (bile duct cell marker), and DNA was visualized with DAPI. Representative images of AFP^+, $CK\text{-}19^+$, and $AFP^+/CK\text{-}19^+$ cells are shown. (B) (Top) Representative dot plot showing the frequencies of AFP^+, $CK\text{-}19^+$, $AFP^+/CK\text{-}19^+$, and $AFP^-/CK\text{-}19^-$ cells on DIV2. AFP and CK-19 immunoreactivities were indirectly monitored by PE-Cy7-conjugated anti-rabbit IgG (Cy7PE) and Alexa Fluor® 647-conjugated anti-mouse IgG (AF647), respectively. (Bottom) Pie chart showing the percentages of AFP^+, $CK\text{-}19^+$, $AFP^+/CK\text{-}19^+$, and $AFP^-/CK\text{-}19^-$ cell populations in $Ubc^{+/-}$ ($n = 10$) and $Ubc^{-/-}$ FLCs ($n = 7$) on DIV2 and DIV5. (C) Mean GFP fluorescence of total, AFP^+, $CK\text{-}19^+$, $AFP^+/CK\text{-}19^+$, and $AFP^-/CK\text{-}19^-$ cell populations in $Ubc^{+/-}$ FLCs ($n = 5$) on DIV2 and DIV5, as determined by flow cytometry. (D) Normalized cell numbers of AFP^+, $CK\text{-}19^+$, $AFP^+/CK\text{-}19^+$, and $AFP^-/CK\text{-}19^-$ populations in $Ubc^{+/-}$ ($n = 10$) and $Ubc^{-/-}$ FLCs ($n = 7$) on DIV2 and DIV5. All data are expressed as the means ± SEM from the indicated number of samples. $^{\#}P < 0.1$; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ *vs.* DIV2 (C) or control ($Ubc^{+/-}$) on each day (D). Scale bar, 10 μm.

cells increased about 1.8-fold. On the other hand, the number of $Ubc^{-/-}$ hepatocytes increased about 1.7-fold ($P = 0.1$) from DIV2 to DIV5, although it did not reach the number of control hepatocytes. Therefore, the reduced cellularity of $Ubc^{-/-}$ FLCs on DIV5 can be attributed to the lower numbers of hepatocytes and bipotent FLEPCs.

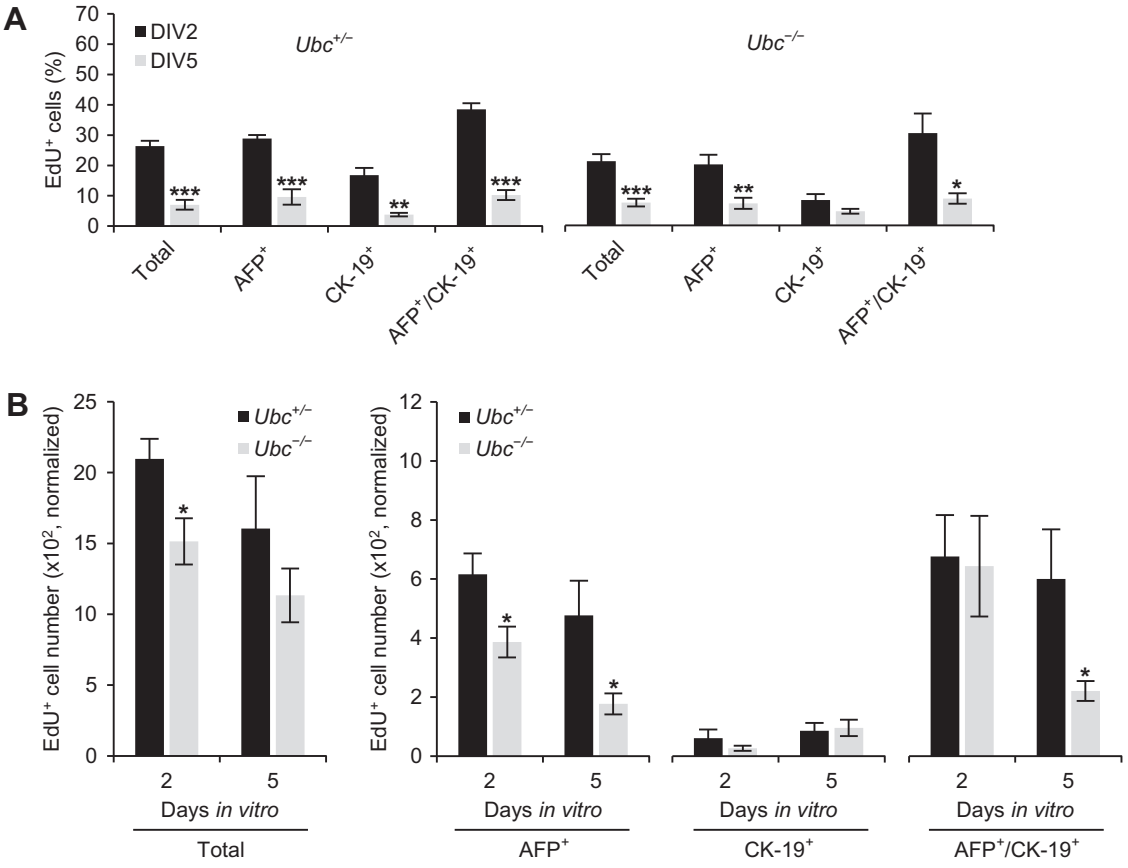

Fig. 4. Impaired proliferation of hepatocytes and bipotent FLEPCs due to disruption of *Ubc* during culture *in vitro*. (A) Percentages of proliferating (EdU$^+$) total, AFP$^+$, CK-19$^+$, and AFP$^+$/CK-19$^+$ cell populations in *Ubc*$^{+/-}$ (n = 4) and *Ubc*$^{-/-}$ FLCs (n = 5) on DIV2 and DIV5. EdU incorporation assay was performed as described in Section 2. (B) Normalized proliferating (EdU$^+$) cell numbers of total, AFP$^+$, CK-19$^+$, and AFP$^+$/CK-19$^+$ populations in *Ubc*$^{+/-}$ (n = 4) and *Ubc*$^{-/-}$ FLCs (n = 5) on DIV2 and DIV5. All data are expressed as the means ± SEM from the indicated number of samples. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ *vs.* DIV2 (A) or control (*Ubc*$^{+/-}$) on each day (B).

AFP$^-$/CK-19$^-$ cells are non-epithelial cells such as hematopoietic cells (see Fig. S1C). Although we already demonstrated that the hematopoietic cell population was reduced in *Ubc*$^{-/-}$ embryos on E13.5 [9], it is not surprising that the number of hematopoietic cells that survived during the *in vitro* culture period was similar between control and *Ubc*$^{-/-}$ cells, as equal numbers of cells were initially plated for the two different genotypes (Fig. 3D). In fact, the frequency of hematopoietic cells was similar between control and *Ubc*$^{-/-}$ FLCs from E13.5 embryos [9], whereas the number of hematopoietic cells was expected to decrease in *Ubc*$^{-/-}$ embryos on E13.5 due to a reduced total liver cell number (specific cell number = total cell number × frequency of specific cells).

It is currently unknown why GFP fluorescence, i.e., *Ubc* transcriptional activity, in *Ubc*$^{+/-}$ AFP$^+$ cells was much lower than that in bipotent cells despite a higher number of AFP$^+$ cells than bipotent cells, especially on DIV5 (Fig. 3C and D). A possible explanation is that the dramatic increase in AFP$^+$ cell number from DIV2 to DIV5 (about 3-fold) was due not only to proliferation of AFP$^+$ cells but also to differentiation of bipotent cells into unipotent AFP$^+$ cells.

3.4. Proliferation of hepatocytes and bipotent FLEPCs is significantly impaired by disruption of Ubc during culture in vitro

To determine whether or not the decreased numbers of *Ubc*$^{-/-}$ hepatocytes and bipotent FLEPCs can be attributed to reduced proliferation of specific cell types *per se*, we measured the proliferating cells, i.e. in S-phase of cell cycle, by labeling with EdU, which is incorporated into DNA during replication. Using flow cytometric analysis, we gated cells positive for specific markers and then determined the frequency of EdU$^+$ cells in each cell population. Bipotent cells showed the greatest proliferation on DIV2 regardless of genotype (Fig. 4A). However, the proliferation capacity of every cell type had decreased on DIV5 regardless of genotype, suggesting that cells no longer proliferated in late phase.

By multiplying the frequency of EdU$^+$ cells, as determined by flow cytometry, by the total numbers of cells in specific growth stages, we confirmed that the results of the EdU incorporation assay (total cells) were in accordance with our proliferation data as determined by cell counting (Fig. 4B left and see Fig. 2B). In examining the number of EdU$^+$ cells, proliferating hepatocytes were significantly reduced in number upon disruption of *Ubc* on both DIV2 and DIV5 (Fig. 4B right). This result exactly reflected the difference in cell number between control and *Ubc*$^{-/-}$ cells on DIV2 and DIV5 (see Fig. 3D). Interestingly, the number of EdU$^+$ bipotent cells was maintained even upon disruption of *Ubc* on DIV2, whereas it was significantly reduced on DIV5. This result also exactly reflected the difference in cell number between control and *Ubc*$^{-/-}$ cells on DIV2 and DIV5. From DIV2 to DIV5, the number of *Ubc*$^{-/-}$ EdU$^+$ bipotent cells decreased dramatically by 66%, whereas *Ubc*$^{-/-}$ EdU$^+$ hepatocytes decreased by 54% (Fig. 4B). In addition, the number of proliferating *Ubc*$^{-/-}$ hepatocytes was lower than that of control cells throughout the culture period. However, the number of proliferating *Ubc*$^{-/-}$ bipotent FLEPCs was maintained in early phase and reduced in late phase.

Since *Ubc* expression levels in bipotent FLEPCs remained high throughout the entire culture period, we speculate that maintaining the number of bipotent FLEPCs is important for fetal liver

development. Accordingly, abrupt reduction of the number of proliferating bipotent FLEPCs and chronic reduction of the number of proliferating hepatocytes could have a detrimental effect on fetal liver development in *Ubc*$^{-/-}$ embryos, resulting in reduced fetal liver size and potentially embryonic lethality.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.05.003.

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