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Y-box binding protein 1 於肝癌幹細胞之功能解析

Characterization of Y-box binding protein 1 in Hepatocellular Carcinoma Stem Cells

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Characterization of Y-box binding protein 1

in Hepatocellular Carcinoma Stem Cells

本論文係_黃泓軒_君（學號 R01B22022）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

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Abstract

Hepatocellular Carcinoma (HCC) is the third cause of cancer mortality in the world. The important issues for the treatment of HCC are the high recurrence rate, easy metastasis and drug resistance. Recently, the concept of cancer stem cells (CSCs) provides a new consideration in the cancer therapy including HCC. Cancer stem cells are a subpopulation of cells in the tumor, which have the capability of self-renewal and differentiation and drug resistance in chemotherapy. Previous studies showed that many properties of CSCs, such as high cell mobility, evading immune destruction and reprogramming of energy metabolism, are very different from the original understanding of cancer. As a result, development of the therapy targeting CSCs is one of the novel therapeutic strategies for the cancer in the future. YB-1 is a protein with multiple functions, which has been found associated with many kinds of cancers. YB-1 can increase the expression of stemness marker genes, enhance cell mobility and up-regulate MDR gene expression. Besides, YB-1 is known as a significant regulator during liver development and regeneration.

To investigate the regulatory function of YB-1 in CSCs of HCC, I used sphere forming method to enrich CSCs. In the sphere cells, the expression of YB-1 and some pluripotent genes was up-regulated. In addition, the sphere forming ability of

YB-1-shRNA knockdown HuH7 HCC cell line was decreased. YB-1 would translocalize to the nucleus of sphere forming cells or side-population cells, and the cancer stem cell population sorted from HCC cell line. These results indicated YB-1 might be involved in the transcriptional regulation in the cancer stem cell-like cells in HCC. Additionally, knock down of YB-1 also down-regulated the stemness, drug resistance and epithelial-mesenchymal transition (EMT) genes expression by qPCR analysis. The detail mechanism would be regulated by Wnt/β-catenin pathway and epigenetic regulation. From these results, YB-1 may play a key role in HCC cancer stem cells.

Key Words: Hepatocellular Carcinoma, Cancer Stem Cell, YB-1

中文摘要

肝癌是世界上致死率前三名的癌症，而其高復發率、高轉移率及抗藥性是現今治療上的主要難題。而近年來快速發展的癌幹細胞理論，提供了一個新的癌症治療觀點。癌幹細胞是腫瘤中存在的類幹細胞群落，具有生長、分化及自我更新的能力。這些癌幹細胞能夠獨立生長分化為腫瘤細胞進而形成腫瘤，為癌症的起始細胞。同時其對傳統癌症藥物具有較高的耐受性，能在化療過程中維持其細胞群落，而在化療結束後繼續分化生長，造成腫瘤復發；除此之外，近年來的研究亦顯示這些癌幹細胞的種種特性如高遷移性、免疫特異性和代謝特殊性，都遠不同於我們原本對癌細胞的認識，成為癌症治療上的缺口，因此發展針對癌幹細胞的治療方法將是未來癌症治療的方向。而多功能蛋白質 YB-1 已被發現在許多癌症中，具有促進幹細胞標記表現、提高癌細胞的移動性和提升抗藥基因表現等癌幹細胞常見的特性；此外在肝臟的發育及再生的過程中， YB-1 亦被發現是重要的調控者，顯示其在幹細胞性質調控上的重要性。

為了進一步研究 YB-1 是否為肝癌幹細胞中的重要調控者，我利用 Sphere Forming 的方式培養肝癌幹細胞，發現在 Sphere 形成過程中， YB-1 和其他多能基因的表現是提升的；進一步利用 RNA 干擾的方式降低 YB-1 表現後，亦發現形成 Sphere 及細胞自我更新的能力明顯下降，而 YB-1 抑制細胞株中癌幹細胞的比例也有所下降；而在肝癌細胞在形成 Sphere 後 YB-1 的核轉移，暗示了其在肝癌幹細胞種基因調控的功能。此外在抑制 YB-1 表現後肝癌細胞株的幹細胞標記、細胞移動力還有抗藥基因的表現皆下降；而細胞凋亡的基因反而上升。這些結果可能由 YB-1 影響 Wnt/β-catenin 路徑和表觀遺傳調控因子有關。從以上結果可知 YB-1 在肝癌幹細胞中具有關鍵的角色。

關鍵字：肝癌、癌幹細胞、YB-1

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Chapter 1. Introduction

1.1 Cancer and Cancer Stem Cell

1.1.1 Cancer Initiation

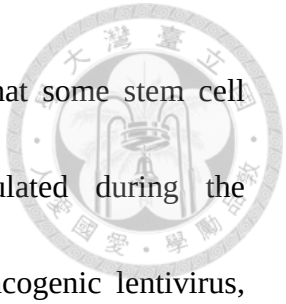
Cancer has been described as a developmental disease, and the balance of regulation in cellular division and death in cancer cells is broken, which results in abnormal proliferation and finally tumour formation (Hanahan and Weinberg, 2011). As a result, how normal cells become cancer cells and how tumour initiates is an important issue in cancer biology. There are two significant models of tumour initiation as the clonal evolution model and the cancer stem cell model. In the clonal evolution model, tumor cells arise from any normal cells, which occur in genetic mutation and generate abnormal offspring that also inherit the mutation, then forming a heterogeneous tumour (Greaves and Maley, 2012). However, this theory cannot well explain the phenomenon that not all of the cells in the tumors can form a new tumor. Cancer stem cell (CSCs) model indicates that the cells in the tumor are hierarchical, including hepatocellular carcinoma (HCC) (Chiba et al., 2006). Only few cells, so-called cancer stem cells (CSCs), have the potential of self-renewal and differentiation and the ability to give rise to all cell types in the tumor (Magee et al., 2012).

1.1.2 Characteristics of cancer stem cells

CSCs possess characteristics associated with normal stem cells. Like normal stem cells, CSCs stay in a quiescent state and seldom divide, and lead to resist some anti-cancer drugs that target on the rapidly replicating cells. Moreover, CSCs may participate in metastasis, which tumor cells leave original site to another organ through circulation. CSCs' properties such as detachment, transport and invasion are related to this process (Mani et al., 2008). Furthermore, CSCs can differentiate to different clones of tumor cells in the particular tumor. They can divide symmetrically to expand its population, or asymmetrically to generate more differentiated cells and form a new tumour (Sell, 2004). Additionally, CSCs express some ABC transporter to resist and survive under the anti-cancer drug treatment and cause cancer recurrence (Liao et al., 2014; Zhao et al., 2014). All characteristics of CSCs may shed light on the treatment or even the cure for the cancer. Hence, investigation of the physiological properties of cancer stem cells may uncover the novel therapeutic strategy for the cancer.

1.2 Origin of cancer stem cells

Although CSCs exhibit the stem cell properties such as self-renewal and differentiation, they do not necessarily originate from the transformation of normal stem cells. Normal cells in tissues may also dedifferentiate and acquire CSC-like properties



under certain conditions (Visvader, 2011). Recent studies show that some stem cell characteristics are acquired and stemness genes are up-regulated during the tumourigenesis. In glioblastoma tumors, which are induced by oncogenic lentivirus, differentiated markers such as Tuj1 and GFAP are decreased during tumour progression, and some neural stem/progenitor genes such as nestin and Sox2 become predominantly expressed (Soda et al., 2011). However, how these factors affect the transformation of normal somatic cells or stem cells still needs further investigation.

1.2.1 Isolation of cancer stem cells

First of all, detection and isolation of CSCs are necessary for cancer stem cell research. Some cell surface biomarkers are identified for CSCs detection in leukemia and the solid tumors. It was reported that CD133 serves as a brain CSC marker (Yang et al., 2011), while CD44 can be ovarian CSC marker (Casagrande et al., 2011). Since the cell linages, origins, and microenvironment of tumours are divergent, thus these surface markers of CSCs are different in the different cancer cell types (Beier and Beier, 2011). As a result, application of surface cell marker for detection still remains uncertain. Owing to the physiological properties of CSCs, such as, drug resistance or self-renewal, there are other ways to isolate the CSCs. One is side population flow cytometry. CSCs show drug resistance by efflux of anti-tumour medicines (Zhou et al., 2001). Based on

this property, researchers divide tumour cells into one group which can transport the dye out of the cells, and the other group which cannot do it. The small portion of tumor cells which can expel the dye is able to form tumours in the immunodeficient mice with very low cell numbers and also has the CSCs capabilities, such as invasion and metastasis (Asakura and Rudnicki, 2002). The other way is sphere forming assay which is based on self-renewal ability of CSCs. This method was first described and performed to isolate the brain tumour CSCs (Singh et al., 2003). Those sphere cells are capable to form tumours in the immunodeficient mice with low cell numbers. To date, there are some ways available to isolate CSCs. However, the exact mechanisms of regulating stemness behaviors are largely unknown.

1.3 Molecular Regulation of CSC

1.3.1 Self-renewal

The transcription factors OCT4, SOX2, KLF4 and MYC are well-known for their roles in embryonic stem cell pluripotency and self-renewal (Stadtfeld and Hochedlinger, 2010). Additionally, some clues indicate the dysregulation of these genes occurs in many cancers. Recently several researchers have used omics method to compare the transcriptional programs activating in embryonic stem cells (ESC) and different cancers (Gunaratne, 2009). Activation of ESC-like gene sets are systematically observed in

aggressive human epithelial cancers (Trosko, 2014). Especially, the MYC oncogene has a crucial effect on both tumor initiation and reactivation of the ESC-like module in normal and cancer cells (Civenni et al., 2013). These data emphasize the importance of the MYC gene in normal and cancer stem cell regulation. Moreover, many regulatory pathways are known to be involved in stem cell maintenance as well as self-renewal and pluripotency, including WNT/β-catenin (Galuppo et al., 2014), TGF-β (Cao et al., 2012), Notch (Abel et al., 2014), MAPK (Balko et al., 2013) etc. The disruption of these functionally overlapping pathways showed an association with CSCs regulation.

1.3.2 Differentiation

Researchers can force CSCs to differentiate with retinoic acid (RA). RA reacts with nuclear receptors in embryonic carcinoma cells leading to inhibition of proliferation and induction of differentiation (Park et al., 2014; Yang and Shinkai, 2013). RA and related compounds pass into the nucleus where they bind to nuclear receptors (RAR-RXR) that form a transcription complex with c-Fos, c-Jun, AP-1, and most likely other activation proteins resulting in up-regulation of a number of genes related to inhibition of proliferation and induction of differentiation (Sell, 2004), such as p21, p27 and Disabled 2. Those results can provide some new molecular targets for CSCs differentiation.

1.3.3 Metastasis

Presence of metastasis or not affects the prognosis of patients with cancers. Important events in this process are acquisition of the ability of migration, invasion through the ECM into the blood vessels, escaping from immune system, and re-establishment at the target tissue. These properties are very similar to CSCs and thought to be regulated by the signals from their specific niche. Although mechanisms of metastasis are not fully understood, the epithelial-mesenchymal transition (EMT) may play a critical role (Zubeldia et al., 2013). Some researchers demonstrated that CSCs or CSC gene signatures strongly correlate with patient metastasis (Kang, 2009). EMT gene Snail1 has been reported as a novel stemness gene (Zhou et al., 2014), which suggests the relationship between CSCs and metastasis.

1.4 Wnt/β-catenin Signaling

In stem cell study, Wnt/β-catenin signaling is an important signaling cascade. Wnt/β-catenin signaling pathways were one key regulator during embryogenesis and developmental process (Serio, 2014,Van Camp, 2014). Recent studies have shown that Wnt/β-catenin signaling pathways are also involved in many cancer cell physiological phenomenon, such as proliferation, anti-drug, metastasis, survival and even stemness (Bisson and Prowse, 2009; He et al., 2005; Vangipuram et al., 2012). There are three

active types in Wnt/β-catenin signaling pathways. In the canonical Wnt pathway, Wnt protein binds onto its receptor LRP and Frizzled, β-catenin would escape 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 arresting, metastasis and anti-drug properties (Shenoy et al., 2012). In molecular research of liver, recent research has shown that β-catenin was overexpressed in inflammation of liver and inhibition of Wnt/β-catenin signaling would obstruct liver progenitor cells activation by down-regulating proliferation genes, altering liver progenitor cell fate in liver regeneration (Strazzabosco and Fabris, 2013). In addition, Wnt ligand have been proved to increase generation efficiency of stem/progenitor cells differentiation to hepatocytes, indicating that Wnt signaling may also play an important role during liver stem/progenitor cell maturation (Bogaerts et al., 2014). Finally, the research in 2008 detected dysfunctional Wnt signaling in most of HCC, which indicated its special roles in HCC regulation (Yang et al., 2008a).

1.4.1 Cancer Therapies of Targeting Cancer stem cells

Like cancer stem cells research, the cancer therapies which target cancer stem cells also under being research. In 2006, some scientist showed that bone morphogenetic proteins (BMPs), which can induce normal neural precursor cells to differentiate into mature astrocytes, can also lead to the differentiation of $CD133^+$ brain tumor stem cells and decreasing their tumor-forming ability. The results also mean that Cancer stem cells have behaviors like stem cells, and are able to respond to the normal signals that induce stem cells to differentiation (Piccirillo et al., 2006). Furthermore, the other group targets the other signal pathway, Wnt pathway, and shows that deletion of the signal transduction component of Cancer stem cells may attenuate mice tumorigenesis (Zeilstra et al., 2008). This evidence indicates that the component in stem cell regulation will be a good target for us to destroy the Cancer stem cells in tumour.

1.5 YB-1

YB-1, Y box binding protein 1, a pleiotropic protein and member of the cold-shock domain (CSD) protein superfamily, has been reported to participate in many cellular functions (Kohno et al., 2003). The mRNA of YB-1 is approximately 1.5 kb long and encodes a 43 kDa protein (324 amino acids) which contains three domains: a variable N-terminal domain, a highly conserved nucleic-acid-binding domain (the CSD) and a

C-terminal tail domain (Makino et al., 1996; Wolffe, 1994). YB-1 was originally identified as a factor that repressed gene expression by binding to the Y-box (an inverted CCAAT box)

1.5.1 YB-1, Cancer and Stem Cell Regulation

YB-1 is known to be transcriptional regulation, translational regulation, DNA repair and stress responses to extracellular signals. Recently, scientists investigating YB-1 mostly focus on its ability to influencing various cancer phenotypes, such like cell-cycle regulation, activating invasion or metastasis, resisting cell death, drug resistance and stress response (Asakuno et al., 1994; Fujii et al., 2009; Jurchott et al., 2003; Ohga et al., 1998). It has been reported that YB-1 directly activates cap-independent translation of mRNA ,which is encoding Snail1 and other transcription factors, and down-regulate epithelial as well as growth-related genes while activate of mesenchymal genes (Evdokimova et al., 2009a). Furthermore, YB-1 can promote cancer cell growth and drug resistance through its induction of CD44 and CD49f in breast cancer, which has been seen as the cancer stem cell marker (To et al., 2010). In brain cancer stem cell, YB-1 was also reported as an important regulator (Fotovati et al., 2011).Notably, YB-1 had been identified to bind on promoters of several epigenetic regulators (Finkbeiner et al., 2009), which are important during cell reprogramming

(Apostolou and Hochedlinger, 2013). This implied that YB-1 would be a stem cell modulator.

1.5.2 YB-1, liver development and regeneration

YB-1 has been reported to have high-expression level in animal fetal and regenerating liver, and would gradually decrease during the liver development (Chen et al., 2009). YB-1 has also been shown to regulate hepatocyte regeneration by inducing the expression of Cyclin A and Cyclin B. As a result, YB-1 might paly a role in the stem/progenitor cells of the liver.

1.5.3 YB-1, Liver disease, and Malignancy

Liver is the largest organ in the human body and participates in carbohydrate storage, lipid synthesis, amino acid metabolism, serum secretion and detoxification of poisonous. (Lu and Mato, 2008) Due to the importance in our body, severe liver illness usually leads to mortality. Liver cancer is one of the most common cancers in the world, however, the molecular mechanisms about HCC initiation are not very clear. The common risk factors of HCC are HBV/ HCV infection and fatty liver, which usually results in hepatitis that usually leads to hepatic fibrosis, cirrhosis and even HCC. In previous study, YB-1 increases expression during liver damage, which causes hyperammonemia by regulating the expression of carbamoyl phosphate synthetase (Chen et al., 2009). Recently, studies on YB-1 mostly focus on its ability to influence

various cancer phenotypes, such as cell-cycle regulation and stress response. However, accepting the research of prognosis marker (Yasen et al., 2005), the molecular studies of YB-1 in hepatocellular carcinoma were few. To date, the role of YB-1 in the CSCs of HCC remains to be clarified. In our lab recent data, YB-1 was considered as an oncogene of hepatocellular carcinoma and may translocate to different subcellular area in different HCC cells conditions.

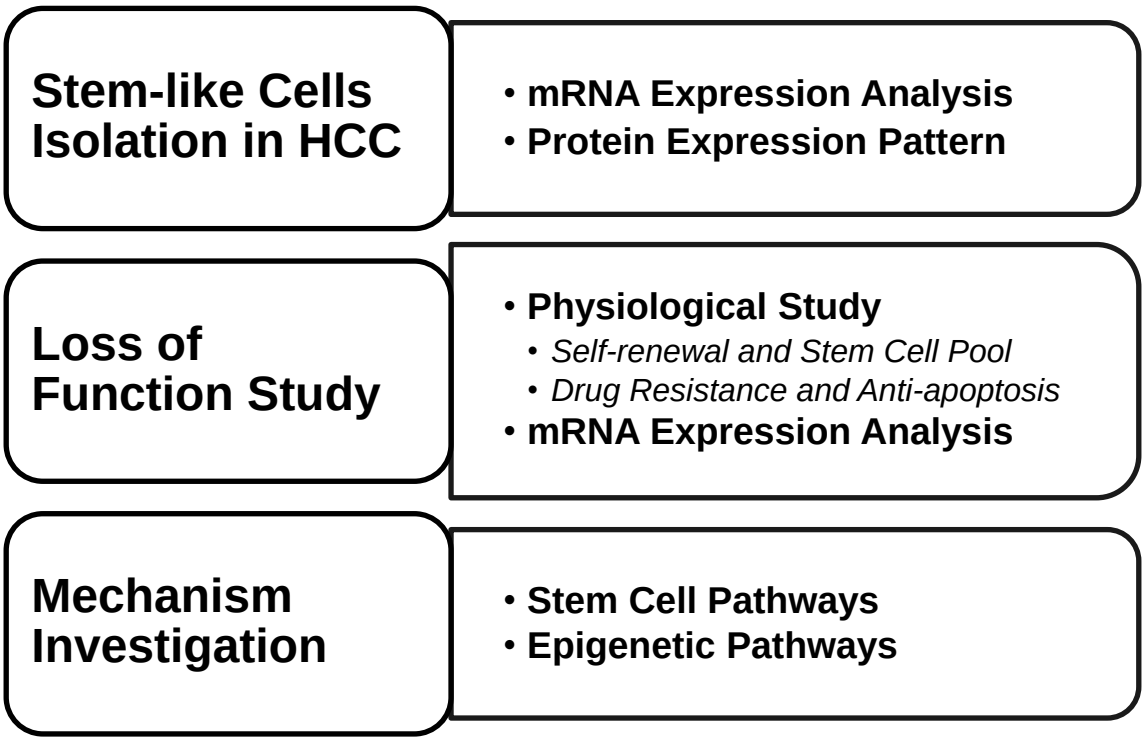
1.6 Motivation and aim

CSCs are the key cells participating in tumour recurrence, drug resistance and metastasis. As a result, investigating the molecular regulatory network of CSC is important. The knowledge of CSC regulation can help us to design more effective therapy strategy. In many kinds of cancer, YB-1 play important roles, including regulating cellular proliferation, drug resistance, cancer progression and EMT (Lasham et al., 2013), which were also seen as characteristics of cancer stem cells (Singh and Settleman, 2010). Also, YB-1 was known to express during liver development and regeneration (Chen et al., 2009; Lu et al., 2005).

Many clues support that CSCs were derived from normal cells which acquiring stemness properties but the mechanism is still unclear. The population of CSCs in tumour will influence the cancer aggressiveness. In our lab recent data, YB-1 may

translocate to different subcellular area in different HCC cells conditions, which intimating regulating role of YB-1 in HCC. Combined its abilities to regulate cell cycle, EMT, drug resistance, anti-apoptosis and liver stem cells, I speculate that YB-1 would affect the CSC properties in tumour through regulating CSC population. In my master thesis, I would investigate the biological function of YB-1 in HCC CSCs and the regulatory network between YB-1 and other stemness pathways.

1.7 Flow Chart



Chapter 2. Materials and Methods

2.1 Cell lines

Huh7 and Hep3B 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.

2.2 Sphere formation assay

For obtaining single cell, Huh7 or Hep3B cells were treated with 0.5% trypsin. Cells were then resuspended in DMEM/F12 medium (Life Technologies) consisting of B27 (Life Technologies), human recombinant EGF (20 ng/ml; Sigma), bFGF (20 ng/ml; Sigma), plated at a density of 103 live cells/ml medium on ultra-low attachment dish or plate and cultured for 6 days.

2.3 RNA Extraction

The cells were scraped using Tripure (Roche) by tips after the removal of culture medium and washed once by PBS. 200 μL/mL Tripure chloroform (Wako) 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 12000 g 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/ddH2O. The quality of RNA was determined by Nanodrop 2000 (Thermal) and electrophoresis before reverse transcribed into cDNA.

2.4 cDNA preparation and Quantitative PCR

2μg or 1μg total mRNA was first reverse transcribed into cDNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystem) according to the manual followed the protocol: 25℃/10 min→37℃/ 120 min→85℃/10 min→4℃. qPCR was performed using iQ SYBR green detection system in Bio-Rad. The expression level of target genes was normalized to β-Actin and GAPDH expression. The primers used for qPCR were shown in Table 4.

2.5 Cellular protein extraction

The cells were scraped using RIPA buffer 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 12000 g in 4℃ for 10 minutes to remove the pellet. The concentration of total cell protein was determined by Bradford method using Bio-Rad protein dye reagent.

2.6 Western blot

The protein samples were mixed with 5x sample buffer (250 mM Tris-HCl, pH 6.8, 10% SDS, 0.5% (w/v) bromophenol blue, 50% glycerol, 5% β-mercaptoethanol) and placed on dry bath (Major Science Dry Bath Incubator) under 100℃ for 10 min. SDS-polyacrylamide gel (10% separation gel and 4% stacking gel) and TGS buffer system (50 mM Tris-HCl, pH 8.3, 380 mM glycine, 0.1% SDS) were used to separate total proteins. Electrophoresis was 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/cm2. The membrane was washed with TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20) twice before blocking with gelatin-NET (Wako) for 1 hour. Primary antibody solution (diluted in gelatin-NET) 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 ddH2O before rinsed in Luminata Classico Western HRP substrate (Millipore WBLUC0500) for 1 min and analyzed by BioSpectrum 2D Imaging System

(UVP BioSpectrum 800). The antibodies used in this study and its dilution condition were shown in Table 3.

2.7 Stable knock down clone establishment

The pLKO.1-YB1 shRNA plasmid, purchased from RNAi Core, Academia Sinica, was transfected into Huh7 with Jet-prime transfection reagent (Polyplus). After 2 days, cells would be selected by 10% FBS DMEM with 1 μg/ml puromycin. 14 days later, single colonies would be isolated and amplified. Knock down efficiency would be checked by qPCR and Western blot.

2.8 Immunofluorescent staining and image processing

After the removal of culture medium, Huh7 were first fixed with 4% paraformaldehyde for 15 minutes then permeabilized with 0.1% Triton X100 for 7 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 (5 μg/ml) 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 2. All immunofluorescence images were first obtained by IN Cell

Analyzer 2000 (GE Healthcare), and the local background were subtracted before merging channels and cropped by ImageJ.

2.9 Colony formation assay

For obtaining single cell, Huh7 or Hep3B cells were treated with 0.5% trypsin. Cells were then resuspended in 3% FBS DMEM, plated on cell culture dish and culture for 14 days. For counting colonies, cells were washed twice with cold PBS, fixed with cold methanol for 5 minutes, stained with 0.5% crystal violet and destained with PBS. Then, picture was taken and analyzed by ImageJ.

2.10 Anti-cancer drug treated and MTT assay

1×10^5 Cells were seeded on 96-well plate. Next day, cells were treated with 1500 - 15 ng/ml Doxorubicin for 3 days. The process of MTT assay followed the condition provided by SIGMA. MTT stock solution (5 mg/ml) was added to each culture being assayed to equal one tenth the original culture volume and incubated for 3 hours. At the end of the incubation period the medium would be removed and dye might be solubilized with acidic isopropanol (0.08 N HCl in absolute isopropanol). Absorbance of converted dye is measured at a wavelength of 570 nm with background subtraction at 630-690 nm.

2.11 Flow Cytometry

For cellular 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 antibodies (or isotype control) were added into each samples and shaken for an hour at 4 ℃. Cells were then stained with propidium iodide for 10 minutes (PI, Life Technologies) and went trough 0.40 μm mesh filter (BD) before washed with PBS and resuspended with 3% FBS (diluted in PBS). Cell flow cytometer analyzer (BD FACSCanto II) was used to analyze the expression of cell surface markers. The antibodies used in this study and its dilution condition were shown in Table 2.

2.12 Statistics

Quantitative PCR 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, a *p* value of less than 0.01 were considered really significant and a *p* value of less than 0.01 were considered extremely significant.

Chapter 3. Results

3.1 HCC cell lines could form spheroid cells

For studying the physiological properties of stem like cells in HCC, a large number of stem-like cells were needed. As a result, sphere forming assay was performed with the protocol of neuron sphere (Singh et al., 2003). After culturing 6 days, two different kinds of HCC cell lines, Huh7 and Hep3B could both form sphere cells on ultralow-attachment dish (Figure 1).

3.2 YB-1 was up-regulated during sphere forming

To analyze the changes of genes expressing pattern between normal and sphere cultures, I performed quantitative PCR test. Some CSCs related genes, CD133, CD90 and EpCAM, were up-regulated in sphere cells (Marquardt et al., 2010). YB-1 mRNA was also up-regulated during sphere forming (Figure 2).

3.3 YB-1 translocated into nucleus in sphere cells

The previous studies have shown that the different subcellular localization of YB-1 may indicate its different regulation functions, such as tumour recurrence or drug resistance (Stein et al., 2001). Compared with normal HCC cells, YB-1 would translocate into nucleus in immunofluorescent staining of sphere HCC cells (Figure 3 A-B).

3.4 The self-renewal ability of YB-1 KD cells decreased

Self-renewal ability leaded CSCs to maintain their cells pool in tumor tissues. Self-renewal ability of CSCs could be evaluated by sphere and colony formation (Hu and Smyth, 2009; Oppitz et al., 2002; Singh et al., 2003). To investigate whether YB-1 affects the self-renewal ability of HCC CSCs, YB-1 KD Huh7 cells were established (Figure 4A). In Figure 4B and 4C, YB-1 KD Huh7 cells formed fewer colonies in colony formation assay and revealed lower stem cell frequency in extreme limiting dilution assay (Table 1 and Figure 5).

3.5 The stem cell population would be regulated by YB-1

Owing to functions of YB-1 in liver development and regeneration, I wanted to know the relation between YB-1 and stem cells in HCC. First, I performed side population flow cytometry, which could distinguish the stem-like cells from heterogeneous cells by the ability to pump out hoechst 33342 dyes (Zhou et al., 2001). While YB-1 was knocked down, the ratio of stem-like cells was decreasing from 0.596% to 0.31%. On the contrary, the ratio of stem-like cells in sphere cells would increase from 0.596% to 1.65% with YB-1 up-regulation (Figure 6). Next, I analyzed the liver stem/progenitor cell surface marker, EPCAM, which had been reported also expressing in HCC cancer stem cells (Yamashita et al., 2009) by flow cytometry. The

ratio of $EPCAM^+$ cells in shYB1 cells would decrease with YB-1 down-regulation (Figure 7).

3.6 The pluripotent genes were downregulated in YB-1 KD cells

Because knock-down YB-1 would decrease stem-like cells in HCC, I would like to know how YB-1 mediates self-renewal ability of HCC cells. First, I measure the typical stemness gene related to self-renewal, Oct4, Nanog and Sox2, which was highly expressed in embryonic stem cells. My results showed that those genes were down regulated in YB-1 KD HCC cells, so did the well-known cancer stem cell marker as well as hepatic lineage stem cell marker, AFP (Figure 8A). On the contrary, the liver lineage differentiated marker, albumin, was up-regulated (Figure 8B). I also tried to overexpress YB-1 and test Nanog transcription activity but the result is negative. (Supplementary figure 1)

3.7 The pluripotent genes were up-regulated in sphere cells

Beside the YB-1 KD HCC cells, I also investigate some pluripotent genes, such as OCT4, cMYC and NANOG in sphere cells. Those genes are up-regulated in sphere cells (Figure 8C). In the mean while, the mRNA expression of CSCs marker genes, BMI1, CD44, EPCAM and CD133 are also up-regulated in sphere cells (Figure 9).

3.8 EMT related genes were altered in different YB-1 expression pattern

Epithelial-mesenchymal transition (EMT) and cancer metastasis are considered as the properties of CSCs in tumor progression.(Zubeldia et al., 2013). From my previous data, YB-1 was up-regulated in sphere cells, and stemness genes were down-regulated in YB-1 KD cells. Thus, YB-1 may also affect EMT and metastasis-related genes. In YB-1 KD cells, I found BMI1, Vimentin and SNAIL1, which have been known to participate in cancer metastasis and EMT were also down-regulated by qPCR analysis. (Figure 10).

3.9 KD YB-1 cells had low ability of drug resistance

Many studies suggest that cancer stem cells had stronger ability of drug resistance and YB-1 seemed to play important role in cancer stem cells (Singh and Settleman, 2010) so I want to investigate the relationship with YB-1 and drug resistance in Huh7. After treated with traditional anti HCC drug, doxorubicin (0.15 μg/ml) or sorafenib (0.15mM) for 3 days, cell viability of YB-1 KD cells was decreased compared to shLac cells (Figure 11).

3.10 Increase the proapoptotic genes in YB-1 KD cells

Some studies suggested that repression of proapoptotic gene might enhance the survival rate of cells under a stress condition (Bertrand et al., 2009). In order to know

whether YB-1 affects the anti-stress ability in HCC cells, proapoptotic genes, E2F1 and BAX were evaluated in normal and YB-1 KD HCC cells. As shown in figure 12, mRNA expression level of E2F1 and BAX were up-regulated in mitomicyin C treated YB-1 KD cells.

3.11 Wnt/β-catenin pathway might be regulated by YB-1

From my results, YB-1 may alter the expression of genes related to self-renewal, CSCs markers, EMT and apoptosis in HCC CSCs. How YB-1 modulates these properties was still unclear. Recently, a ChIP-on-Chip bioinformatics data indicated YB-1 may affect Wnt/β-catenin pathway, which regulates several key stemness properties of normal and tumor stem cells in breast cancer (Finkbeiner et al., 2009). In HCC, whether YB-1 alters Wnt signaling needs to further investigate. By immunofluorescence staining, active Wnt signaling led β-catenin accumulation and translocation in the cell nucleus of HCC sphere cells (Figure 13A-B). Furthermore, the expression of Wnt signaling target genes, AXIN1 and AXIN2, as well as important signal transducer, β-catenin, were down-regulated in YB-1 KD cells (Figure 14A-B). On the contrary, β-catenin protein would express more in sphere cells (Figure 14B).

3.12 Epigenetic modulators were affected after Knock down of YB-1

There was an article reporting that YB-1 would play important role in mechanism of controlling Wnt/β-catenin pathway (Finkbeiner et al., 2009). In this article, the author also shown that some epigenetic modulators were regulated by YB-1. In the other hand, stem-like properties acquisition also had a relationship with epigenetic regulation (Apostolou and Hochedlinger, 2013). As a result, I combined their results in table 2 and believed that some of epigenetic modulators would be regulated by YB-1. After knock down YB-1 with siRNA-oligo, the mRNA level of SOX2 decreased while the genes related to DNA methylation pattern change, DNMT1 and DNMT3A, as well as histone modification protein, HDAC1, were up-regulated (Figure 15).

Chapter 4. Discussion and Conclusion

In my present data, YB-1 gene expression pattern in sphere cells of HCC were up-regulated and nucleus-translocating. These YB-1 highly expressing sphere cells also expressed typical CSCs markers. YB-1 knockdown would decrease the self-renewal ability and stem cell pool of HCC cells. Further gene expression profile showed that pluripotent, CSC-related and EMT genes were down-regulated in YB-1 KD HCC cells. On the contrary, these patterns were exactly opposite to sphere cells. In addition, ability of anti-drug and anti-apoptosis would both decline in YB-1 KD HCC cells. In the end, I found YB-1 knockdown would affect the expression profile of Wnt/β-catenin pathway and some epigenetic modulators.

In many kinds of cancer, YB-1 played important regulation roles, which were also seen as characteristics of cancer stem cells (Lasham et al., 2013; Singh and Settleman, 2010). However, the CSC regulation studies of YB-1 in hepatocellular carcinoma were very few. My sphere forming data of immunofluorescent staining showed that more than 15% HCC cells containing nucleus-translocating YB-1 (Figure3.). This pattern was similar to the hepatocytes of bad prognostic HCC patients (Yasen et al., 2005). It suggested that CSC might be the reason causing the bad prognostic, which would be controlled by YB-1.

Although YB-1 was known to highly express during liver development and

regeneration (Chen et al., 2009; Lu et al., 2005), the exactly roles of regulating stem cell in HCC were unknown. I found that knock of YB-1 would decrease self-renewal ability of HCC cells (Figure 4 and 5) and further experiment showed that side population in HCC cells might altered by YB-1 expression profiles (Figure 6 and 7). Combination of both data, YB-1 was necessary to maintain self-renewal ability of HCC cells through affecting the size of cancer stem cell pool.

My experiment data also explained the more detail mechanism in YB-1 regulating CSC pool. Firstly, the sphere cells of HCC expressed pluripotent genes and stemness surface marker higher, such CD44, CD133, EPCAM, BMI1, OCT4, SOX2, NANOG and SNAIL1 (Atlasi et al., 2007; Chiou et al., 2008; Sarvi et al., 2014; Yang et al., 2008b; Zhou et al., 2014; Zhu et al., 2014). After knockdown of YB-1, these patterns would be reversed, which suggested that YB-1 was a regulator in CSCs. Especially, SNAIL1, which have been known to be highly regulated by YB-1(Evdokimova et al., 2009b), were also reported a significant pluripotent regulator (Garibaldi et al., 2012). This route might explain why YB-1 was critical to CSCs exist.

Secondary, protein translocalization usually meant the functional switch of YB-1, such as reaction of UV or DNA damage (Cohen et al., 2010). Consider with the correlation of YB-1 and pluripotent gene, nucleus-translocating would suggest the

idea that YB-1 could regulate some molecular events in CSCs of HCC. According to my data, EMT, anti drug and anti-apoptotic pathway could be further investigated through this way in the future work.

Finally, No matter in normal stem cells or in cancer stem cells, YB-1 seemed to alter the stem cell behavior through many ways (Lu et al., 2005). In consequence, I investigate proper pathways would be regulated by YB-1. My data show YB-1 might activate Wnt/β-catenin pathway through stabilizing β-catenin (Figure 14). In the mean while my data also suggested the recent study, YB-1 playing an important role in epigenetic regulation of CSC genes (Davies et al., 2014).

In summary, I found that YB-1 would be up-regulated during sphere forming, which would enhance self-renewal ability in HCC cell lines. Further experiment suggested the importance of YB-1 in CSC pool maintenance, EMT genes regulation, anti-apoptosis and anti-drug ability. Those properties would be caused by YB-1 mediated pluripotent or other genes activation. Moreover, Wnt/β-catenin pathway and some epigenetic pathways would regulated by YB-1 and participate in this regulatory process (Figure 16.). My data presented here offer a new approach to study the functions of YB-1 in HCC progression.

Figures and Tables

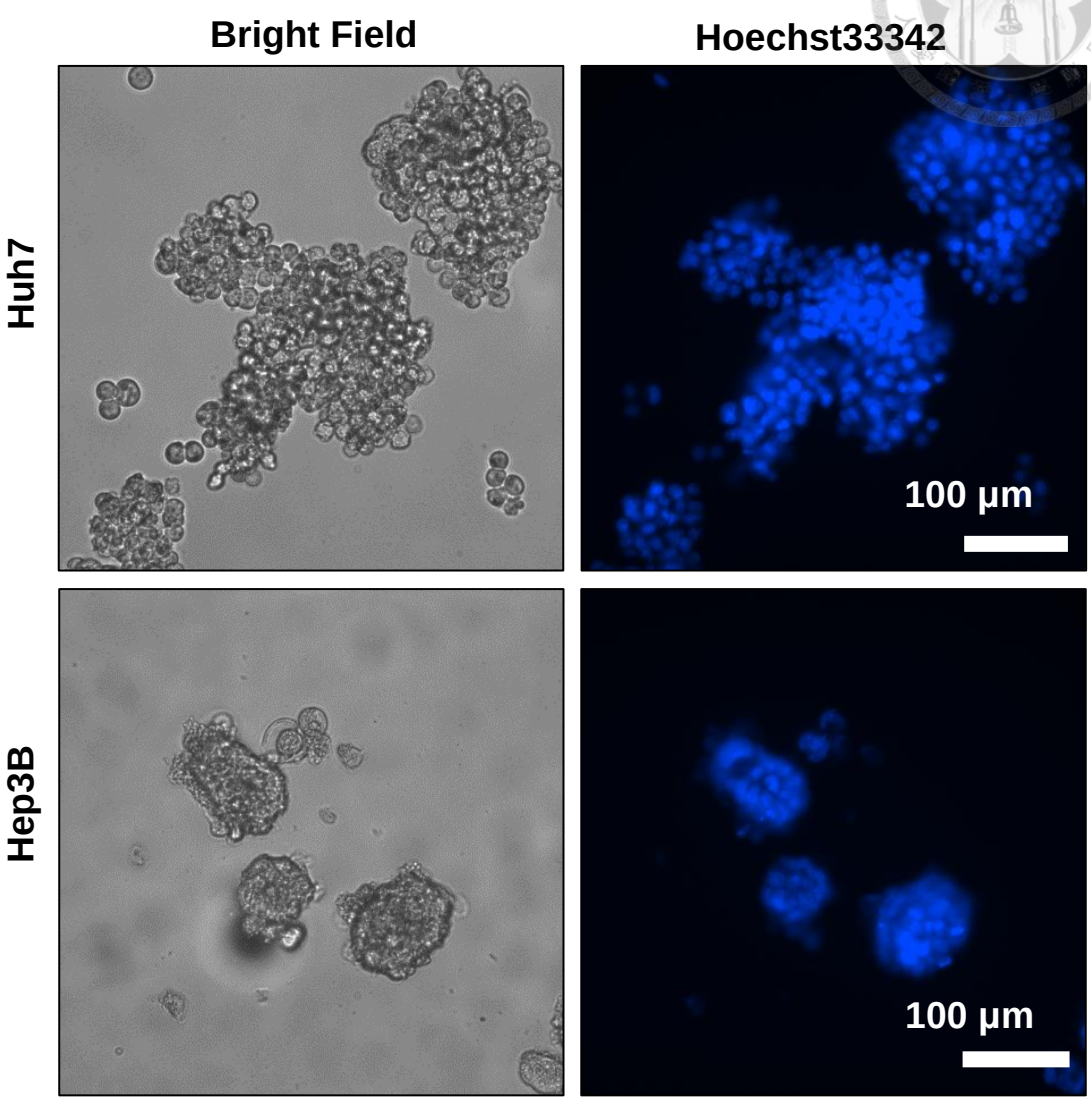


Figure1

Figure 1. Huh7 and Hep3B could form sphere cells.

Huh7 and Hep3B could form sphere cells on ultralow-attachment dish after 6 days of culture. Sphere cells were stained with hoechst33342 in final concentration 5μg/ml for 5 minutes and picture was taken by in cell 2000 image system (Gray, bright field ;Blue, nucleus)

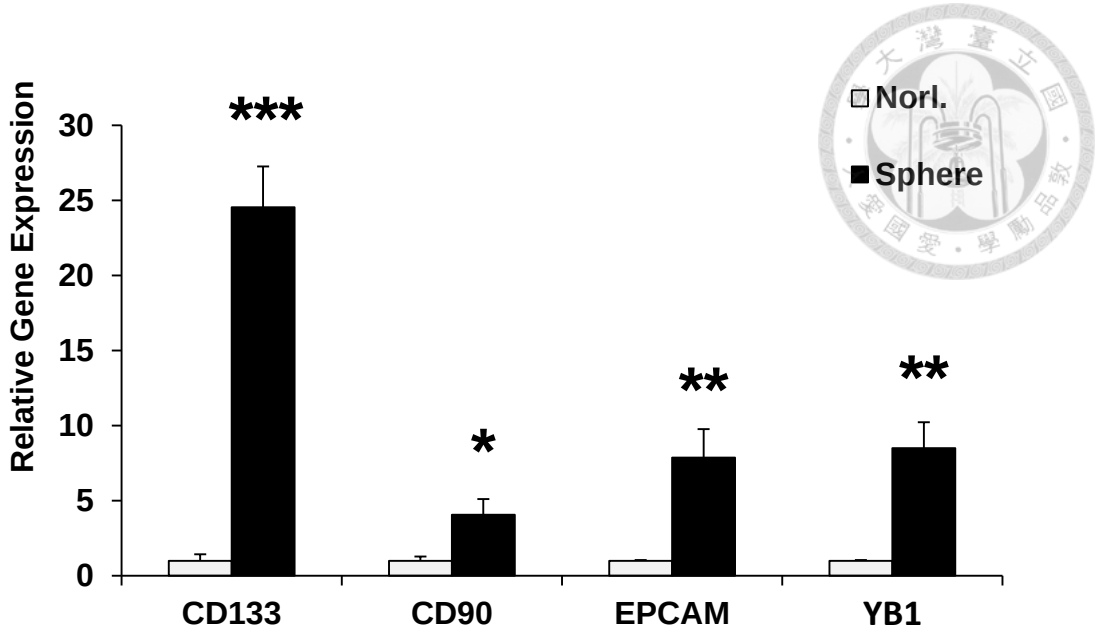


Figure 2. Stemness markers were up-regulated during sphere forming.

The mRNA expression level of 6 days sphere cells of Huh7 were analyzed by quantitative PCR. CD133, CD90 and EPCAM, the stemness genes were up-regulated in sphere cells. In the mean while, YB-1 mRNA was up-regulated during sphere forming. Relative to Norl., *, $p<0.05$;**, $p<0.01$;***, $p<0.001$.

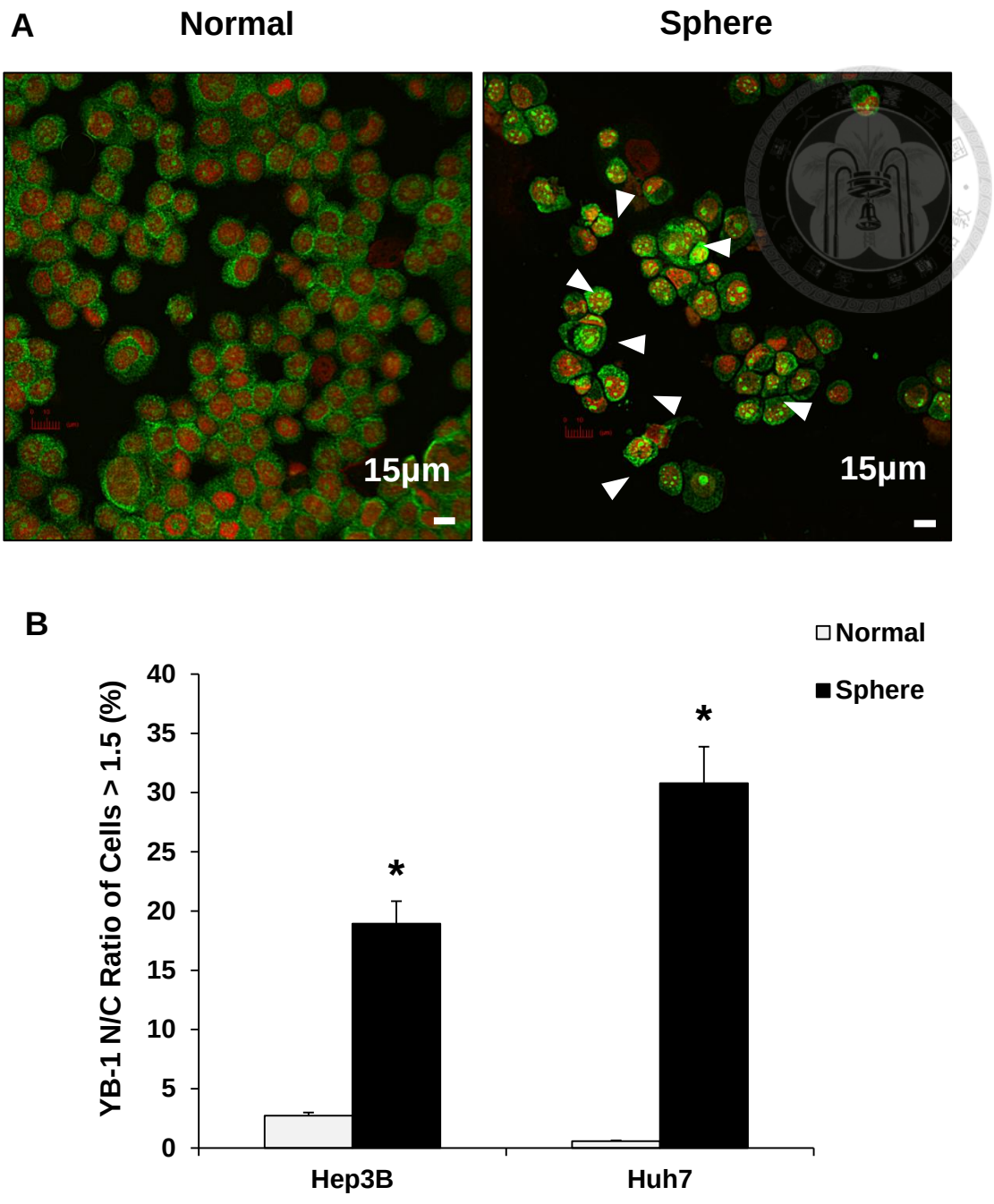

A
Normal
Sphere
15μm
15μm
B
Normal
Sphere
YB-1 N/C Ratio of Cells > 1.5 (%)
40
35
30
25
20
15
10
5
0
*
*
Hep3B
Huh7

Figure3

Figure 3. YB-1 would translocate into nucleus during sphere forming.

Huh7 cells were cultured with normal or sphere forming condition, and then were detached with trypsin. The detached cells were fixed and undergone immunofluorescent staining. Comparing to normal HCC cells, there was more YB-1 translocating from cytosol into nucleus in sphere HCC cells (A). It was interesting that YB-1 accumulated in nucleolus. Quantitative data also shown that there were more than 15% HCC cells contained nucleus-translocating YB-1 (B). Relative to normal *, $p<0.05$ (Red, nucleus; Green, YB-1)

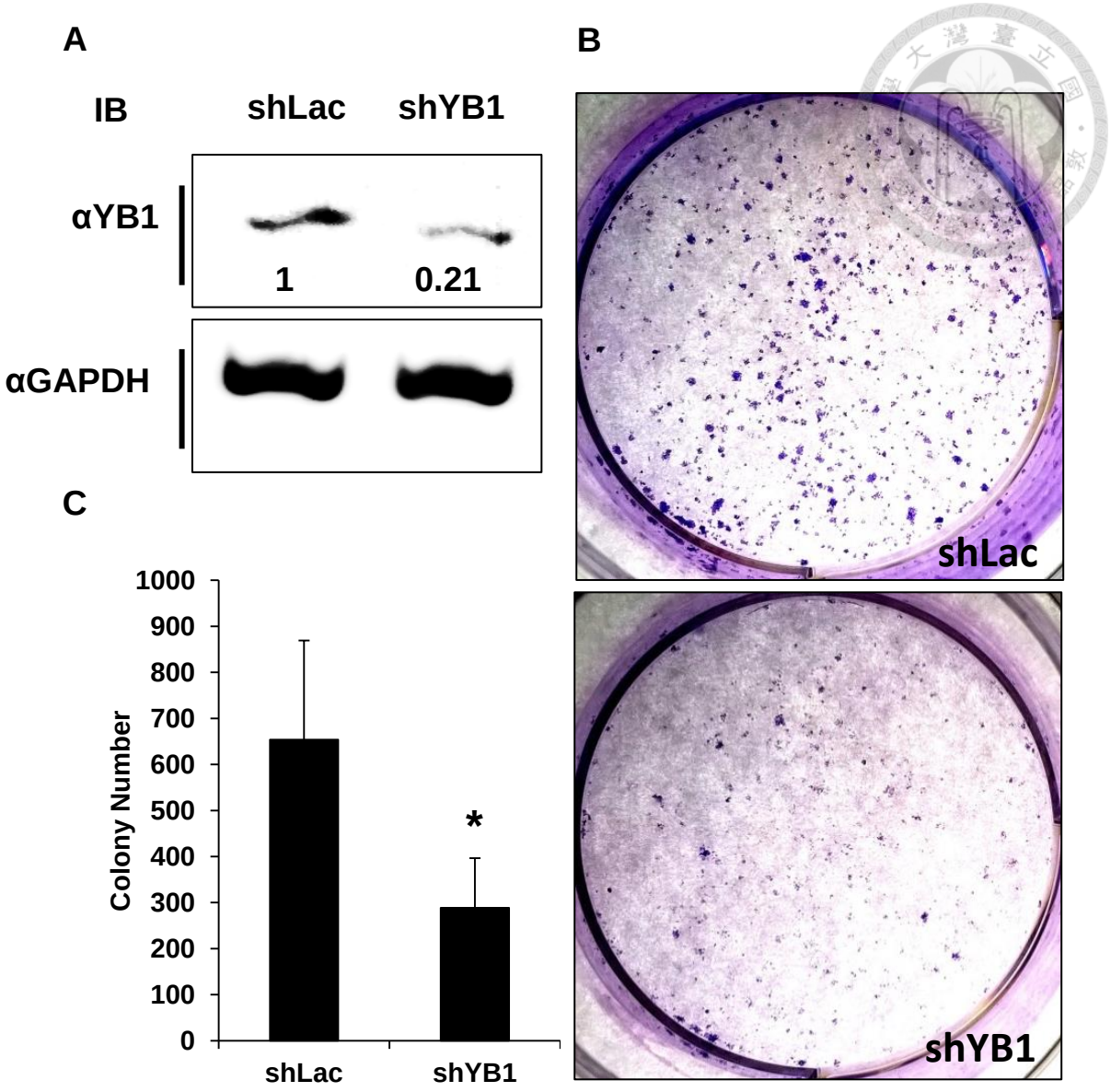


Figure 4. YB-1 KD Huh7 cells formed fewer colonies.

I successfully established YB-1 KD Huh7 cells (A), which formed fewer colonies on the cell culture dish in 3% FBS DMEM (B, C). Relative to shLac *, $p<0.05$

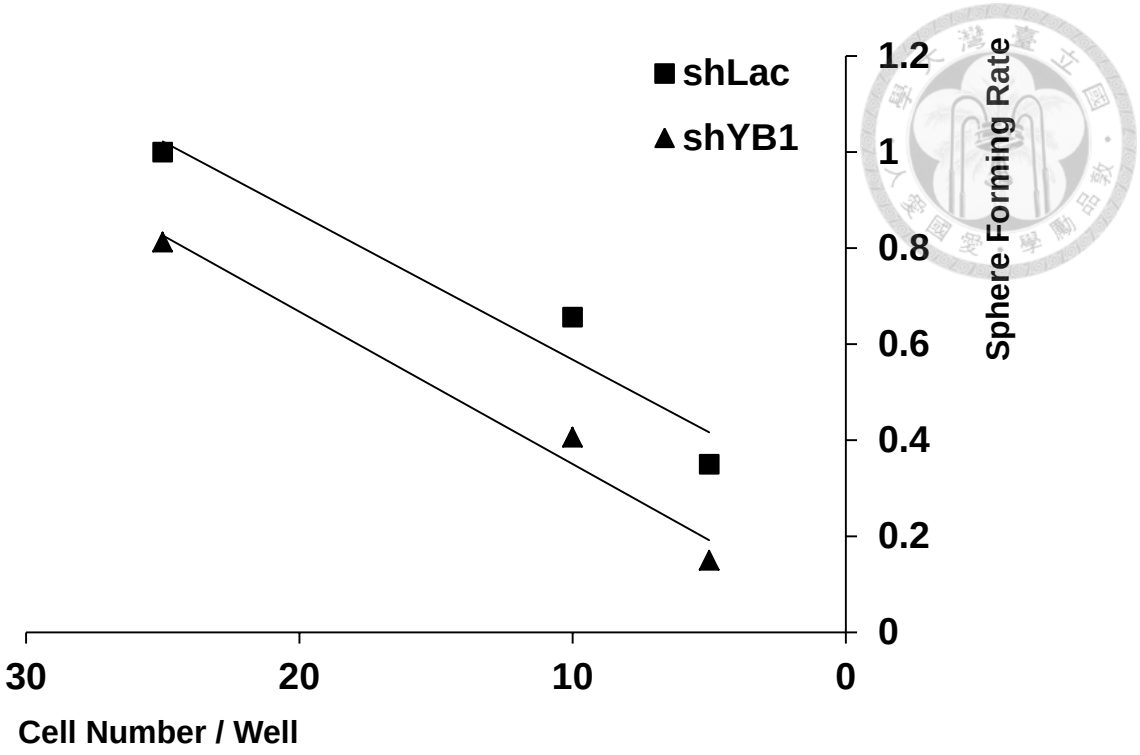


Figure 5. YB-1 knock down decreased stem cell frequency of Huh7.

In extreme limiting dilution assay, shYB1 cells could not form the sphere cells well. Comparing to shLac cells, the stem cell frequency of shYB1 cells was decreased (Table 1.) and the ELDA scatter plot indicates significant differences in stem cell frequencies between the shYB1 and shLac (p<0.05).

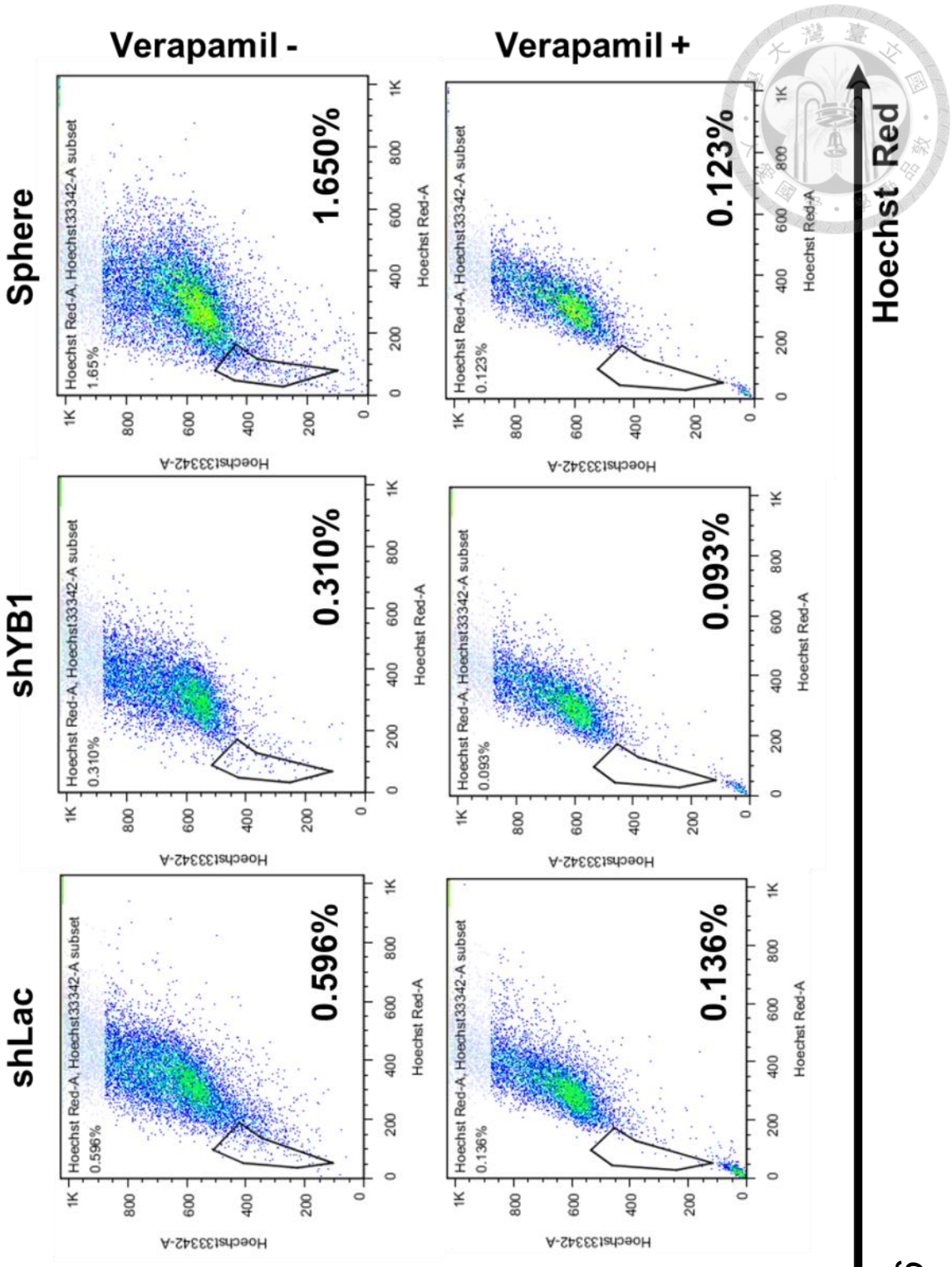

Verapamil -
Verapamil +
Sphere
shYB1
shLac
1.650%
0.123%
0.310%
0.093%
0.596%
0.136%
Hoechst Blue
Hoechst Red

Figure6

Figure 6. Side population would be altered by different gene expression profile.

The results of side population flow cytometry indicated that ratio of stem-like cells in cell lines was altered by YB-1 expression profiles. While YB-1 knock down, the ratio of stem-like cells was decreasing. On the contrary, the ratio of stem-like cells in sphere cells would increase with up-regulated of YB-1.

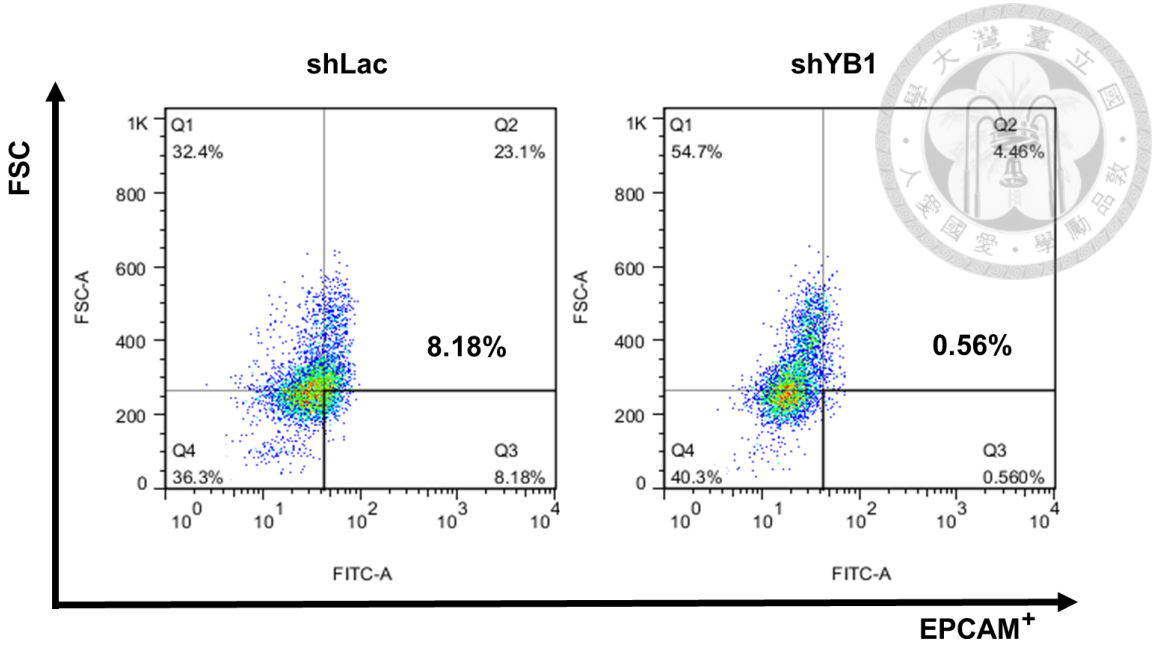


Figure7

Figure 7. EPCAM$^+$ cells would decrease after YB-1 knockdown.

The results of flow cytometry indicated that the ratio of EPCAM$^+$ cells, which was recognized as tumour initiation cell, was altered by YB-1. The ratio of EPCAM$^+$ cells in shYB1 cells would decrease with down-regulated of YB-1.

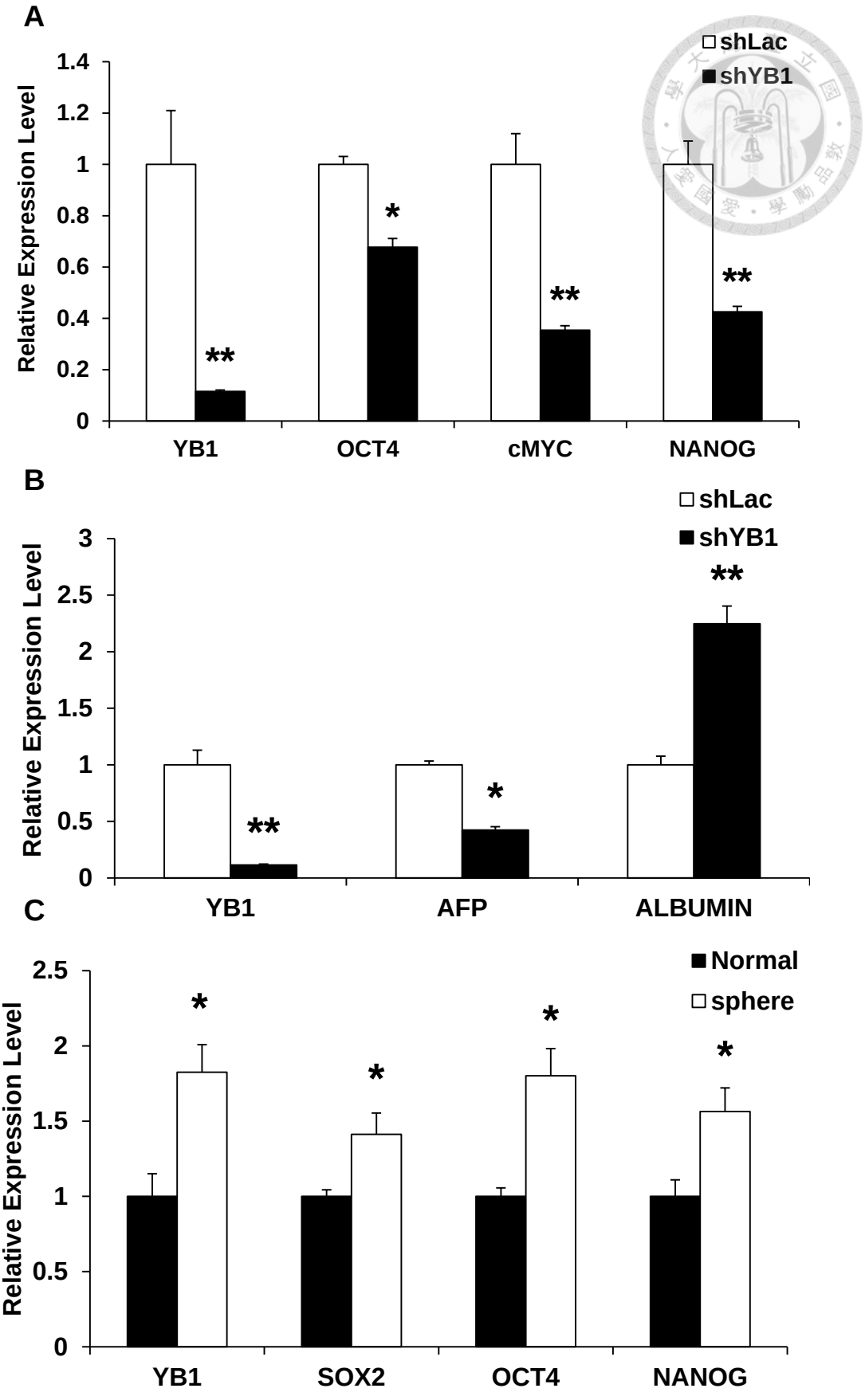

A
shLac
shYB1
Relative Expression Level
1.4
1.2
1
0.8
0.6
0.4
0.2
0
**
*
**
**
YB1
OCT4
cMYC
NANOG
B
shLac
shYB1
Relative Expression Level
3
2.5
2
1.5
1
0.5
0
**
*
**
YB1
AFP
ALBUMIN
C
Normal
sphere
Relative Expression Level
2.5
2
1.5
1
0.5
0
*
*
*
*
YB1
SOX2
OCT4
NANOG

Figure8

Figure 8. Pluripotent genes were altered by YB-1.

The gene expression analysis of YB-1 KD cells indicated that some pluripotent genes, such as OCT4, cMYC and NANOG were down-regulated (A). Moreover, the HCC marker AFP was also down-regulated in YB-1 KD cells, while liver lineage differentiated marker, albumin, was up-regulated (B). However, the pluripotent genes such as OCT4, SOX2 and NANOG were co-upregulated with YB-1 in sphere cells (C). Relative to shLac or Normal *, $p<0.05$; **, $p<0.01$

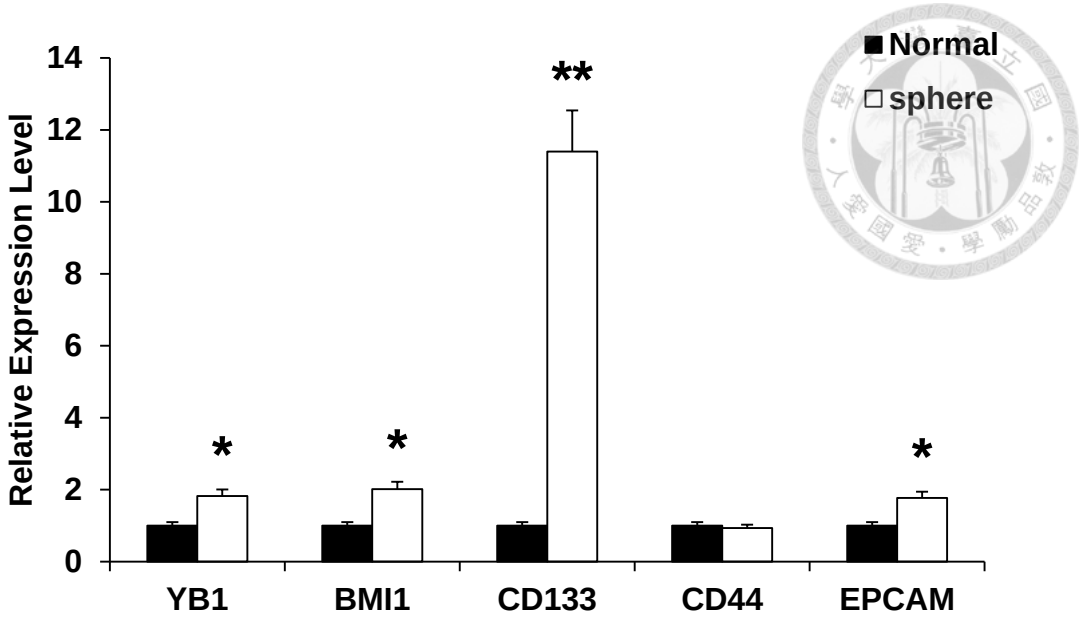


Figure 9. Stem cell marker and differential state marker were regulated by YB-1.

The gene expression analysis of YB-1 KD cells indicated that some gene markers related to stem cells behavior were up-regulated, such as BMI1, CD133, CD44 and EPCAM. Relative to Normal *, $p<0.05$; **, $p<0.01$

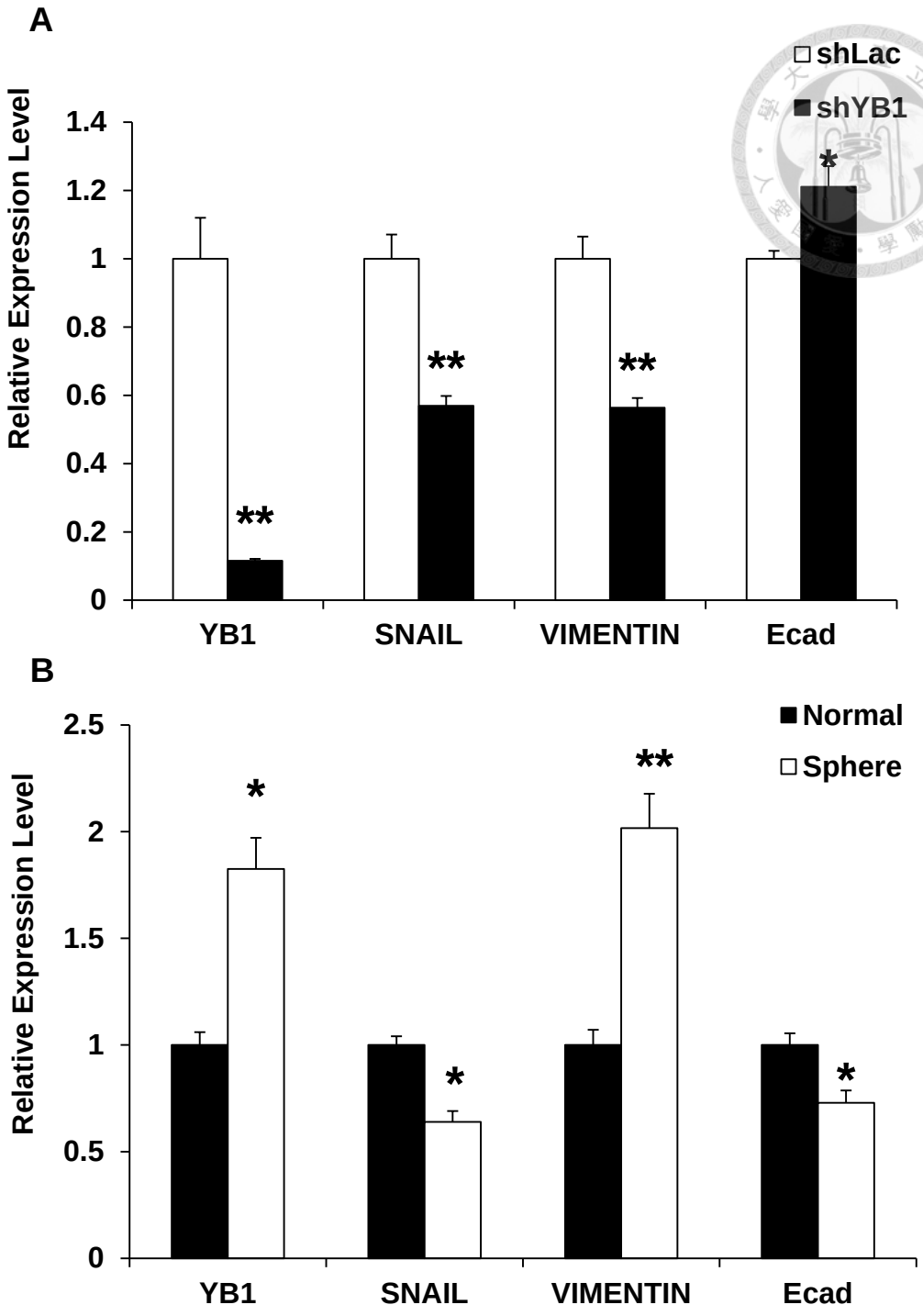

A
shLac
shYB1
Relative Expression Level
1.4
1.2
1
0.8
0.6
0.4
0.2
0
**
**
**
*
YB1
SNAIL
VIMENTIN
Ecad
B
Normal
Sphere
Relative Expression Level
2.5
2
1.5
1
0.5
0
*
*
**
*
YB1
SNAIL
VIMENTIN
Ecad

Figure10

Figure 10. Metastasis-related genes were down-regulated in YB-1 KD cells.

In gene expression analysis of YB-1 KD cells, the EMT and metastasis-related genes, SNAIL and VIMENTIN were down-regulated, while E-cadherin, important epithelial marker, was up-regulated. On the other hands, gene expression profiles of sphere cells reveal the opposite results to YB-1 KD cells, excepted SNAIL. Relative to shLac *, $p<0.05$; **, $p<0.01$

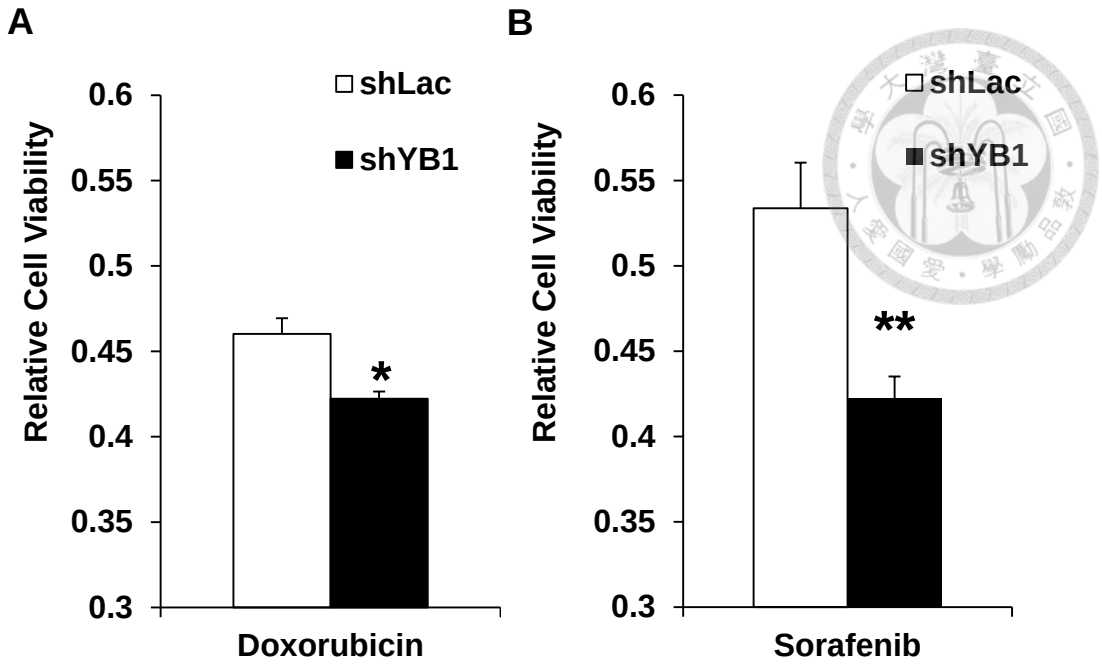


Figure 11. YB-1 KD cells revealed lower cell viability after treatment of anti-cancer drug.

After treated with doxorubicin (0.15 μg/ml) or sorafenib (0.15mM) for 3 days in 3% FBS DMEM, the cell viability of YB-1 KD cells, compared to shLac cells, were decreased. Relative to shLac *, $p<0.05$; **, $p<0.01$

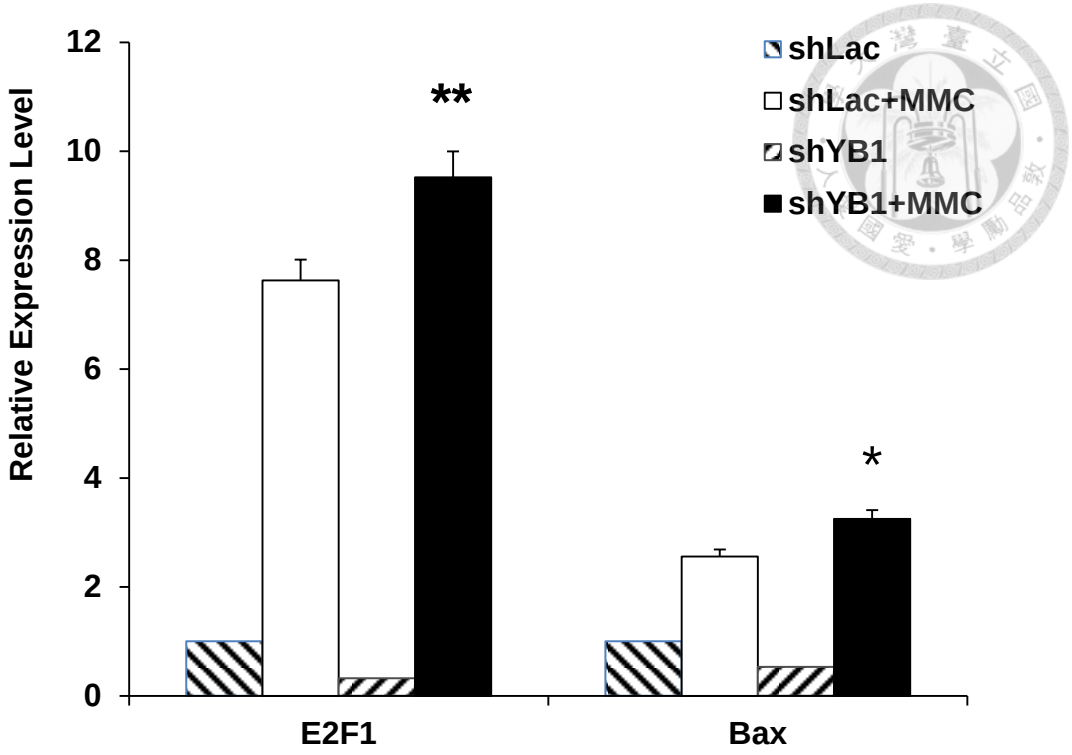


Figure 12. Proapoptotic genes were up-regulated in YB-1 KD cells.

After 1.5mM MMC treated, proapoptotic genes were up-regulated in YB-1 KD cells, which indicated YB-1 KD cells could not survival easily. Relative to shLac+MMC *, $p<0.05$; **, $p<0.01$

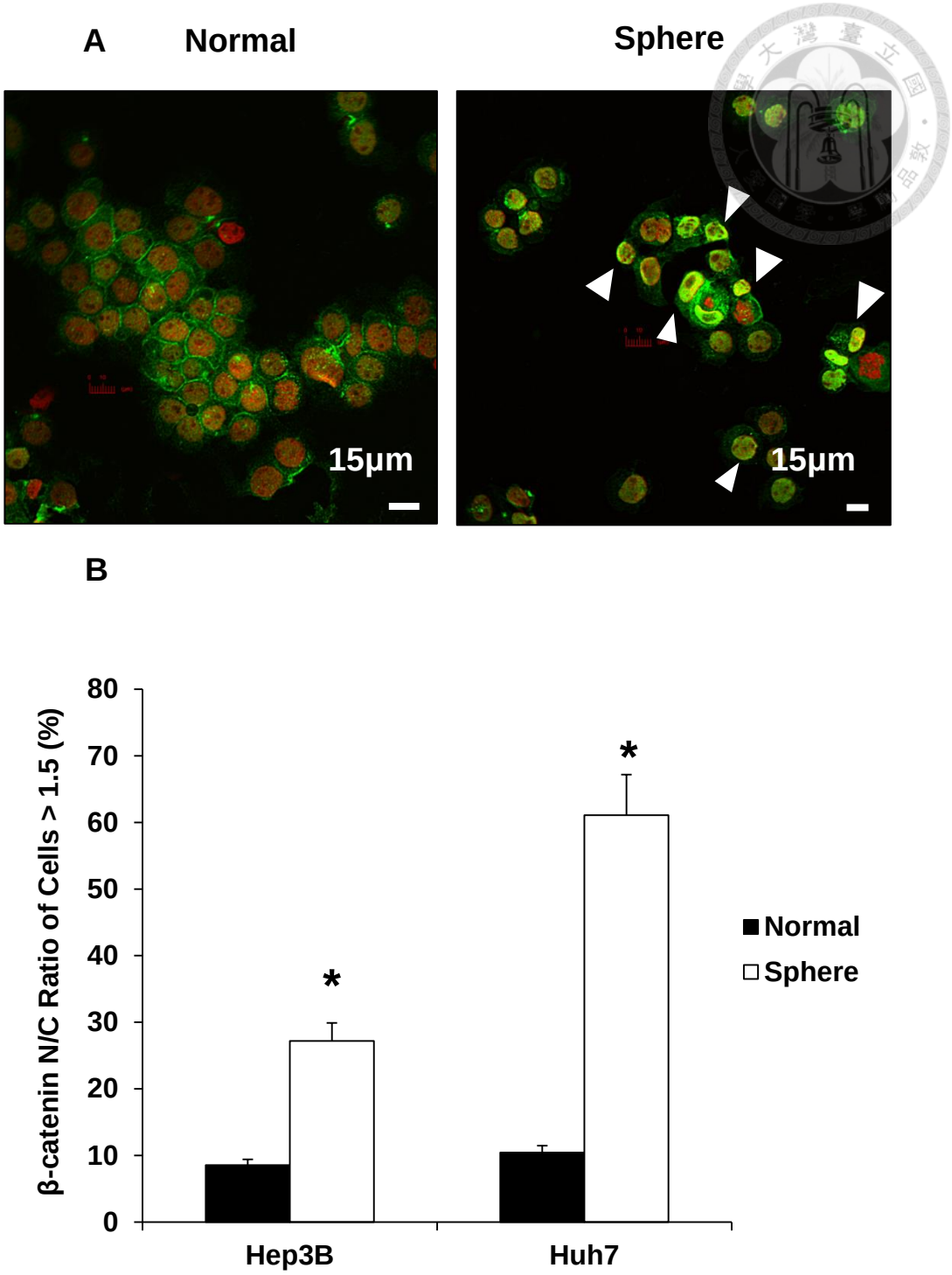

A
Normal
Sphere
15μm
15μm
B
β-catenin N/C Ratio of Cells > 1.5 (%)
80
70
60
50
40
30
20
10
0
*
*
Normal
Sphere
Hep3B
Huh7

Figure13

Figure 13. Sphere forming would lead Wnt signaling activation.

Huh7 cells were cultured with normal or sphere forming condition, and then were detached with trypsin. The detached cells were fixed and undergone immunofluorescent staining. Comparing to normal HCC cells, there was more β-catenin translocating from membrane into nucleus in sphere HCC cells (A). Quantitative data also shown that there were more than 15% HCC cells contained nucleus-translocating β-catenin (B). This result suggested that Wnt signaling would be activated during sphere forming. Relative to normal *, $p<0.05$ (Red, nucleus; Green, β-catenin)

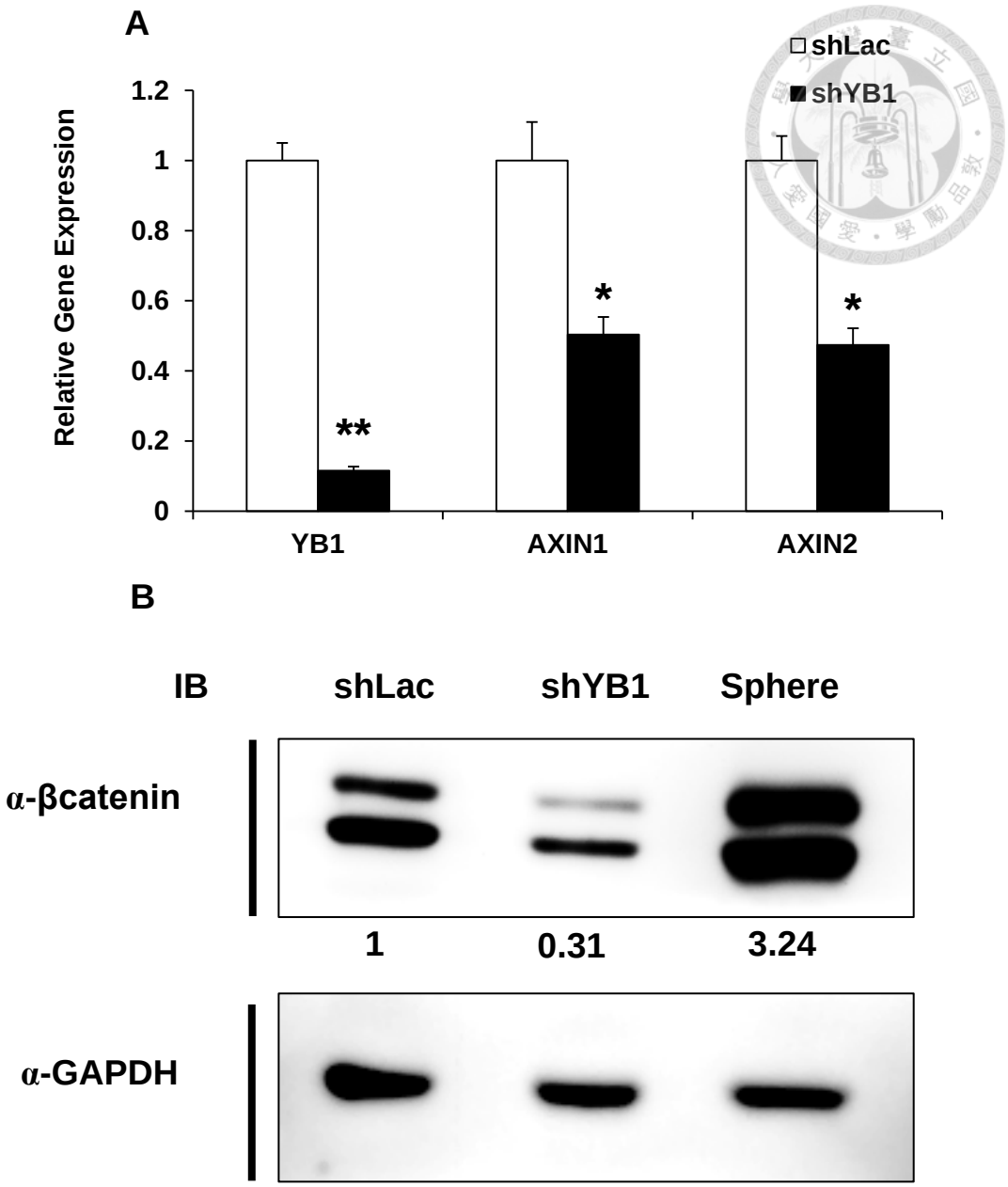

A
shLac
shYB1
Relative Gene Expression
1.2
1
0.8
0.6
0.4
0.2
0
**
*
*
YB1
AXIN1
AXIN2
B
IB
shLac
shYB1
Sphere
α-βcatenin
1
0.31
3.24
α-GAPDH

Figure14

Figure 14. YB-1 KD would lead to down-regulating of Wnt signaling.

YB-1 KD would lead to down-regulating AXIN1 and AXIN2, down stream of Wnt signaling (A). Furthermore, β-catenin was up-regulated in translational level during sphere forming and was down-regulated in YB-1 KD cells (B). Relative to shLac *, $p<0.01$; **, $p<0.001$

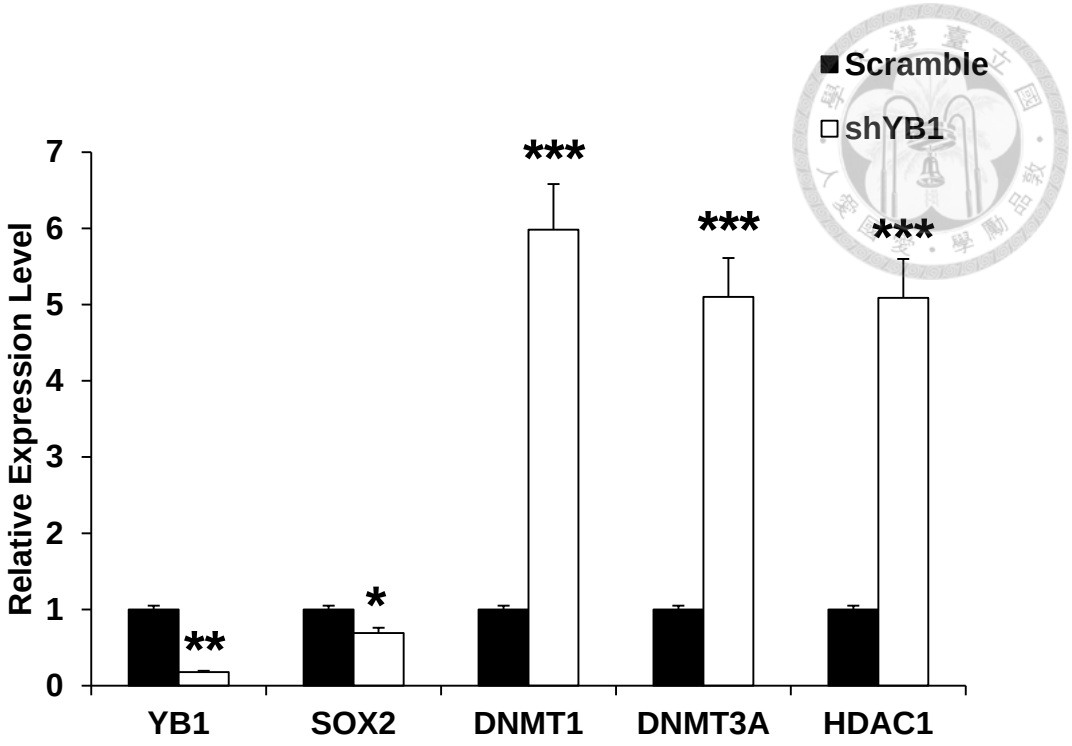


Figure15

Figure 15. Epigenetic modulators were affected by YB-1.

During knock down YB-1 mRNA, the mRNA level of SOX2 decreased while the genes related to DNA methylation pattern change, DNMT1 and DNMT3A, as well as histone modification protein, HDAC1, were up-regulated. Relative to shLac *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$

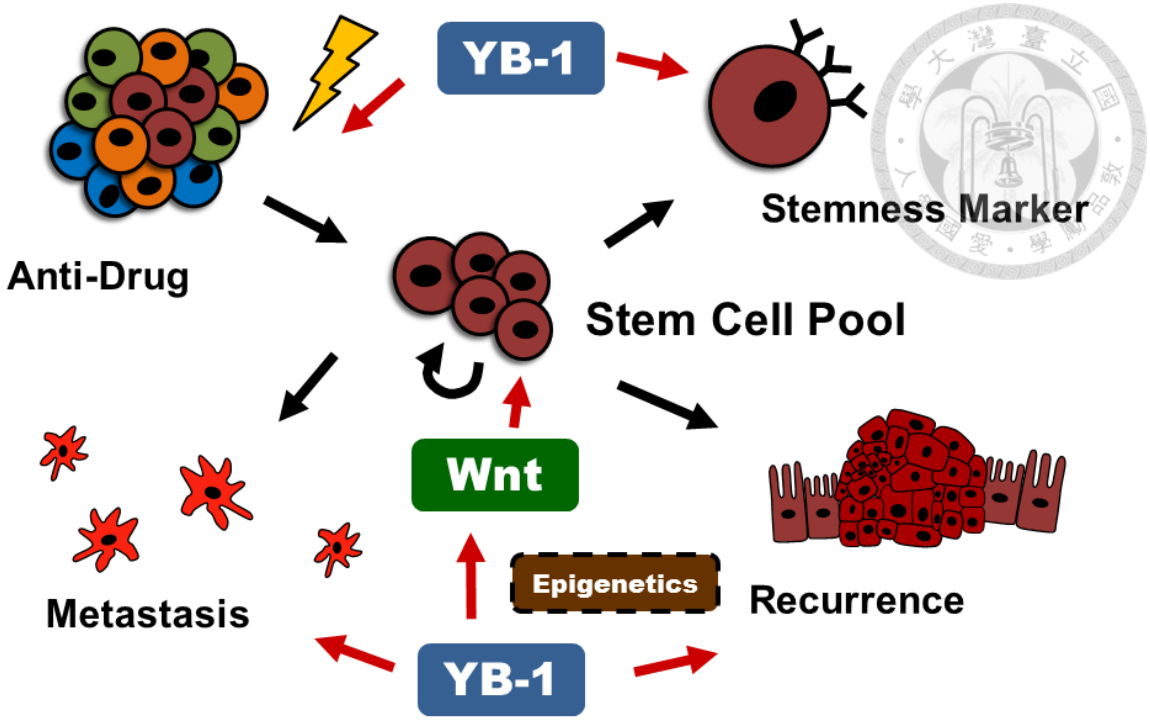


Figure 16. Putative YB-1 Regulatory Pattern in HCC Cancer Stem Cells

YB-1 would be up-regulated during sphere forming, which would amplify the stem cell pool in HCC cell lines. Further data suggested the importance of YB-1 in self-renewal, stem cell pool maintenance, EMT genes regulation, anti-apoptosis and anti-drug ability. Those properties would be caused by YB-1 mediated pluripotent gene activation, which co-regulated with Wnt/β-catenin pathway and some epigenetic pathways.

Table 1. Extreme limiting dilution assay (ELDA) of Sphere forming.

Cell Number (per well)	25	10	5	Frequency (cell / stem cell)	*p* Value*
shLac	**16**/16	**21**/32	**14**/40	**9.39**	
shYB1	**14**/16	**13**/32	**6**/40	**15.52**	**0.028**

***relative to shLac**

Table 2. YB-1 targeting epigenetic modulators

mice	human	symbol	accession
Wdr5	WDR5	WDR5	NM_017588
Dot1L	DOT1L	DOT1L	NM_032482
Setdb1	SETDB1	SETDB1	NM_001145415
Suv39h1	SUV39H1	SUV39H1	NM_003173
Suv39h2	SUV39H2	SUV39H2	NM_001193424
Ehmt2	EHMT2	EHMT2	NM_006709
Jmjd1b	JMJD1B	KDM3B	NM_016604
Jmjd2b	JMJD2B	KDM4B	NM_015015
Hdac1	HDAC1	HDAC1	NM_004964
Dnmt1	DNMT1	DNMT1	NM_001130823
Dnmt3a	DNMT3A	DNMT3A	NM_022552

Table 3. List of antibodies

Antibody	Manufacturer	Catalog Number	Application	Dilution Fold
Primary Antibodies				
Anti-YB-1	Abcam	ab12148	IF	1/300
Anti-YB-1	Abcam	ab12148	IB	1/10000
Anti-GAPDH	Millipore	MAB374	IB	1/10000
Anti-β-catenin	Abcam	Ab-2365	IF	1/300
Anti-β-catenin	Abcam	Ab-2365	IB	1/10000
Anti-EPCAM	Biolegend	324203	FC	1/250
Mouse Isotype Ctrl IgG	eBioscience	11-4714	FC	1/250
Secondary Antibodies				
Anti-rabbit AlexaFlouro488	Life Technologies	A21206	IF	1/500
Anti-mouse HRP	Jackson ImmunoResearch	115-035-003	IB	1/10000
Anti-rabbit HRP	Jackson ImmunoResearch	111-035-003	IB	1/10000

Table 4. List of qPCR primer sequence

Gene	Primers	
GAPDH	Forward	GGAGCGAGATCCCTCCAAAAT
	Reverse	GGCTGTTGTCATACTTCTCATGG
β-Actin	Forward	CATGTACGTTGCTATCCAGGC
	Reverse	CTCCTTAATGTCACGCACGAT
YB-1	Forward	TAGACGCTATCCACGTCGTAG
	Reverse	CCCCACTCTCACTATTCTGGT
CD133	Forward	GGCCCAGTACAACACTACCAA
	Reverse	CGCCTCCTAGCACTGAATTGATA
CD90	Forward	ATCGCTCTCCTGCTAACAGTC
	Reverse	CTCGTACTGGATGGGTGAACT
CD44	Forward	GCACAGACAGAATCCCTGCTA
	Reverse	GCCATTTGTGTTGTTGTGTGAA
EPCAM	Forward	GCGGGCTCTTTAAGGCCAA
	Reverse	CCCCAGCAGTGTTCACACA
Albumin	Forward	GAGACCAGAGGTTGATGTGATG
	Reverse	AGTTCCGGGGCATAAAAGTAAG
AFP	Forward	CTTTGGGCTGCTCGCTATGA
	Reverse	GCATGTTGATTTAACAAGCTGCT
OCT4	Forward	GACAGGGGGAGGGGAGGAGCTAGG
	Reverse	CTTCCCTCCAACCAGTTGCCCCAAAC
SOX2	Forward	GGGAAATGGGAGGGGTGCAAAAGAGG
	Reverse	TTGCGTGAGTGTGGATGGGATTGGTG
cMYC	Forward	GTCAAGAGGCGAACACACAAC
	Reverse	TTGGACGGACAGGATGTATGC
Nanog	Forward	CGTGTGAAGATGAGTGAAACTGA
	Reverse	CTCGCTGATTAGGCTCCAAC

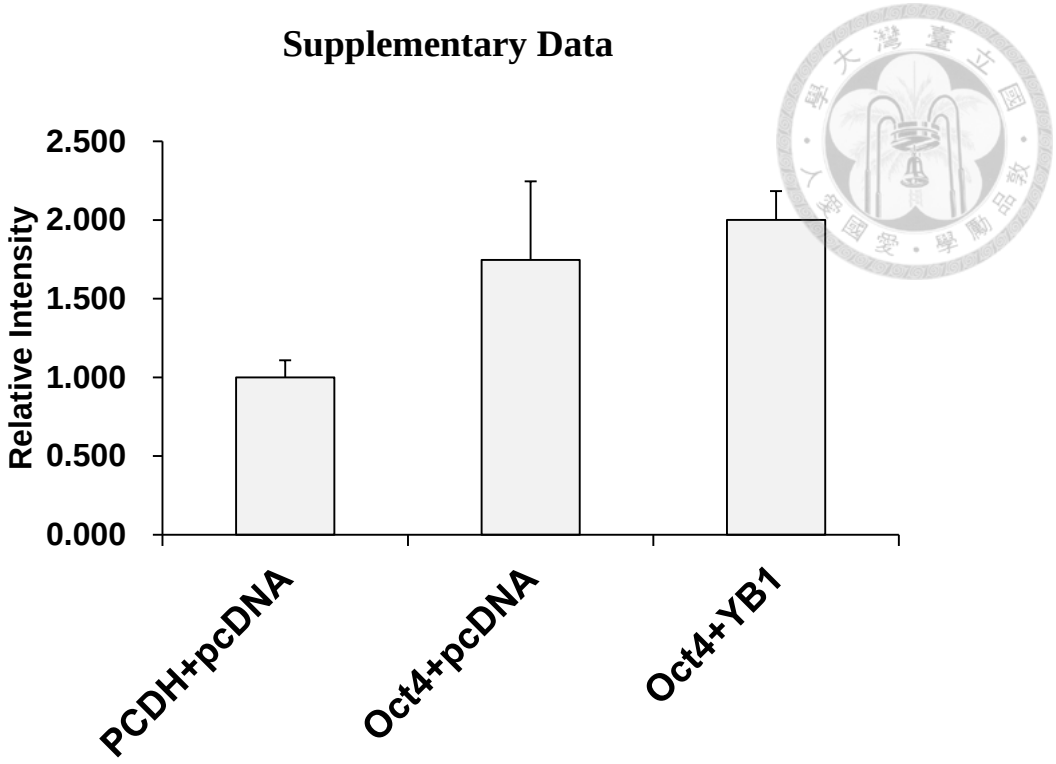
List of qPCR primer sequence (continued)

BMI1	Forward	CGTGTATTGTTCGTTACCTGGA
	Reverse	TTCAGTAGTGGTCTGGTCTTGT
SNAIL1	Forward	TCGGAAGCCTAACTACAGCGA
	Reverse	AGATGAGCATTGGCAGCGAG
Vimentin	Forward	AGTCCACTGAGTACCGGAGAC
	Reverse	CATTTCACGCATCTGGCGTTC
E-cadherin	Forward	ATTTTTCCCTCGACACCCGAT
	Reverse	TCCCAGGCGTAGACCAAGA
E2F1	Forward	ACGTGACGTGTCAGGACCT
	Reverse	GATCGGGCCTTGTTTGCTCTT
BAX	Forward	CCCGA GAGGT CTTTT TCCGA G
	Reverse	CCAGC CCATG ATGGT TCTGA T
Axin1	Forward	TGGAGCCCTGTGACTCGAA
	Reverse	GGGACACGATGCCATTGTTA TC
Axin2	Forward	CT TAAAG GTCTT GAGGG TTGAC
	Reverse	CAACA GATCA TCCCA TCCAA CA
DNMT1	Forward	CCTAGCCCCAGGATTACAAGG
	Reverse	ACTCATCCGATTTGGCTCTTTC
DNMT3A	Forward	CCGATGCTGGGGACAAGAAT
	Reverse	CCCGTCATCCACCAAGACAC
HDAC1	Forward	CCGCATGACTCATAATTTGCTG
	Reverse	ATTGGCTTTGTGAGGGCGATA

Appendix

List of Abbreviations

Abbreviation	Full term
KD	Knock down
AFP	α-fetoprotein
EPCAM	Epithelial cell adhesion molecule
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
Lef1	Lymphoid enhancer-binding factor 1
HCC	Hepatocellular carcinoma
CSC	Cancer Stem Cell
EMT	Epithelial-Mesenchymal Transition
FC	Flow cytometry
IF	Immunofluorescence staining
IB	Immuno Blotting



Supplementary figure 1. YB-1 would not enhance Nanog transcription.

YB-1 (overexpressed by pcDNA), Oct4 (overexpressed by PCDH) and Nanog promoter driven luciferase pGL4 were transfected to HEK293T for 48 hours. Then I would measure the light intensity of samples. The results showed that YB-1 would not affect the Oct4 driven Nanog gene transcription activation.

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