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碩士論文

Department of Biochemical Science and Technology
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Master Thesis

於小鼠肝纖維模式下探討低氧環境對肝臟細胞之影響

The effect of hypoxia on liver cells in mouse liver fibrosis model

涂琬瑛

Woan-Ing Twu

指導教授：陳彥榮 博士

Advisor: Yen-Rong Chen, Ph.D.

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口試委員審定書

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本論文係涂琬瑛（R01B22009）在國立臺灣大學生化科技學系完成之碩士學位論文，於民國 103 年 7 月 9 日承下列考試委員審查通過及口試及格，特此證明

口試委員：陳亮等（簽名）

（指導教授）

系主任、所長 黃慶璨（簽名）

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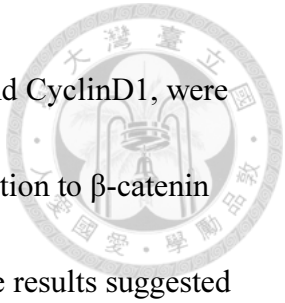
Abstract

Hepatic fibrosis is the response of liver encountering chronic injury, which would often progress to liver cirrhosis or even hepatocellular carcinoma (HCC). Chronic liver disease and liver fibrosis were in the list of top tenth cause of death in both Taiwan and the US, but no effective drug was developed for the treatment of fibrosis.

The accumulation of extracellular matrix (ECM) during liver fibrosis would lead to hypoxia due to the blockage of oxygen diffusion, and hypoxia inducible factor 1 α (HIF1α) is the main modulator of hypoxic response. Here in my research, I have detected upregulated expression of HIF1α, in mRNA as well as in protein level in fibrotic liver. The cells expressing HIF1α also expressed epithelial cell adhesion molecule (EpCAM), Albumin (Alb) and cytokeratin 19 (CK19), suggesting that these $HIF1\alpha^+$ cells were hepatic progenitor cell (HPC)-like. I also observed translocated β-catenin in fibrotic liver, indicating the activation of canonical Wnt signal. These data proved that HPCs were localized in hypoxic area during liver fibrosis, and HIF1α may interact with Wnt/β-catenin signaling to regulate HPC.

As for the *in vitro* experiment, I cultured HCC cell line Huh7 under normoxic and hypoxic conditions to compare the expression levels of the genes mentioned in the *in vivo* experiment and found out that the mRNA expression level of HPC markers,



EpCAM and CK19, and Wnt/β-catenin downstream targets, Axin2 and CyclinD1, were all elevated. Increased EpCAM and CK19 protein expression, in addition to β-catenin translocation could also be observed in hypoxic cultured Huh7. These results suggested that the increased expression of HPC marker and Wnt/β-catenin signaling pathway observed *in vivo* could be regulated by hypoxic condition.

Keywords: liver fibrosis, hypoxia, HPC, EpCAM, HIF, Wnt/β-catenin

中文摘要

當肝臟受到長期損傷會產生纖維化的現象，而肝纖維化往往會更進一步惡化成肝硬化甚至是肝癌。慢性肝病及肝纖維化在台灣及美國都是前十大主要死因之一，但目前並沒有有效的治療方式。

在肝纖維化時，胞外基質的過度累積會影響氧氣擴散造成組織內產生缺氧的情形，而 hypoxia inducible factor 1 α (HIF1α) 是體內主要調控缺氧反應的分子。在本篇研究中，我發現在纖維化的肝臟當中， HIF1α 的 mRNA 和蛋白質表現量都會上升。這些會表現的細胞同時也會表現 epithelial adhesion molecule (EpCAM) 、Albumin (Alb) 和 Cytokeratin 19 (CK19) ，顯示這些細胞很有可能是肝臟前驅細胞。同時我也發現 β-catenin 在纖維化的肝臟中有進核的情形，表示典型 Wnt 訊息傳遞路徑是有被活化的。這些實驗結果顯示肝臟前趨細胞在纖維化的肝臟當中是處於低氧的狀態，而且 HIF1α 可能會透過 Wnt 訊息傳遞路徑調控肝臟前趨細胞。

在細胞實驗方面，我在正常氧濃度及低氧環境下培養肝癌細胞株 Huh7 ，並分析在動物實驗中有提到的基因及蛋白質的表現量。實驗結果顯示在低氧環境下，肝臟前驅細胞的標記分子 EpCAM 和 CK19 ，以及 Wnt/β-catenin 訊息傳遞路徑的下游基因 Axin2 和 CyclinD1 的 mRNA 表現量都有上升的情形； EpCAM 和 CK19 的蛋白質表現量也有上升，也可以看到 β-catenin 有進核的情形。這些研究結果顯示在動物實驗中觀察到的肝臟前趨細胞標記以及 Wnt/β-catenin 訊息傳遞路徑活化的情形很有可能是被低氧狀態所誘導的。

關鍵字：肝纖維化、低氧、肝臟前驅細胞、 EpCAM 、 HIF 、 Wnt/β-catenin

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Chapter 1. Literature Review

1.1. Liver fibrosis and disease progression

1.1.1. Liver fibrosis

Hepatic fibrosis is the response of liver encountering chronic injury, including virus infection, overmuch alcohol consumption and hepatitis, and may further progress to liver cirrhosis or even hepatocellular carcinoma (HCC). According to the statistics from Taiwan's Department of Health, chronic liver disease and liver fibrosis were the top seventh cause of death in 2011, but no effective drug was developed for the treatment of fibrosis. Therefore, understanding the mechanisms during liver fibrosis may help develop some new treatment towards the disease.

1.1.2. Mechanisms of chronic liver disease progression

Hepatic fibrosis is the response of liver encountering chronic injury, which is characterized by the accumulation of extracellular matrix (ECM). And one of the most vital issues discussing in hepatic fibrosis is the cellular origin of the over-produced ECM.

Hepatic stellate cells (HSCs) are the main source of ECM in damaged liver, while fibroblasts derived from other resources may also have more or less contribution to ECM remodeling during fibrosis (Wells, 2008). HSC is the

primary vitamin A storage site in body, which resides in a subendothelial space called “Disse” between hepatocytes and sinusoidal endothelial cells in a quiescent phenotype in healthy liver (Friedman, 2008; Kumar et al., 2013).

When liver encounters damages, death of hepatocytes (apoptosis, necrosis or other kinds of cell death) would lead to cytokine production of adjacent hepatocytes or immune cells, and trigger inflammatory response. These cytokines would not only recruit more immune cells to injured sites, but they would also activate quiescent HSC into its myofibroblast phenotype, which is the type of fibrogenic HSC (Lee and Friedman, 2011). Activated fibrogenic HSC would lose its vitamin A storage ability, secret fibrotic collagen (type I, III, and IV), and have imbalanced metalloproteinase (MMP)/tissue inhibitor of metalloproteinase (Timp) expression, which controls the homeostasis of ECM in liver (Hemmann et al., 2007). These morphologies of HSC would cause collagen accumulation around periportal area in liver. Then the accumulated collagen fibers would replace normal hepatocytes and disrupt liver architectures and functions, thus causing the formation of regenerative nodules, which mark the presence of liver cirrhosis (Lefton et al., 2009). Cells within regenerative nodules are often dysplastic due to the entrapment of accumulated ECM, and result in neoplastic lesion. Ultimately, these dysplastic cells would transform and might

lead to the formation of HCC (Ramakrishna et al., 2013).

Except for the signals derived from dead or injured hepatocytes and immune cells, HSC also responds to signals originated in other cell types. Stem cells, including bone marrow-derived mesenchymal stem cells and adult stem cells residing in liver, have been reported to regulate HSC activation in damaged liver (Carpino et al., 2013; Chen et al., 2011). More thorough relationships among HSC and other populations in liver, however, require further investigation.

1.2. Hepatic Progenitor cell (HPC) and liver disease

1.2.1. Hepatic Progenitor Cells (HPC)

HPCs are the adult stem cells residing in liver. Under normal circumstances, hepatocytes and cholangiocytes, the two main populations in liver, are the main sources of hepatic regeneration (Michalopoulos, 2007). But under severe damage, when hepatocytes and cholangiocytes alone are insufficient for the restoration of liver function, the hepatic progenitor cells (HPCs) would be activated and differentiate into the injured cell type to restore the population (Williams et al., 2014). HPCs are a group of cells with ability to differentiate into both hepatocyte and cholangiocytes. HPCs express markers specific in both hepatocyte- and cholangiocyte-lineage, such as CD44, CD90, CD133, Albumin (Alb), cytokeratin

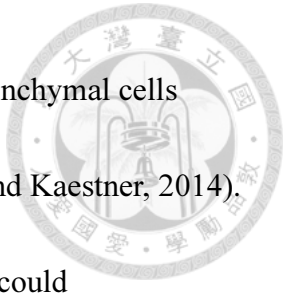
19 (CK19), epithelial cell adhesion molecule (EpCAM), and α-fetoprotein (AFP) (Bogaerts et al., 2014).

1.2.2. HPC in liver disease

Previous studies have shown different roles of HPC under liver damage. In cirrhotic or fibrotic liver followed by hepatectomy, reduced DNA synthesis and cell cycle arrest were observed in hepatocytes, indicating dysfunctional ability of repopulation of hepatocytes (Kanta and Chlumska, 1991; Marshall et al., 2005). An article in 2013 had further suggested that the activated HPC were highly associated with the fibrotic area in a similar liver regeneration model (Kuramitsu et al., 2013). But some fate-tracing researches reported otherwise. In these studies, they found out that HPC only contributed to 1-3% of regenerated hepatocytes during injury (Espanol-Suner et al., 2012; Malato et al., 2011). Though these various results may arise from different approaches they used to cause liver damage, they suggested that the diverse roles of HPC in different models required more intensive and thorough investigation.

1.2.3. Dedifferentiation of hepatocyte into HPC

Though activation of HPC have been observed in damaged liver, the origin of these activated HPC is still controversial (Williams et al., 2014). A review published this year discussed the possible origin of HPC during liver injury,



including both biliary and hepatic epithelial cells, HSCs, mesenchymal cells derived from bone marrow, and HPCs from fetal liver (Shin and Kaestner, 2014). Results of lineage tracing experiment showed that hepatocyte could dedifferentiate into HPC and transdifferentiate into biliary lineage in injured liver (Yanger and Stanger, 2011). Plasticity of both hepatic and biliary lineage could be observed *in vitro* culture of mature hepatocytes, suggesting that the activated HPC could be derived from mature hepatocytes (Chen et al., 2012a).

1.3. Hypoxia, hypoxia-inducible factors (HIFs) and liver disease

1.3.1. Hypoxia and HIF

During the progression of liver fibrosis towards HCC, hypoxic areas often form due to excess accumulation of ECM components (Cannito et al., 2014). Hypoxia-inducible factors (HIFs) are the critical mediators of physiological responses to acute and chronic hypoxia. HIF is a transcription factor consisting of two different subunits, an O_2-regulated α subunit and a constitutively expressed β subunit. There are at least three kinds of HIFs, HIF1, HIF2 and HIF3. Among them, HIF1 has been reported to be the main regulator under hypoxic condition, while the importance of HIF2 has been shown to be more vital recently. Downstream targets of HIFs include genes involved in metabolism,

proliferation, dedifferentiation, angiogenesis, invasiveness and metastasis, and apoptosis (Semenza, 2007). HIF also plays an important role in modulating multiple stem cell properties, such as self-renewal and cell-fate commitment, in a variety of stem cell lineages, including embryonic, hematopoietic, mensenchymal and neural lineage (Cunningham et al., 2012; Imanirad and Dzierzak, 2013; Mohyeldin et al., 2010).

1.3.2. HIF in liver disease

HIFs have been reported to be involved in several signaling pathways during hepatic fibrosis and further HCC tumorigenesis. For instance, HIF positively regulated the activation of HSC via MAPK pathway (Wang et al., 2013). Other studies have shown that HIF promoted the expression of pro-fibrogenic and pro-angiogenic genes in myofibroblast, including vascular endothelial growth factor (VEGF), tumor growth factor β (TGFβ) and several inflammatory cytokines (Cannito et al., 2014). HIFs are also involved in the regulation of proliferation, angiogenesis, metastasis, chemoresistance and radioresistance in HCC (Dong et al., 2013). However, the researches of the role of HIF in hepatic fibrosis focused only on HSC rather than other populations involved, such as HPCs and hepatocytes.

1.4. Epithelial cell adhesion molecule (EpCAM) and liver disease

1.4.1. Epithelial cell adhesion molecule (EpCAM)

EpCAM, one of the HPC markers mentioned above, is also a cell surface marker in several other cancers, including colon and breast cancer (Herlyn et al., 1979; Osta et al., 2004). EpCAM is a glycosylated transmembrane protein composed of three domains, an extracellular domain, EpEX, a single transmembrane domain, and an intracellular domain, EpICD. Upon proteolysis activation, EpICD would be released into cytoplasm, forming a complex with β-catenin and translocating into nucleus. Translocated EpICD and its interaction partner act as transcription factor that induce the expression of genes involved in Wnt signaling pathway (Munz et al., 2009).

1.4.2. EpCAM in liver disease

EpCAM would be upregulated in cirrhotic livers and during liver regeneration, and elevated expression of EpCAM was corresponding to longer telomere lengths in hepatocytes (Yoon et al., 2011; Zhang et al., 2008). High expression of EpCAM in liver was considerably associated with poor prognosis of patients with HCC (Yamashita et al., 2008). EpCAM-targeted immunotoxin has been proved to impair sphere-forming ability in HCC cell lines (Ogawa et al., 2014). But the upstream regulators and downstream targets of EpCAM in chronic liver

disease have not been fully understood.

1.4.3. Relationships between HIF1α and EpCAM

Hypoxic condition has been shown to induce EpCAM expression during renal regeneration and in prostate cancer cell line (Bao et al., 2012; Trzpis et al., 2008). *In silico* analysis could also detect HIF-responsive element (sequence that would be bound by HIF) on EpCAM promoter. A more recent paper has further discovered that HCC with expression of HIF1α and EpCAM were more likely to reoccur after therapy (Yamada et al., 2014). But the relationships between HIF and EpCAM during liver fibrosis remain unclear.

1.5. Wnt signaling pathway and liver disease

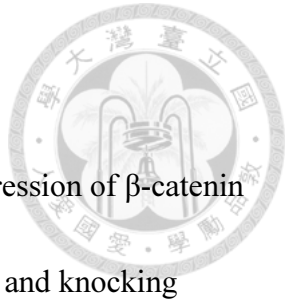
1.5.1. Wnt/β-catenin signaling pathway

Wnt signaling pathways were first discovered as key regulator during embryogenesis and developmental process. Further researches have shown that Wnt signaling pathways are also involved in lots of features of cancer, such as proliferation, dedifferentiation, metastasis and survival (Clevers and Nusse, 2012). In the canonical Wnt pathway, after the binding of Wnt protein onto its receptor Frizzled, β-catenin would be released from its destruction complex and accumulate in cytoplasm. Accumulated β-catenin would translocate into nucleus

and form a transcriptional activation complex with Tcf/Lef to activate Wnt-responsive genes, which include genes mediating cell proliferation, cell cycle progression, migration, invasion and metastasis (Moeini et al., 2012). Wnt/β-catenin signaling is crucial for maintaining stemness properties during embryonic development, and the ablation of components in this pathway would lead to dysregulated signaling network and further affect the developmental process (Holland et al., 2013; Katoh, 2011; Kuhl and Kuhl, 2013; Schepers and Clevers, 2012).

1.5.2. Wnt/β-catenin in liver disease

Resent research has shown that β-catenin was overexpressed in hepatic fibrosis and inhibition of Wnt/β-catenin signaling would obstruct HSC activation by down-regulating collagen synthesis and inducing apoptosis (Ge et al., 2014). A study in 2008 detected dysfunctional Wnt signaling in about 95% of HCC (Bengochea et al., 2008). Results from later research suggested that Wnt signaling would alter HPC cell fate to regenerate hepatocyte in chronic liver disease (Boulter et al., 2012). In addition, Wnt ligand have been proved to increase generation efficiency of stem cells differentiation to hepatocytes, indicating that Wnt signaling may also play an important role during liver regeneration (Chen et al., 2012b).



1.5.3. Relationships between HIF1α and Wnt/β-catenin

In addition to EpCAM, HIF1α would also regulate the expression of β-catenin to enhance epithelial-mesenchymal transition (EMT) in HCC, and knocking down β-catenin would impair EMT and metastatic properties induced by hypoxia in prostate cancer (Zhang et al., 2013; Zhao et al., 2011). The activation of β-catenin by HIF1α could also be observed in hypoxic-cultured stem cells (Mazumdar et al., 2010). However, similar relationships have not yet been reported during liver fibrosis.

1.5.4. Relationships between EpCAM and Wnt/β-catenin

EpCAM has been proved to be a direct downstream target of Wnt signaling in normal hepatocytes and HCC cell lines, and the presence of EpCAM makes HCC more sensitive to Tcf/β-catenin binding inhibitors (Yamashita et al., 2007). Crosstalk between EpCAM and Wnt signaling pathway has also been reported before (Maetzel et al., 2009). Same research has shown that they shared common signal transduction component, β-catenin. These results from previous research suggested that Wnt/β-catenin signaling pathway may also interact with EpCAM in fibrotic liver.

Chapter 2. Aim and Experimental Flowchart

2.1 Motivation and Aim

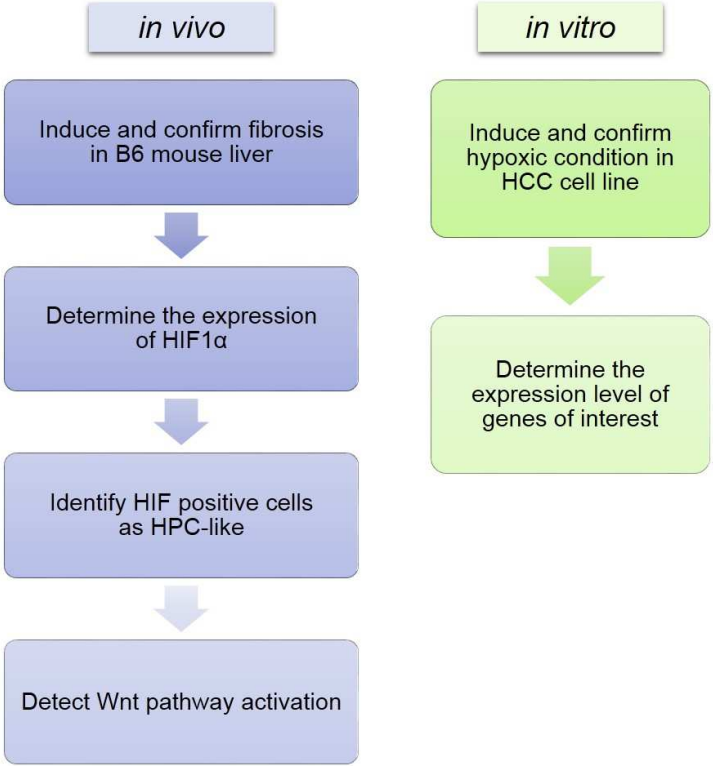
Though the roles of HIF1α, EpCAM and Wnt/β-catenin have all been reported in the cellular events during liver fibrosis, no research examines the interaction among them. And since they have all been observed to directly or indirectly interact with each other, I suspect that they may all participate together in fibrotic liver. Therefore in this study, I hypothesized that the upregulated HIF1α in hepatic fibrosis would modulate liver repair and regeneration through regulating the expression of EpCAM and Wnt/β-catenin. And I would use human HCC cell line as a pre-test for further experiment.

2.2 Flowchart

As shown in the following figure, this project was divided into two parts, an *in vivo* part and an *in vitro* one. For the *in vivo* part, first I would induce liver fibrosis in C57BL/6 mice and confirm the fibrosis induction. Then I would detect the mRNA and protein expression pattern of HIF1α in fibrotic liver. After that I identified the HIF1α positive cells as HPC-like. At last I analyzed β-catenin translocation in these HPC-like cells to determine the activation of Wnt signaling and tried to draw up to a possible mechanism.

As for the *in vitro* part, I tested the gene expression pattern involved in my *in*

vivo data in human HCC cell line to see if they were regulated by hypoxic culture. This part would serve as a pre-test for future experiment, which would be to isolate HPC from mice embryo and culture them under hypoxia to examine the effect of oxygen concentration during liver regeneration.



Chapter 3. Methodology

3.1. Animal and CCl_4 model

Six-week-old C57BL/6 mice (BioLASCO) were intraperitoneally injected with 0.5 μL/g mouse CCl_4 (Alps Pharmaceutical Industry, 1/10 diluted in corn oil, WAKO) twice a week for six weeks to induce liver fibrosis (Taura et al., 2010). Five mice were sacrificed every two weeks one day after the last injection and the livers were removed. The biggest lobe of mice liver were removed from the organ and equally dissected into three parts, one for total RNA extraction and the other two for cryosection and further analysis.

3.2. Whole liver RNA extraction

Liver tissue was homogenized in 3 mL Tripure (Roche) to extract total RNA. Homogenized tissue was centrifuged at 12000g in 4℃ for 15 minutes to remove the pellet. 200 μL/mL Tripure chloroform (MP Biomedical) was added into the supernatant then the mixture was centrifuged at 12000g in 4℃ for 15 minutes. The upper aqueous phase was transferred into a new microcentrifuge tube before 500 μL/mL Tripure isopropanol (Wako) was added and centrifuged at 12000g in 4 ℃ for 15 minutes to precipitate the RNA. Precipitated RNA was washed twice with 70% ethanol before air-dried and dissolved in 50 μL DEPC/ddH_2O. The quality of RNA was determined by Nanodrop 2000 (Thermo) and electrophoresis

before reverse transcribed into cDNA.

3.3. Hematoxylin and eosin (H&E) staining

Liver tissue were fixed in 10% formalin (Sigma Aldrich, diluted in PBS [137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4]) for 10 minutes, embedded in paraffin and dissected. The sections were then deparaffinized and rehydrated before stained with hematoxylin. After washed by distilled water, the samples were immersed in eosin solution, washed again, dehydrated, cleared with xylene and mounted for image analysis.

3.4. Masson's Trichrome staining

Liver tissue were fixed in 10% formalin for 10 minutes, embedded in paraffin and dissected. The sections were then deparaffinized and rehydrated before stained with Weigert's hematoxylin solution. After washed by distilled water, the samples were immersed in Biebrich scarlet-acid fuchsin solution, washed again, and immersed in phosphomolybdic-phosphotungstic acid solution to remove the red color. After washed, aniline blue solution was used to stain collagen in the tissue. Finally, the sections were decolorized in 1% acetic acid, washed, dehydrated, cleared with xylene and mounted for image analysis.

3.5. Liver immunofluorescence staining

Liver tissue was fixed in OCT (Sakura Finetek) and cryosectioned into 8 μm

thick sample using Leica CM 1900 (Leica). The cryostats were then fixed with 5% paraformaldehyde (Sigma Aldrich) for 15 minutes before permeabilized with 0.1% Triton X100 (Riedel-de Haën) for 5 minutes. 4% fetal bovine serum (FBS, diluted in PBS) were used as blocking buffer. After blocking for an hour in room temperature, the samples were incubated in primary antibody solution overnight in 4℃. Then the samples were incubated in secondary antibody at room temperature for an hour, stained with Hoechst 33342 for 10 minutes and mounted before analyzed by IN Cell Analyzer (GE Healthcare). PBS served as wash buffer between each staining process. The antibodies used in this study and its dilution condition were shown in **Table 1**.

3.6. Laser microdissection (LMD) and RNA isolation

Liver tissue was fixed in OCT and cryosectioned into 8μm thick sample using Leica CM 1900 (Leica). The samples were then placed onto PET slide (Leica) for laser microdissection (LMD) using Leica LMD 7000 (Leica). Around 50mm^2 of selected region were cut for 1 preparation of RNA. The RNA was isolated using High Pure FFPET RNA Isolation Kit (Roche). First the tissue pellet were digested in 100 μL RNA Tissue Lysis Buffer, 16 μL 10% SDS, and 40 μL Proteinase K for 30 minutes at 85℃, and further digested in 80 μL Proteinase K at 55℃ for another 30 minutes. Then 325 μL RNA Binding Buffer and 325 μL 100% EtOH

were added into the clear tissue lysate before the lysate were transferred onto the High Pure Collection Tube and centrifuged for 30 seconds at 6000g. The High Pure Collection Tube was centrifuged again for 2 minutes at 14000g to dry the filter. 100 μL DNase was added into the High Pure Collection Tube and incubated for 15 minutes at room temperature before 500 μL Wash Buffer I was added into the Collection Tube and centrifuged for 20 seconds at 6000g. Then the High Pure Collection Tube was washed twice by adding 500 μL Wash Buffer II and centrifuge for 20 seconds at 6000g. Finally the High Pure Collection Tube was dried by centrifuged at 14000g for 2 minutes before 25 μL RNA Elution Buffer were added and centrifuged at 6000g for 1 minutes. The quality of RNA was determined by Nanodrop 2000 before reverse transcribed into cDNA.

3.7. Cell culture

Huh7 HCC cell line were cultured in Dulbecco's modified Eagle's medium (DMEM, Life Technologies) supplemented with 10% FBS (GE Healthcare) and 1% penicillin/streptomycin/glutamine (PSG, Life Technologies) and maintained at 37℃ in a humidified incubator with 5% CO_2. Hypoxic culture were conducted with same culture medium in incubator with 5% CO_2 and 1% O_2.

3.8. Cellular RNA extraction

The cells were scraped using Tripure by tips after the removal of culture

medium and washed once by PBS. 200 μL/mL Tripure chloroform was added into the supernatant then the mixture was centrifuged at 12000g in 4℃ for 15 minutes. The upper aqueous phase was transferred into a new microcentrifuge tube before 500 μL/mL Tripure isopropanol was added and centrifuged at 12000g in 4℃ for 15 minutes to precipitate the RNA. Precipitated RNA was washed twice with 70% ethanol before air-dried and dissolved in 50 μL DEPC/ddH_2O. The quality of RNA was determined by Nanodrop 2000 and electrophoresis before reverse transcribed into cDNA.

3.9. Cellular protein extraction

The cells were scraped using RIPA buffer (50 mM Tris-HCl, 1% NP-40, 0.5% Na-deoxycholate, 0.1% SDS, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, pH7.4) supplemented with cOmplete Cock Tail Protease Inhibitor (Roche) by tips after the removal of culture medium and washed by PBS. The total cell lysate were then centrifuged at 12000g in 4℃ for 10 minutes to remove the pellet. The concentration of total cell protein were determined by Bradford method (Bio-Rad Protein Assay) in ELISA reader (Thermo Multiskan FC).

3.10. Quantitative reverse transcription PCR (qRTPCR)

2 μg or 1 μg (LMD sample) total RNA was first reverse transcribed into cDNA using High-Capacity cDNA Reverse Transcription Kit (Applied biosystem)

according to the manual using the following protocol: 25℃/10min→37℃/ 120min→85℃/10min→4℃. qPCR was performed using iQ SYBR green detection system in Bio-Rad CFX96 (Bio-Rad) using the following protocol: 95℃ /2min the℃/ 5sec→65℃/30sec]X39 cycles→cy℃/ 5sec→65℃/5secc→℃. The expression level of target genes was normalized to β-Actin and 18S rRNA (for Huh7 sample) or β-Actin and glyceraldehyde-3-phosphate dehydrogenase (GAP, for LMD sample) expression. The primers used for qPCR were shown in **Table 2**.

3.11. Western blotting

10 μg protein samples were mixed with 5x sample buffer (250 mM Tris-HCl, 10% SDS, 0.5% (w/v) bromophenol blue, 50% glycerol, 5% β-mercaptoethanol, pH6.8) and placed on dry bath (Major Science Dry Bath Incubator) under 100℃ for 10 minutes. SDS-polyacrylamide gel (10% separation gel and 4% stacking gel) and TGS buffer system (50 mM Tris-HCl, 380 mM Glycine, 0.1% SDS, pH8.3) were used to separate total proteins. Electrophoresis were performed according to the following protocol: 100V for 20 minutes and 150V for 1.5 hour. Then the proteins were transferred onto pre-rinsed PVDF membrane (Roche) in western transfer buffer (25 mM Tris-HCl, 192 mM Glycine, pH 8.3) with Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad) for 1 hour. The current used for transfer was 1.5 mA/cm^2. The membrane was washed with TBST (10 mM

Tris-HCl, 150 mM NaCl, 0.1% Tween-20, pH8.0) at least twice between each step. 4% skim milk (diluted in TBST) was used for blocking process, and the time duration was an hour at room temperature. Primary antibody solution (diluted in gelatin-NET [0.25% gelatin, 0.15 M NaCl, 5 mM EDTA, 0.05% Tween-20, 50 mM Tris, pH8.0] or 4% skim milk) were added after blocking and following wash and incubated in 4℃ overnight. Then the membrane was washed thrice before replaced into second antibody solution and incubate for 1 hour. At last the membrane was washed twice with TBST and once with ddH_2O before rinsed in HRP substrate (WBKLS0500, Millipore) for 1 minutes and analyzed by BioSpectrum 2D Imaging System (UVP BioSpectrum 800). The antibodies used in this study and its dilution condition were shown in **Table 1**.

3.12. Flow cytometry

For surface marker analysis, cells were harvested with Trypsin solution (Life Technologies), recovered and washed in culture medium before shaked in FcR blocking solution (Miltenyi Biotec) for 20 minutes at 4℃. Primary conjugated antibody (or isotype control) were added into each samples and shaked for an hour at 4℃. Cells were then stained with propidium iodide (PI, Life Technologies) for 10 minutes before washed with PBS and resuspended with 3% FBS (diluted in PBS). Cell analyzer (BD FACSCanto II) was used to analyze the expression of

markers. The antibodies used in this study and its dilution condition were shown in **Table 1**.

For intracellular antigen analysis, cells were first harvested with Trypsin solution (Life Technologies), fixed with 4% paraformaldehyde for 15 minutes and permeabilized with 0.5% Tween-20 (Shimakyu's pure chemical) for another 15 minutes. Then the cells were blocked with 4% FBS for an hour, incubated in primary antibody solution for an hour, and finally mixed with secondary antibody solution for another hour. After stained with PI for 10 minutes and resuspended with PBS, cells were analyzed using cell analyzer. The antibodies used in this study and its dilution condition were shown in **Table 1**.

3.13. Cell immunofluorescence staining

After the removal of culture medium, Huh7 were first fixed with 5% paraformaldehyde for 7 minutes then permeabilized with 0.1% Triton X100 for 15 minutes. 4% FBS (diluted in PBS) were used as blocking buffer. After blocking for an hour in room temperature, the samples were incubated in primary antibody solution overnight in 4℃. Then the samples were incubated in secondary antibody at room temperature for an hour, stained with Hoechst 33342 for 10 minutes before analyzed by IN Cell Analyzer (GE Healthcare). The cells were washed by PBS between each staining steps. The antibodies used in this study and its dilution

condition were shown in **Table 1**.

3.14. Image processing

The histochemical staining images were taken by Dr. Hsiao-Mei Chao of Taipei Medical University Hospital. All immunofluorescence images were first obtained by IN Cell Analyzer (GE Healthcare), and the local background were subtracted before merging channels and cropped by ImageJ. Results of flow cytometry were analyzed using FlowJo software and displayed in overlayed histogram.

3.15. Statistics

qRTPCR data in the bar charts represent means ± SEM and were obtained from average data of three independent experiments. Statistical significance was calculated using a two-tailed Student's *t*-test. Differences with a *P* value of less than 0.05 were considered significant, and a *P* value of less than 0.01 were considered really significant.

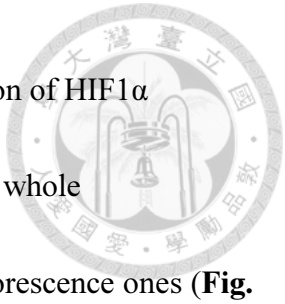
Chapter 4. Results

4.1 CCl_4 induced fibrosis in C57BL/6 mice liver

CCl_4 model was used to induce fibrosis in C57BL/6 mice liver. H&E staining of livers showed degenerated and ballooned hepatocytes around portal vein in CCl_4-treated livers. Infiltration of immune cells could also be observed in the injured area (**Fig. 1**). The results of Masson's trichrome staining further confirmed that CCl_4 treatment induced fibrosis in C57BL/6 mice liver, indicated by the blue fibrosis septa in the periportal area (**Fig. 2**). According to the Masson's trichrome staining results, livers from six weeks of CCl_4 treatment were considered to be more fibrotic than the others, so only the results from six weeks CCl_4-treated livers would be shown in the following data. Besides histochemical staining, I also homogenized the liver to obtain total mRNA and performed qRTPCR. The expression of fibrosis-related genes, α smooth muscle actin (αSMA) and collagen type 1 α1 (Col1a1), were upregulated in fibrotic liver (**Fig. 3**). These results showed that CCl_4 induction was successful.

4.2 Upregulated HIF1α in fibrotic liver

In order to determine the role of hypoxia or HIF1 regulating liver regeneration in fibrotic liver, first I performed immunofluorescence staining to investigate the



expression pattern of HIF1α. As shown in **Fig. 4**, the expression of HIF1α increased in CCl_4-treated liver. But the qRTPCR results of the whole liver-digested sample could not correspond to the immunofluorescence ones (**Fig. 5**). So I conducted laser microdissection (LMD) from the cryosectioned sample, isolated the mRNA and repeated qRTPCR. The expression levels of αSMA, Col1a1, and tissue inhibitor of metalloproteinase 2 (Timp2, another fibrosis-related gene) were all upregulated, as well as HIF1α mRNA (**Fig. 6**).

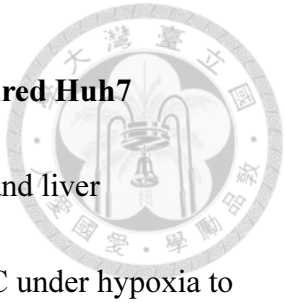
4.3 Identification of HIF1α positive cells as HPC-like cells

To identify these HIF1α expressing cells, immunofluorescence staining were performed to stain the cell specific marker. According to previous studies, there are three populations that would increase during liver fibrosis, HSC, HPC and immune cell. Since HIF1α have been reported to participate in the regulation of HPC (Cannito et al., 2014), and that this regulation have not been observed in fibrotic liver, I would like to know if these HIF1α expressing cells were HPC. **Fig. 7A** showed that all the cells expressed Alb, marker of the hepatocyte lineage in HPC. The expression of EpCAM, a surface marker expressed only by HPC rather than hepatocyte nor cholangiocyte, increased in fibrotic liver, and were colocalized with HIF1α (**Fig. 7B**). Only the cells surrounding vascular expressed

CK19, marker of the cholangiocyte lineage, and colocalization of HIF1α and CK19, and EpCAM and CK19, could only be observed in fibrotic liver (**Fig. 7C, D**). Data of qRTPCR from LMD sample showed increased EpCAM and CK19 expression in CCl_4-treated liver (**Fig. 8**). These results suggested that the HIF1α positive cells were HPC-like.

4.4 Upregulated Wnt in HPC-like cell

Previous researches have shown that hypoxia/HIF would regulate Wnt signaling in stem cells and HCC, and canonical Wnt signaling have been reported to direct differentiation fate in HPC. So I wished to investigate Wnt signaling in these HPC-like cells and determined the relationship between HIF1α, EpCAM and Wnt. Immunofluorescence staining results showed translocation of β-catenin after CCl_4 treatment, indicating the activation of Wnt signaling (**Fig. 9**). Colocalization of β-catenin with EpCAM (**Fig. 9A**), β-catenin with HIF1α (**Fig. 9B**) and β-catenin with CK19 (**Fig. 9C**) could also be observed in fibrotic liver. LMD followed by qRTPCR also suggested Wnt signaling were activated in the injured area in fibrotic liver (**Fig. 10**).



4.5 Upregulated EpCAM, Wnt and CK19 under hypoxic cultured Huh7

To further investigate the role of hypoxia or HIF1 in HPC and liver regeneration, I planned to culture primary hepatocyte and HPC under hypoxia to examine the expression patterns of HPC markers and Wnt signaling. Before obtaining primary cells from mouse, I first used HCC cell line Huh7 as a pre-test. The hypoxic culture were confirmed by HIF1α and HIF2α protein expression (**Fig. 11**), as well as VEGFa and platelet-derived growth factor a (PDGFa) mRNA expression (**Fig. 12**). After cultured for 24 or 48 hours, the mRNA excpression level of HPC markers, EpCAM and CK19, increased significantly (**Fig. 13**). Downstream target genes of Wnt/β-catenin, Axin2 and Cyclin D1 were also upregulated under hypoxic culture (**Fig. 14**). As for protein expression level, results of flow cytometry showed that EpCAM expression were upregulated after 48 hours hypoxic culture (**Fig. 15**). Similar results were also observed in the expression of CK19 (**Fig. 16**). On the other hand, translocation of β-catenin could be seen after 24 hours of hypoxia induction (**Fig. 17**). The *in vitro* data above suggested that the observation *in vivo* could be modulated by hypoxic condition and the expression of HIF1α.

Chapter 5. Discussion and Conclusion

Chronic hepatic fibrosis is one of the risk factors for HCC, but the mechanisms of disease progression remain unclear. In this research, I have found out that during liver fibrosis, the expression of HIF1α would be upregulated, in mRNA as well as in protein level. These HIF1α^{+} cells also expressed Alb, CK19 and EpCAM, which were the markers of HPC, suggesting that these cells were HPC-like. I also investigated the activation condition of Wnt/β-catenin signaling in these HPC-like cells, and observed translocated β-catenin in fibrotic liver, indicating the activation of canonical Wnt signal. At last, before obtaining HPC and mature hepatocytes from mouse embryo or adult mouse, I cultured HCC cell line Huh7 under normoxic and hypoxic conditions to compare the expression levels of the genes mentioned in the *in vivo* experiment. The mRNA expression of HPC markers, EpCAM and CK19, and Wnt/β-catenin downstream targets, Axin2 and CyclinD1, were all elevated. Increased EpCAM and CK19 protein expression, in addition to β-catenin translocation could also be observed in hypoxic cultured Huh7.

Hypoxia and HIF have already been proved to alter HSC activation during liver fibrosis, but the effects of low oxygen concentration on other cell types in liver are still unknown. Except for HSC, HPC would also be activated in fibrotic liver. Previous research has shown that hypoxia would increase efficiency of HPC differentiation into

hepatocytes (Si-Tayeb et al., 2010), suggesting that the differentiation fates of HPC could be directed by oxygen concentration. In addition to HPC differentiation, hypoxia may also affect HPC activation. Studies have shown that hypoxia would increase stem cell marker expression in HCC cell line, and the HPC population in liver may be contributed by the dedifferentiation of hepatocytes (Chen et al., 2012a; Mathieu et al., 2011). In my study, I detected upregulated HIF1α in HPC-like cells in fibrotic liver and HPC marker in HCC under hypoxic culture. These results suggested that hypoxia may cause dedifferentiation of hepatocytes during liver fibrosis.

Apart from hypoxia and HIF, Wnt signaling has also been proved to mediate HPC activation in damaged liver (Apte et al., 2008). This study showed that conditional knockout of β-catenin would strongly decrease HPC population. My data also showed translocated β-catenin in fibrotic liver, marking the activation of Wnt signaling. The relationships between HIF and Wnt signaling in liver have also been reviewed (Bogaerts et al., 2014). Colocalization of HIF1α and β-catenin in my research suggested that the fates of HPC would be modulated by synergetic interaction between HIF and Wnt signaling.

So I proposed a possible mechanism based on the results of my research and the former ones. As shown in **Fig. 18**, during liver fibrosis, the accumulation of collagen causes hypoxia occurred in the periportal area, where dormant HPCs often reside.

Hypoxic condition results in the upregulation of HIF1α signaling and β-catenin stabilization, thus leading to translocation of β-catenin into the nucleus. Moreover, elevated HIF1 and Wnt/β-catenin signaling would both induce EpCAM expression. Finally, the transcription factor complex containing EpICD, β-catenin and Lef would drive the expression of Wnt downstream targets to modulate the differentiation direction of HPC. But the mechanism of how EpICD translocate into nucleus was not covered in my research.

For further experiment, I would isolate primary HPCs from both mouse embryo and adult mice and differentiate them *in vitro* to investigate the effect of hypoxia and HIF1α on the fates of HPC. I would also examine whether hypoxia and HIF1α enhance dedifferentiation of primary hepatocytes *in vitro* by isolating primary cells from adult mice. At last, I would try to use cell tracing techniques to detect the origin of the HPC-like cells in fibrotic liver in my experiment.

In summary, I have detected elevated expression of HIF1α in mouse fibrotic liver, and identified these HIF1α positive cells as HPC-like. I also observed activated Wnt/β-catenin signaling in these HPC-like cells. At last, I found out that the HPC cell markers and Wnt/β-catenin signaling would be upregulated in hypoxic cultured HCC cell line, suggesting that the observations *in vivo* were modulated by upregulated HIF1α signal.

Chapter 6. Figures and Tables

Table 1. List of antibodies

Antibody	Manufacturer	Catalog Number	Application	Dilution Fold
Primary Antibodies				
Anti-Actin	Santa Cruz Biotechnology	Sc-1616	WB*	1/5000 (in gelatin-NET)
Anti-Alb	Bethyl Laboratories	A90-234A	IF†	1/300
Anti-β-catenin	Abcam	Ab-2365	IF	1/300
Anti-β-catenin	Santa Cruz Biotechnology	Sc-1496	IF	1/100
Anti-CK19	Santa Cruz Biotechnology	Sc-33119	IF	1/100
Anti-CK19	Home made		FC‡	1/250
Anti-EpCAM	BD Bioscience	552370	IF	1/300
Anti-EpCAM	Biolegend	324203	FC	1/250
Anti-HIF1α	Novus Biologicals	NB-100-105	WB	1/1000 (in 4% skim milk)
			IF	1/300
Anti-HIF1α	Santa Cruz Biotechnology	Sc-10790	IF	1/100
Anti-HIF2α	Novus Biologicals	NB-100-122	WB	1/1000 (in 4% skim milk)
Isotype Ctrl				
Mouse IgG	eBioscience	11-4714	FC	1/250
Rabbit IgG			FC	1/250

*WB: Western blotting ; †IF: immunofluorescence staining ; ‡FC: flow cytometry

Table 1. List of antibodies (continues)

Antibody	Manufacturer	Catalog Number	Application	Dilution Fold
Secondary Antibodies				
Anti-goat AF488[#]	Life Technologies	A11055	IF	1/1000
Anti-goat AF647	Life Technologies	A21447	IF	1/1000
Anti-goat HRP	Bethyl Laboratories	A50-100P	WB	1/10000
Anti-mouse HRP	Jackson ImmunoResearch	115-035-003	WB	1/10000
Anti-rabbit AF488	Life Technologies	A21206	IF	1/1000
Anti-rabbit AF647	Life Technologies	A21443	IF	1/1000
Anti-rabbit HRP	Jackson ImmunoResearch	111-035-003	WB	1/10000
Anti-rat AF488	Life Technologies	A21208	IF	1/1000

[#]AF: Alexa Fluoro

Table 2. List of qRTPCR primers

Gene	Primers (5(ime)	
Mouse		
GAP	Forward	AGGTCGGTGTGAACGGATTTG
	Reverse	TGTAGACCATGTAGTTGAGGTCA
β-Actin	Forward	GGCTGTATTCCCCTCCATCG
	Reverse	CCAGTTGGTAACAATGCCATGT
α-SMA	Forward	GTCCCAGACATCAGGGAGTAA
	Reverse	TCGGATACTTCAGCGTCAGGA
Col1a1	Forward	GCTCCTCTTAGGGGCCACT
	Reverse	CCACGTCTCACCATTGGGG
EpCAM	Forward	GCGGCTCAGAGAGACTGTG
	Reverse	CCAAGCATTTAGACGCCAGTTT
HIF1α	Forward	ACCTTCATCGGAAACTCCAAAG
	Reverse	CTGTTAGGCTGGGAAAAGTTAGG
Timp2	Forward	TCAGAGCCAAAGCAGTGAGC
	Reverse	GCCGTGTAGATAAACTCGATGTC
Albumin	Forward	GTTGAGGACCAGGAAGTGTG
	Reverse	GTCTCAGCAACAGGGATACAG
CK19	Forward	GGGGGTTCAGTACGCATTGG
	Reverse	GAGGACGAGGTCACGAAGC
Axin2	Forward	TGACTCTCCTTCCAGATCCCA
	Reverse	TGCCCACACTAGGCTGACA
Cyclin D1	Forward	GCGTACCCTGACACCAATCTC
	Reverse	CTCCTCTTCGCACTTCTGCTC

Table 2. List of qRTPCR primers (continues)

Gene	Primers (5rime)	
Human		
β-Actin	Forward	CATGTACGTTGCTATCCAGGC
	Reverse	CTCCTTAATGTCACGCACGAT
18S rRNA	Forward	GGAGTATGGTTGCAAAGCTGA
	Reverse	ATCTGTCAATCCTGTCCGTGT
VEGFa	Forward	AGGGCAGAATCATCACGAAGT
	Reverse	AGGGTCTCGATTGGATGGCA
PDGFa	Forward	GCAAGACCAGGACGGTCATTT
	Reverse	GCAAGACCAGGACGGTCATTT
EpCAM	Forward	ATAATCGTCAATGCCAGTGTA
	Reverse	TTTGCTCTTCTCCCAAGTTT
CK19	Forward	AACGGCGAGCTAGAGGTGA
	Reverse	GGATGGTCGTGTAGTAGTGGC
Axin2	Forward	CT TAAAGGTCTTGAGGGTTGAC
	Reverse	CAACAGATCATCCCATCCAACA
Cyclin D1	Forward	CAATGACCCCGCACGATTTC
	Reverse	CATGGAGGGCGGATTGGAA

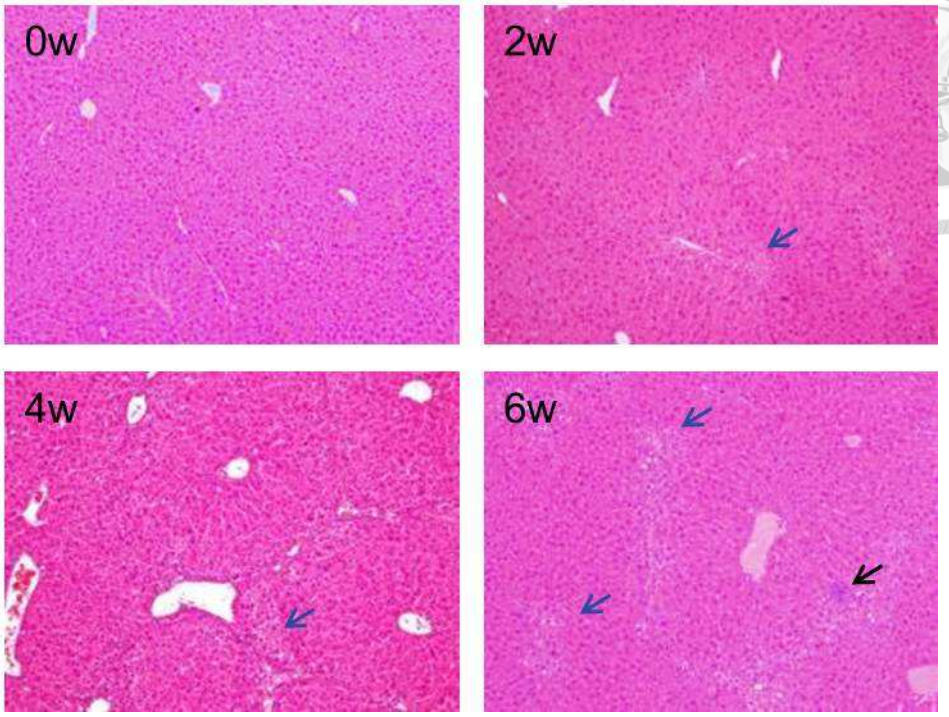


Figure 1. H&E staining of mice liver

H&E staining of mice underwent 0, 2, 4, 6 weeks of CCl_4 injection. Blue arrows represented degenerated and ballooned hepatocytes. Black arrow indicated immune cell infiltration.

Magnification: 100×

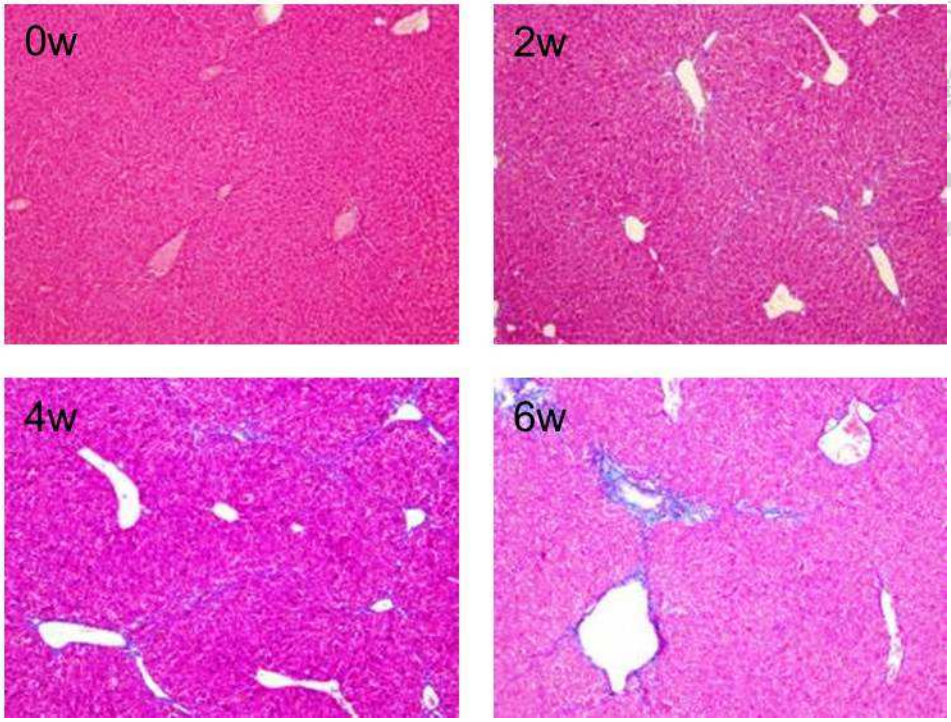


Figure 2. Masson's trichrome staining of mice liver

Masson's trichrome stain of mice underwent 0, 2, 4, 6 weeks of CCl_4 injection.

Collagen fibers were stained blue.

Magnification: 100×

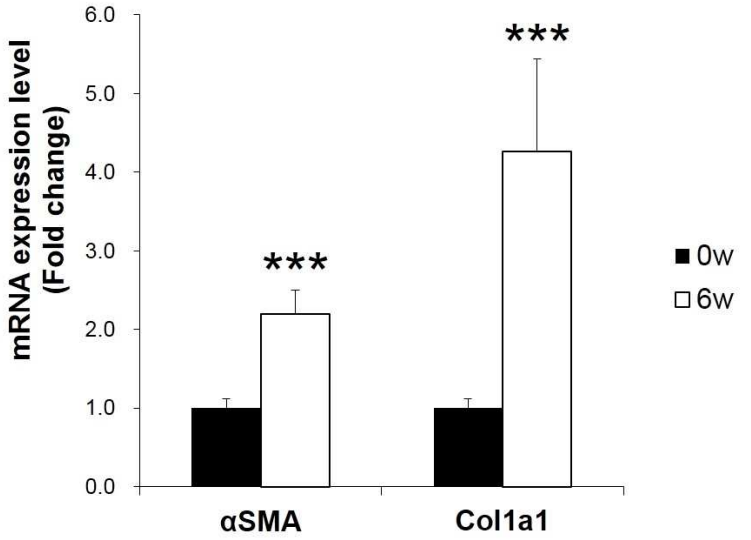


Figure 3. Fibrotic marker mRNA expression level in whole mice liver

qRTPCR analysis of fibrotic marker (αSMA and Col1a1) of RNA isolated from whole mice liver with or without 6 weeks of CCl_4 injection. Results were normalized to the expression of control (0w) groups using GAP and β-actin as reference genes. ***p<0.01; Student's t-test.

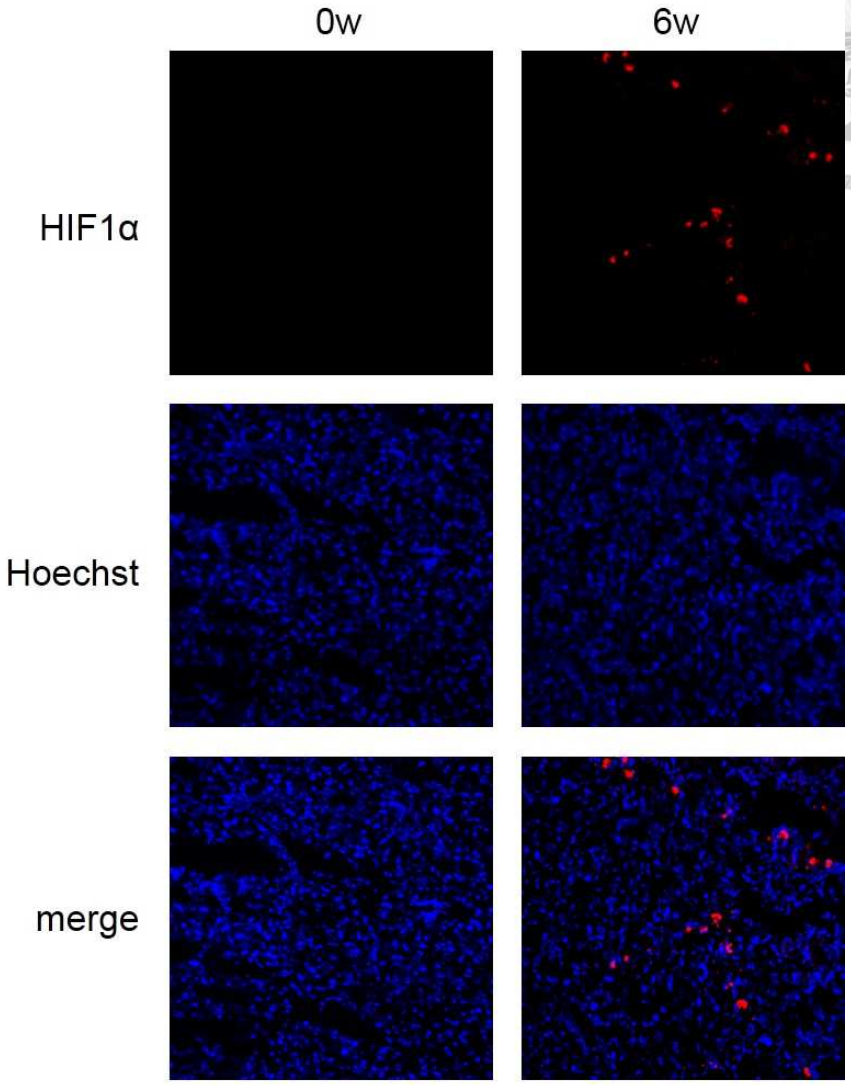


Figure 4. Immunofluorescence staining of HIF1α in mice liver

8μm thick cryostats from non- and 6 week-CCl_4-treated livers were stained with HIF1α antibodies. Hoechst represented Hoechst 33342. Magnification: 200×

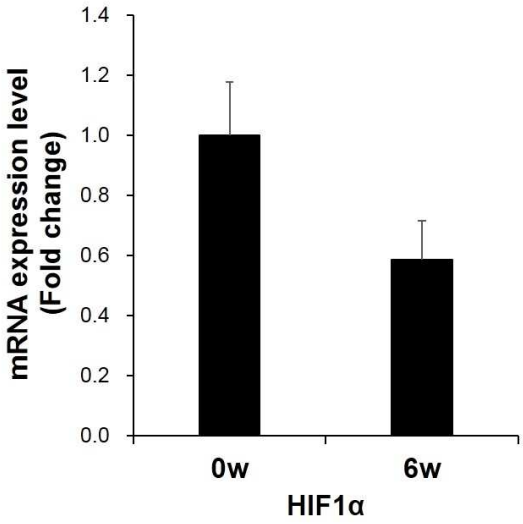


Figure 5. mRNA expression level of HIF1α in whole mice liver

qRTPCR analysis of HIF1α of mRNA isolated from whole mice liver with or without 6 weeks of CCl_4 injection. Results were normalized to the expression of control (0w) groups using GAP and β-actin as reference genes.

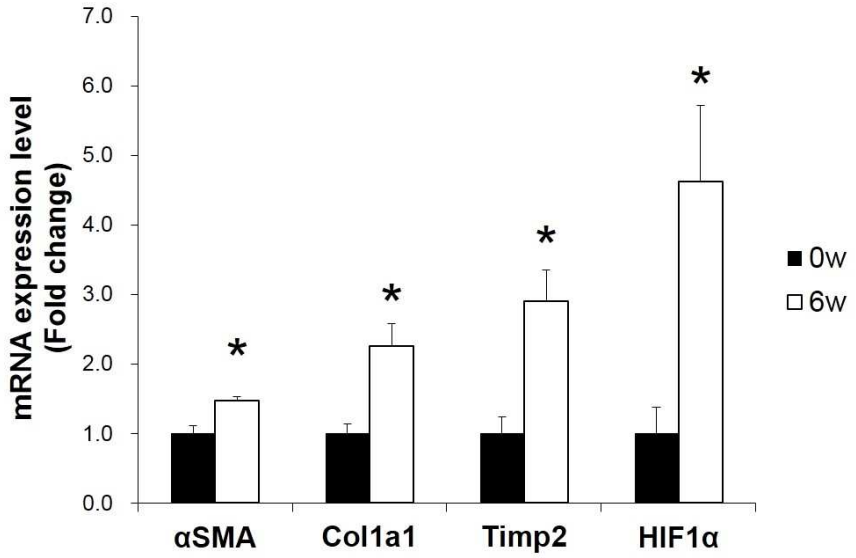


Figure 6. Fibrotic markers and HIF1α mRNA expression level in LMD liver

qRTPCR analysis of RNA isolated from LMD liver with or without 6 weeks of CCl_4 injection. αSMA, Col1a1 and Timp2 represented fibrotic marker. Results were normalized to the expression of Ctrl groups using GAP and β-actin as reference genes. *p<0.05; Student's t-test.

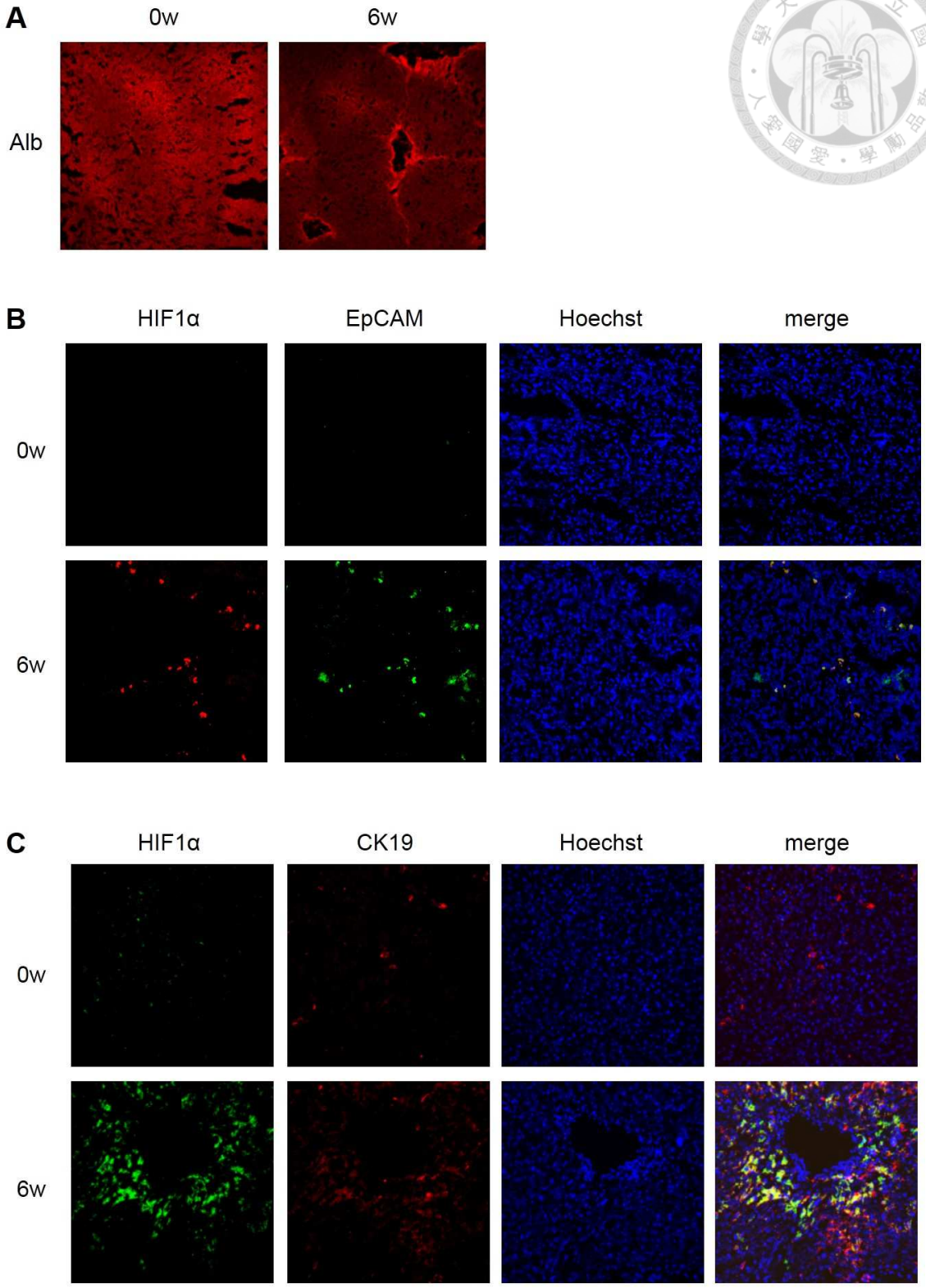


Figure 7. Immunofluorescence staining of HIF1α and HPC markers

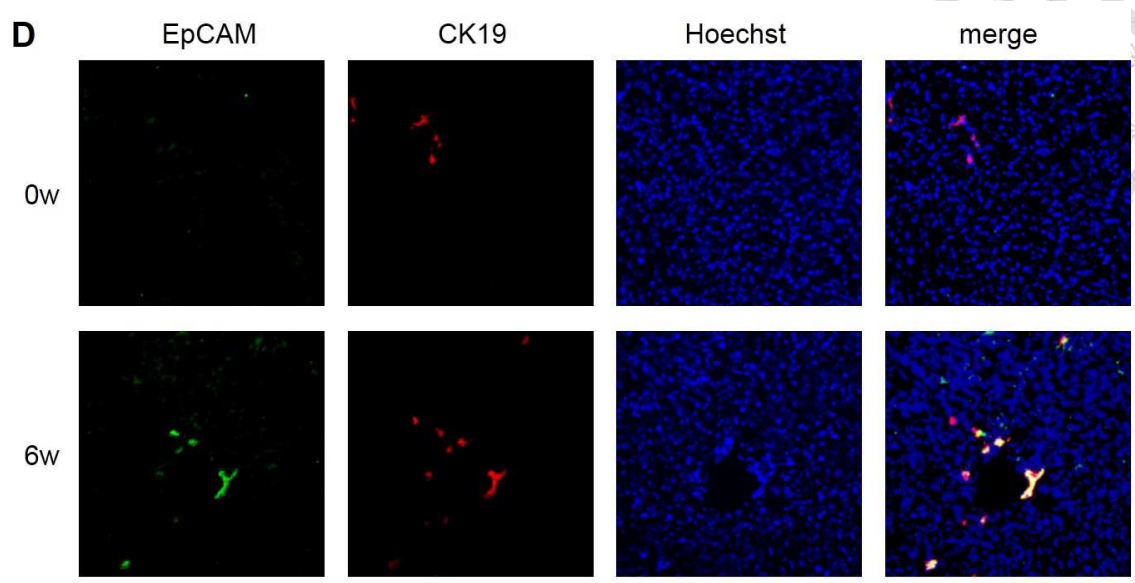


Figure 7. Immunofluorescence staining of HIF1α and HPC markers (continue)

(A) 8μm thick cryostats from non- and 6 week-CCl_4-treated liver were stained with Alb antibody.

(B) 8μm thick cryostats from non- and 6 week-CCl_4-treated liver were stained with HIF1α and EpCAM antibodies.

(C) 8μm thick cryostats from non- and 6 week-CCl_4-treated liver were stained with HIF1α and CK19 antibodies.

(D) 8μm thick cryostats from non- and 6 week-CCl_4-treated liver were stained with EpCAM and CK19 antibodies.

Hoechst represented Hoechst 33342. Magnification: 200×

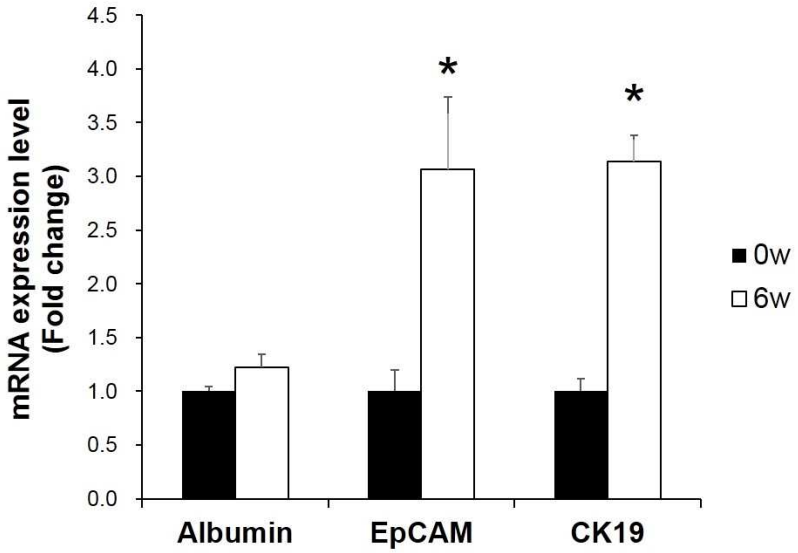


Figure 8. mRNA expression level of HPC markers in LMD liver

qRTPCR analysis of RNA isolated from LMD liver with or without 6 weeks of CCl_4 injection. Results were normalized to the expression of Ctrl groups using GAP and β-actin as reference genes. *p<0.05; Student's t-test.

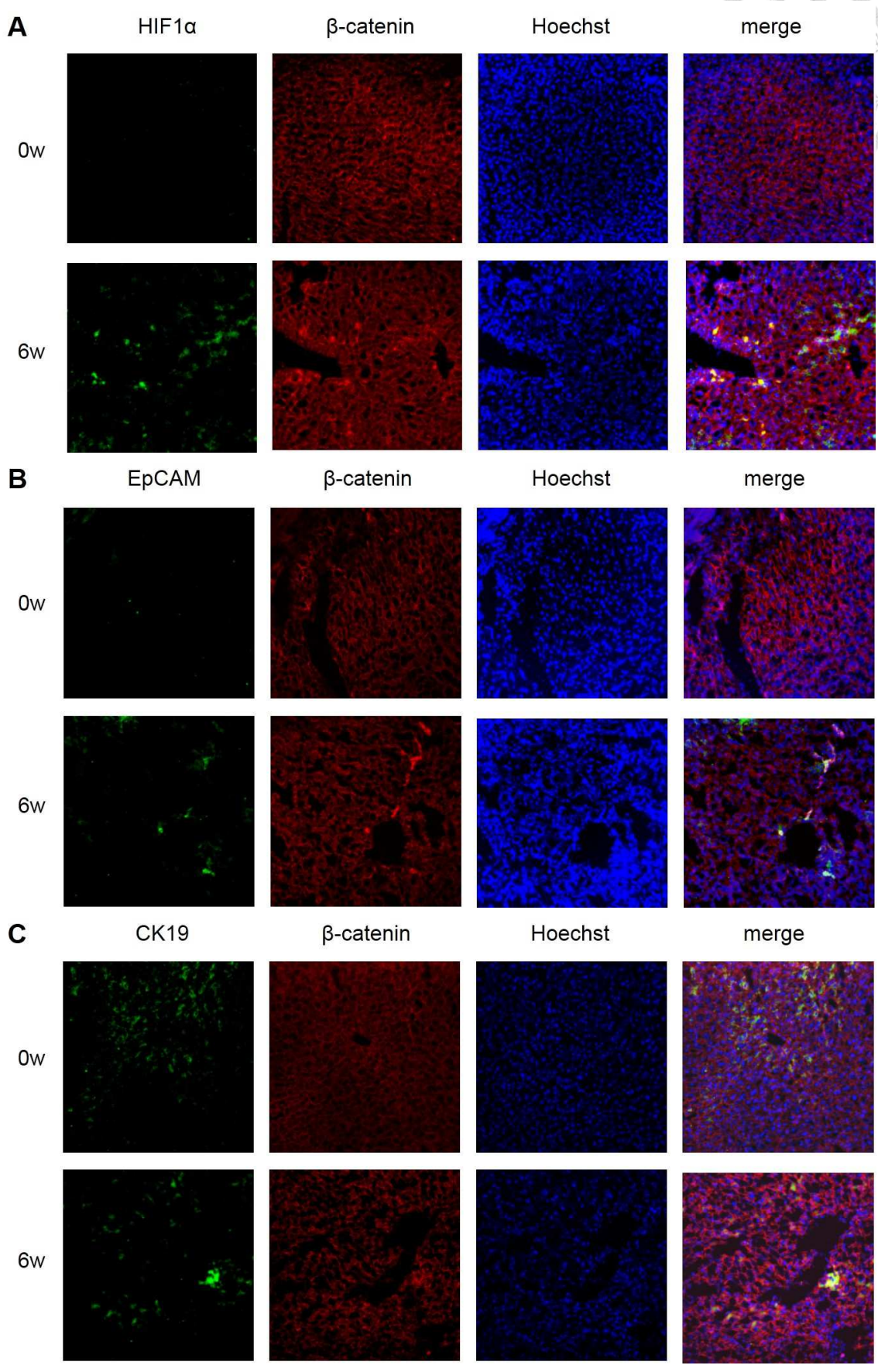


Figure 9. Immunofluorescence staining of Wnt/β-catenin signaling activation

(A) 8μm thick cryostats from non- and 6 week-CCl_4-treated liver were stained with HIF1α and β-catenin antibody.

(B) 8μm thick cryostats from non- and 6 week-CCl_4-treated liver were stained with EpCAM and β-catenin antibodies.

(C) 8μm thick cryostats from non- and 6 week-CCl_4-treated liver were stained with CK19 and β-catenin antibodies.

Hoechst represented Hoechst 33342. Magnification: 200×

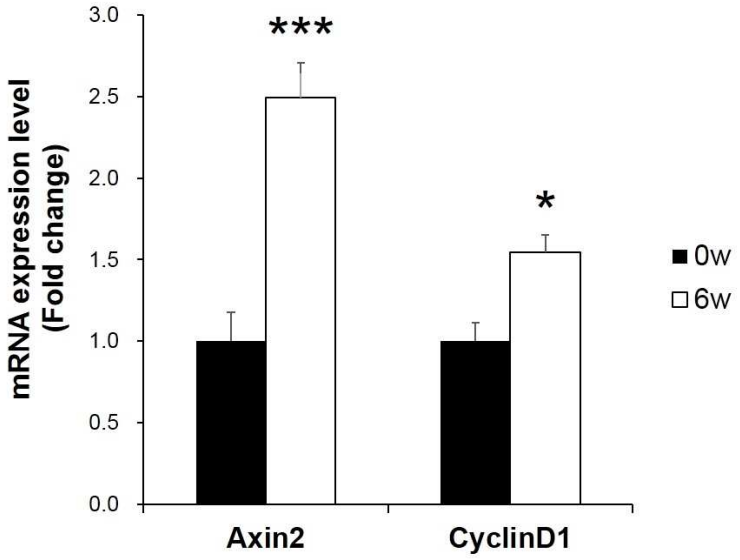


Figure 10. mRNA expression level of Wnt downstream targets in LMD liver

qRTPCR analysis of RNA isolated from LMD liver with or without 6 weeks of CCl_4 injection. Results were normalized to the expression of Ctrl groups using GAP and β-actin as reference genes. *p<0.05; ***p<0.01; Student's t-test.

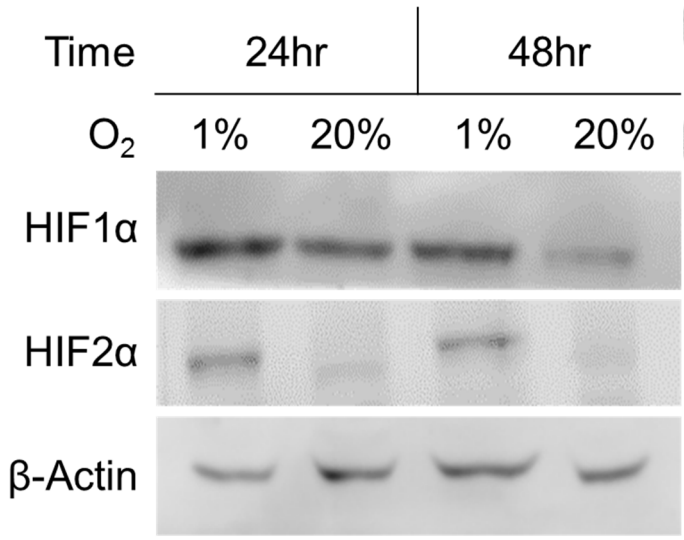


Figure 11. Confirmation of Huh7 hypoxic culture by HIF1α and HIF2α proteins

Western analysis of HIF1α and HIF2α of Huh7 cultured under normoxia or hypoxia for 24 or 48 hours. β-actin served as loading control.

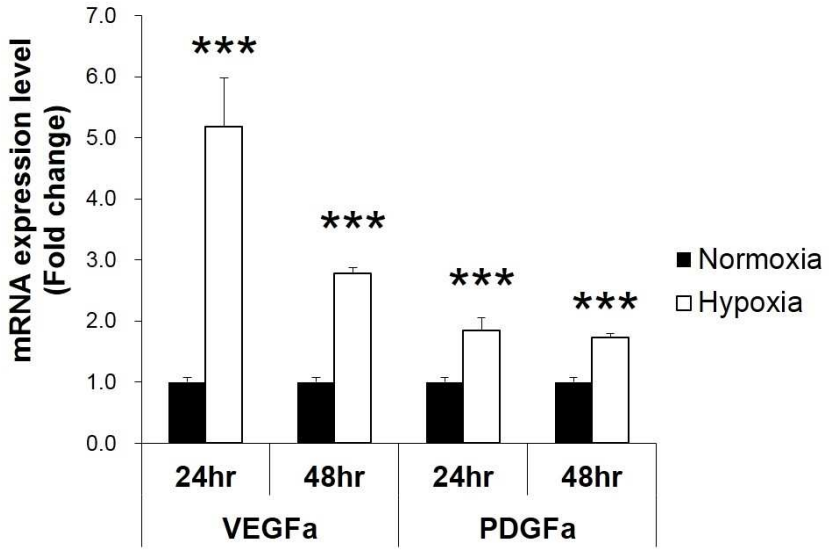


Figure 12. Confirmation of Huh7 hypoxic culture by HIFs downstream targets

qRTPCR analysis of RNA isolated from normoxic or hypoxic cultured Huh7. Duration of cultures were shown in the figure. Results were normalized to the expression of the normoxic groups of each time points using β-actin and 18S rRNA as reference genes. ***p<0.01; Student's t-test.

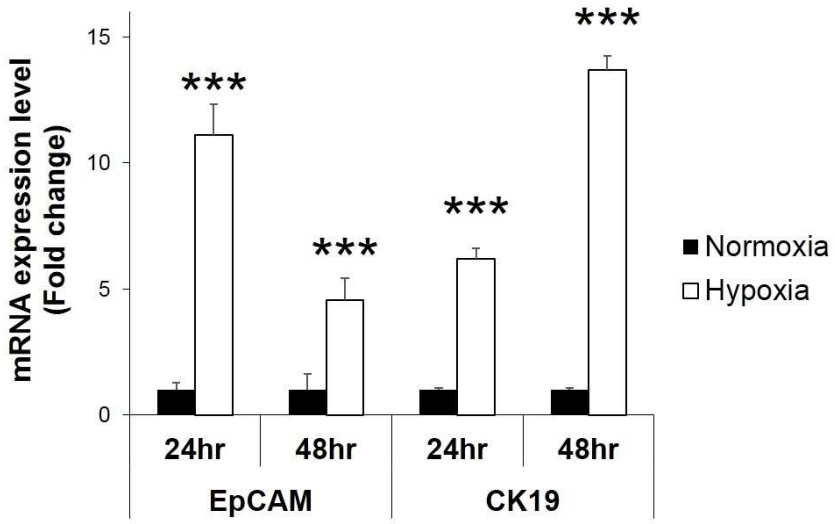


Figure 13. mRNA expression level of HPC markers in Huh7

qRTPCR analysis of RNA isolated from normoxic or hypoxic cultured Huh7. Duration of cultures were shown in the figure. Results were normalized to the expression of the normoxic groups of each time points using β-actin and 18S rRNA as reference genes. ***p<0.01; Student's t-test.

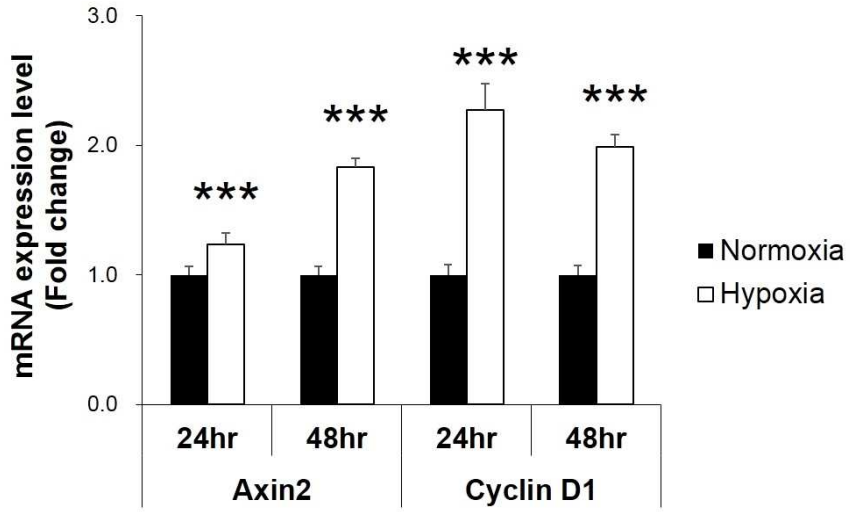


Figure 14. mRNA expression level of Wnt downstream target genes in Huh7

qRTPCR analysis of RNA isolated from normoxic or hypoxic cultured Huh7. Duration of cultures were shown in the figure. Results were normalized to the expression of the normoxic groups of each time points using β-actin and 18S rRNA as reference genes. ***p<0.01; Student's t-test.

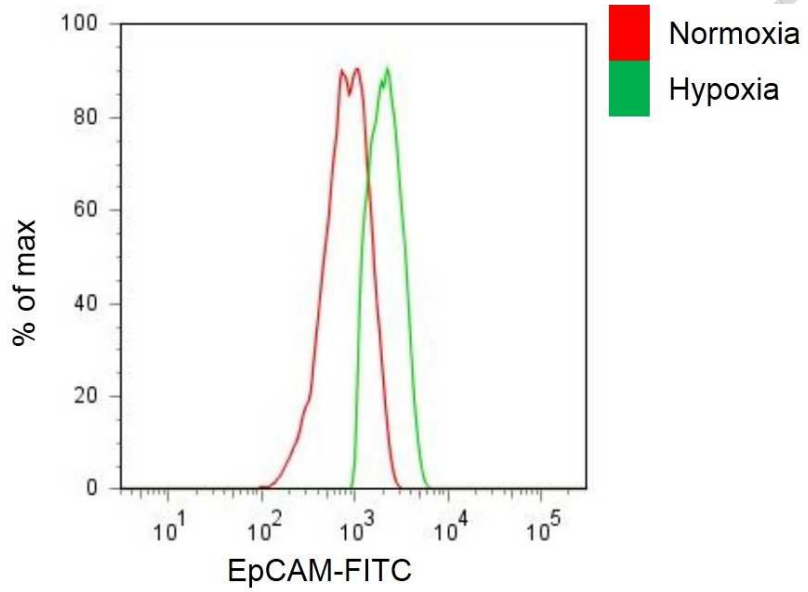


Figure 15. EpCAM protein expression in Huh7

Flow cytometry assay of EpCAM in Huh7 cultured under normoxia or hypoxia for 48 hours. Data were analyzed using FlowJo software and histogram overlays were displayed as %Max, scaling each curve to mode = 100%.

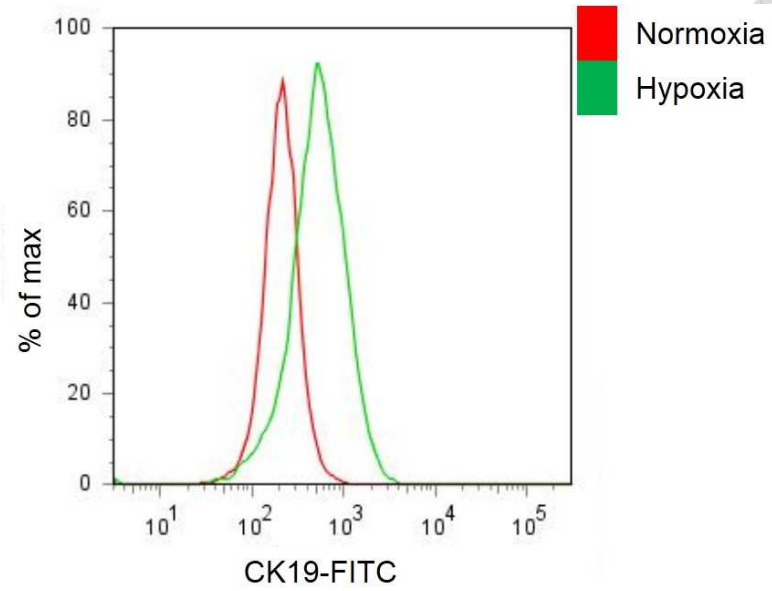


Figure 16. CK19 protein expression in Huh7

Flow cytometry assay of CK19 in Huh7 cultured under normoxia or hypoxia for 48 hours. Data were analyzed using FlowJo software and histogram overlays were displayed as %Max, scaling each curve to mode = 100%.

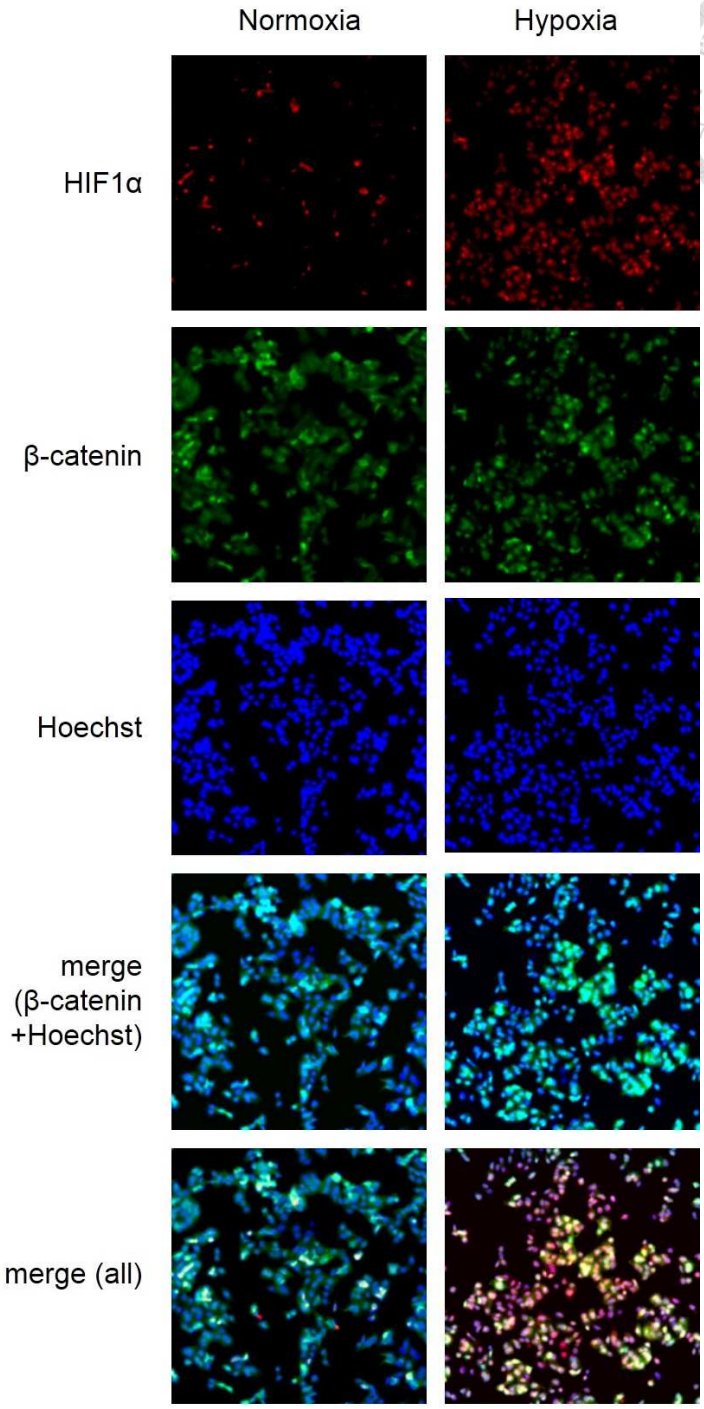


Figure 17. Immunofluorescence staining of β-catenin in Huh7

Huh7 cultured under normoxic or hypoxic conditions for 24 hours were stained with HIF1α and β-catenin antibodies. Hoechst represented Hoechst 33342.

Magnification: 100×

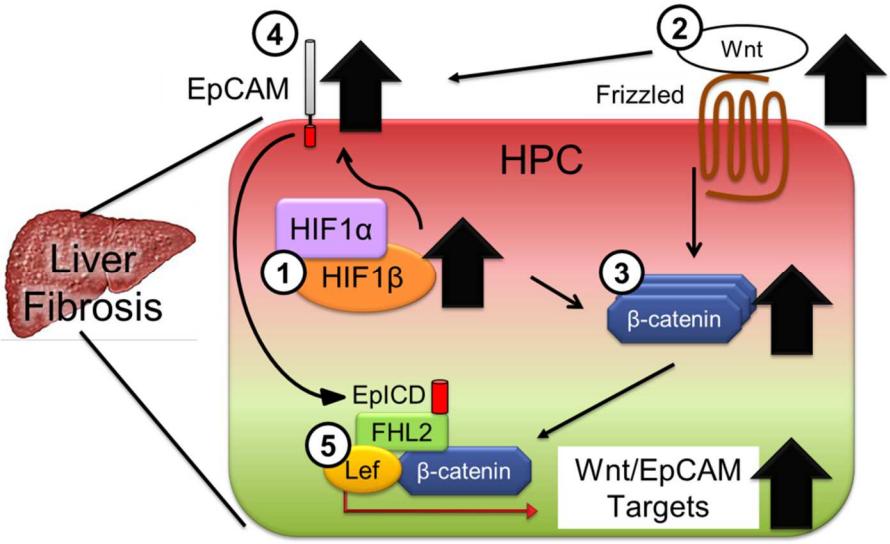


Figure 18. Possible mechanisms

During liver fibrosis, collagen accumulation would cause hypoxic environment around portal vein, where HPC resides, and induce the activation of HIF signaling (①). Wnt signaling would also be stimulated during hepatic fibrosis (②). The activation of both HIF and Wnt signaling would cause stabilize β-catenin, leading to its accumulation in cytoplasm (③). HIF and Wnt signaling would also upregulate EpCAM expression (④). Upregulated EpCAM and accumulated β-catenin would translocate into nucleus and act together with Lef as a transcription complex to induceWnt and EpCAM target genes expression, modulating the differentiation fates of HPC (⑤).

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Appendix

List of Abbreviations

Abbreviation	Full term
Gene	
Alb	Albumin
AFP	α-fetoprotein
CK19	Cytokeratin 19
Col1a1	Collagen type 1 α 1
EpCAM	Epithelial cell adhesion molecule
EpEx	EpCAM extracellular domain
EpICD	EpCAM intracellular domain
GAP	Glyceraldehyde-3-phosphate dehydrogenase
HIF	Hypoxia inducible factor
Lef1	Lymphoid enhancer-binding factor 1
PDGFa	Platelet-derived growth factor a
αSMA	α smooth muscle actin
Tcf7	Transcription factor 7
TGFβ	Tumor growth factor β
Timp2	Tissue inhibitor of metalloproteinase 2
VEGFa	Vascular endothelial growth factor a
Cell	
HSC	Hepatic stellate cell
HPC	Hepatic progenitor cell
Others	
AF	Alexa Fluoro
ECM	Extracellular matrix
FC	Flow cytometry
HCC	Hepatocellular carcinoma
ICC	Intrahepatic cholangiocellular carcinoma
IF	Immunofluorescence staining
LMD	Laser microdissection
WB	Western blotting